Rainbow trout as a model of retinal photoreceptor death and regeneration

by

William Edward (Ted) Allison

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ABSTRACT

Salmonid fishes have been reported to have a remarkable ontogeny of cone photoreceptors in their retina. The ultraviolet-sensitive (UVS) cones are of particular interest, as they disappear from, and reappear into, the retina. These events occur at times associated with migration to marine waters, and the return migration to freshwater spawning grounds, respectively. The primary goal of this thesis was to discover the mechanisms underlying this ontogeny of UVS cones by studying a salmonid, the rainbow trout (*Oncorhynchus mykiss*). Two hypotheses were considered: 1) UVS cones become dormant, similar to speculations regarding light damage of rod photoreceptors in albino trout; 2) UVS cones die and subsequently regenerate from stem cells known to robustly proliferate in trout retina.

I cloned partial cDNAs of each opsin from trout and used them to develop *in situ* hybridization labelling of photoreceptors. I introduced the ability to assess UV sensitivity utilizing electroretinograms, and developed a polyclonal antibody against the UVS opsin, to label UVS cones in immunohistochemistry. I combined these tools to examine trout UVS cones during natural development, and found that it was similar to events during thyroid hormone (TH) treatment. I used labels and inhibitors of programmed cell death to determine that UVS cone death is a major mechanism of UVS cone disappearance. UVS cones reappeared into the retina following termination of TH treatment. Application of cell fate markers indicates that reappearing UVS cones can be generated from proliferating stem

cells. Electroretinograms demonstrated that these regenerated UVS cones sufficiently integrate into the retina to pass signals onto second order neurons. This represents the only known example of cone photoreceptors regenerating from stem cells during natural development. I speculate on the adaptive value of the ontogeny of UVS cones.

I also investigated mechanisms underlying the apparent survival of rod photoreceptors when albino trout retina receive light-induced damage. Previous conclusions in this area had been influential in forming the hypotheses of UVS cone ontogeny. Two hypotheses were envisioned: 1) rod photoreceptors were surviving light damage; 2) rods were being killed by light but quickly replaced by proliferating retinal cells. My results support the latter hypothesis.

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List of Abbreviations

ACC	Accessory Corner Cone
AP	Alkaline Phosphatase
BrdU	5-Bromo-2'-deoxyuridine
CGZ	Circumferential Germinal Zone
CNS	Central Nervous System
D	Dorsal
DIG	Dioxigenin
EM	Electron Microscopy
ERG	Electroretinogram
FL	Fluorescein
FOV	Field of View
GnRH	Gonadotropin Releasing Hormone
IHC	Immunohistochemistry
INL	Inner Nuclear Layer
IRD	Inner Ring Deiodinase
LWS	Long Wavelength Sensitive
MAB	Maleic Acid Buffer
mRNA	messenger RNA
MSP	Microspectrophotometry
MWS	Medium Wavelength Sensitive
N	Nasal
OLM	Outer Limiting Membrane
ONL	Outer Nuclear Layer
OPL	Outer Plexiform Layer
ORD	Outer Ring Deiodinase
PBS	Phosphate Buffered Saline
PCD	Programmed Cell Death
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PINCs	Proliferating Inner Nuclear Layer Cells
PS	Polarization Sensitivity
PTW	PBS plus Tween
ROS	Rod Outer Segment
RPE	Retinal Pigmented Epithelium
RT-PCR	Reverse-Transcriptase Polymerase Chain Reaction
SWS	Short Wavelength Sensitive
T_3	3,5,3'-triiodothyronine
T_4	L-Thyroxine
TH	Thyroid Hormone
THR	Thyroid Hormone Receptor
TUNEL	Terminal deoxyUridine triphosphate Nick-End Labelling
UV	Ultraviolet
UVS	Ultraviolet-Sensitive
λ_{max}	Wavelength of maximal absorbance

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none

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Chapter 1:

Introduction

Aims and Scope of Thesis

Research on the visual systems of salmon and trout has suggested a remarkable ontogeny of photoreceptors, wherein changes in retinal structure appear to be associated with the fish's migration through a variety of habitats. In particular, a group of morphologically identifiable cone photoreceptors, the accessory corner cones (ACC), was noted to be disappearing from some areas of the retina. This was found to be coincident with the disappearance of cones that contain an ultraviolet- (UV-) wavelength-sensitive (UVS) pigment, and also coincident with the loss of UV sensitivity as determined by electrophysiology or psychophysical paradigms. Thus, the disappearing group of cones was putatively identified as ultraviolet-sensitive cones. Manipulations with thyroid hormone (TH) or retinoic acid were able to mimic these events, and the reappearance of the ACC. Examination of sexually mature salmonids showed that ACC were present in a substantial portion of the retina, and together with the data above these were thought to represent ACC that had reappeared as the salmon were preparing for their return migration to freshwater spawning grounds. This ontogeny has implications both for visual ecology and for mechanisms of photoreceptor development relevant to clinical settings.

The aims of this thesis were to resolve several issues stemming from this interesting ontogeny of salmonid photoreceptors. I selected rainbow trout

(*Oncorhynchus mykiss*) as a representative salmonid, and in some instances utilized TH treatments to address the following:

1) To confirm the identity of the ACC, putatively identified as the UVS cone, by examining opsin gene expression. (Chapter 2)
2) To address hypotheses regarding the mechanisms of the UVS cone disappearance and reappearance. (Chapter 3)
3) To examine mechanisms whereby photoreceptors may be surviving light-induced damage in albino trout. Previous observations of this light-damage paradigm had been incorporated in the formation of hypotheses in topic #2 above. (Chapter 4)

Two major topics of this thesis will be retinal development of the rainbow trout, a salmonid fish, and how thyroid hormone treatments alter this development. Thus, in this introduction I will:

1) Review the structure of salmonid retina.
2) Review major concepts of retinal development and regeneration, with special attention to salmonids and thyroid hormone signalling.

1.1 Structure of the salmonid retina

The retina is an accessible part of the central nervous system (CNS) that is particularly useful in studies of CNS function and development. Several features make the retina accessible:

1) It is easy to stimulate. The natural stimulus (light) that can easily be modified in its characters (intensity, wavelength, polarization).
2) It is easy to record from the retina, including *in vivo* recordings.

3) It is easy to dissect. In dark-adapted retina, the attachment points of the neural retina are limited to the optic nerve and a ring at the retinal periphery (near the iris). Disrupting these contacts allows an intact neural retina to be quickly isolated free of pigmented epithelium.
4) The retina has a layered structure, similar to higher CNS nuclei. The layered organization of cell types and plexiform layers facilitates their identification and the understanding of their organization / interactions.

The retinal structure of vertebrates is generally very well conserved. Some additional features of teleost retina make them particularly suitable for studies of CNS function and development:

5) The spatial organization of photoreceptors in the outer retinal layers is patterned in an array. The regularity of this repeated organization of photoreceptors has led to the structure being referred to as a 'mosaic'. This has assisted in identifying photoreceptor classes, and in recognizing spatial relationships that are relevant to formation of cone identity during development.
6) The second order neurons are positioned with similar regularity, which simplifies study of their connectivity and function.
7) The retina continues to grow throughout the life of the fish. Several populations of proliferating cells are present (reviewed in section 1.2, below) and the continual generation of new retina, and new cells within existing retina, have greatly facilitated study of how photoreceptors differentiate and how stem cells can regenerate damaged retina.

The eye of trout has long been considered as a general example of teleost eyes, to which the eyes of other teleosts may be compared (Verrier, 1928; Walls, 1942; Rochon-Duvigneaud, 1943; Polyak, 1957). The retina lines the back of the eye, with ganglion cells being closest to the lens and vitreous humor. Therefore the term 'vitreal' has been adopted for describing the layer of the neural retina closest to the ganglion cells, where light first impinges (Figure 1). The photoreceptors are at the outside of the neural retina, towards the sclera (a cartilaginous tissue on the outside of the eye). Therefore, the term 'scleral' has been adopted for the direction towards the back of the eye. The view of the retina presented in Figures 1 and 2A, along the vitreal-scleral axis is termed a 'radial' section. Orthogonal to this, and orthogonal to the long-axis of the photoreceptors (Figure 2B), is termed a 'tangential' section. Finally, it should be noted that due to variation in the position of the eyes amongst vertebrates, the terms 'nasal' and 'temporal' have been adopted to replace what would be appropriate for 'anterior' and 'posterior' in a fish (these terms are not synonymous in a primate, for example).

The outer segments of photoreceptors express opsin proteins which absorb light for its conversion to chemical and ultimately electrical potential to begin the visual cascade. Opsins are seven-transmembrane G-proteins and can be classed into several opsin classes based upon their amino acid sequence. Changes in amino acid sequence tune the opsin's wavelength of maximal absorbance (λ_{max}). The opsin protein binds a vitamin A derivative, the chromophore, in its binding pocket, amongst the transmembrane helices. The

Figure 1. Structure of the salmonid eye, as represented by a radial slice through the eye of rainbow trout. Modified from a drawing by Mlle. Rochon-Duvigneaud (1863-1952) in her treatise on teleost vision (1943). RPE, retinal pigment epithelium. The double-headed arrow defines the adjectives *vitreal* and *scleral* (see p. 4).

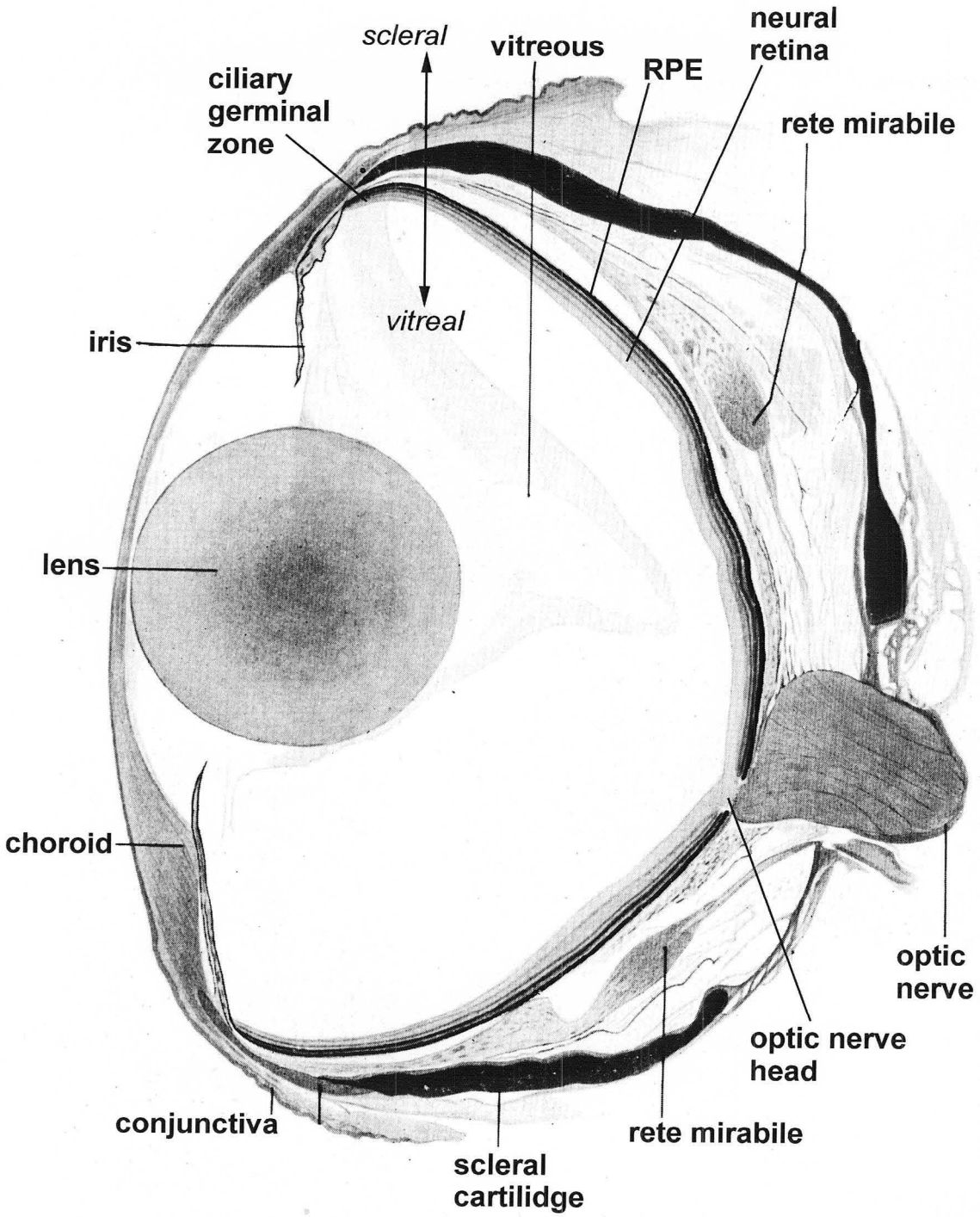
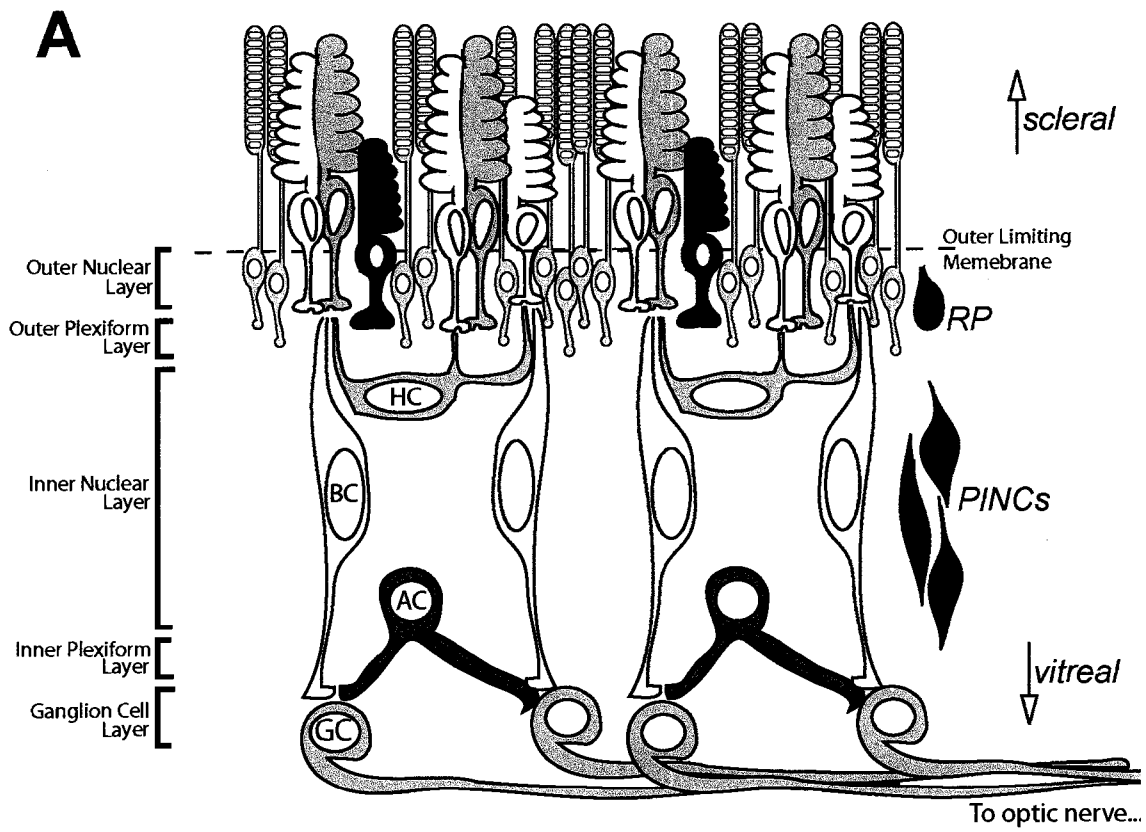

scleral
vitreous
neural retina
RPE
ciliary germinal zone
rete mirabile
vitreal
iris
lens
choroid
optic nerve
optic nerve head
conjunctiva
rete mirabile
scleral cartilidge

Figure 2. Schematic of salmonid retinal cell types. **A.** Radial view of the retina, similar to the double-headed arrow (scleral-vitreal) in Figure 1. Rods and four cone photoreceptors form the most scleral layer of the neural retina (retinal pigment epithelium, not shown here, would appear scleral to the photoreceptors and its pigment intercalates amongst the photoreceptors). The cones take the form of double cones (larges cones, with two members separated only by their cell membranes), which contain long- and medium-wavelength sensitive (LWS & MWS) pigments. Two single cone populations occur, containing ultraviolet-sensitive (UVS) opsin (coloured dark, with nuclei vitreal to the outer limiting membrane) and short-wavelength sensitive (SWS) opsin. The photoreceptor nuclei form the outer nuclear layer. The photoreceptors interact with at least three layers of horizontal cells (HC) and pass signals through bipolar cells (BC) to ganglion cells (GC). GC axons form the optic nerve. Amacrine cells (AC) also mediate the output of ganglion cells. HC, BC, and AC nuclei form the Inner nuclear layer. Proliferating cells, which appear black in this figure, occur as rod progenitors (RP) in the ONL and proliferating inner nuclear layer cells (PINCs) in the INL. The RP and PINCs have been thought to only generate rods during natural development (although see chapter 3). **B.** Tangential view, at the level of cone outer segments or nuclei. Small dots represent rods. The cone photoreceptors (represented by the same colours as in panel A) form a regular pattern with double cone partitioning membranes pointing towards SWS cones. The other single cones, the UVS cones, appear in the corners of the mosaic units.


A
scleral
Outer Limiting Memebrane
RP
Outer Nuclear Layer
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Inner Plexiform Layer
Ganglion Cell Layer
HC
BC
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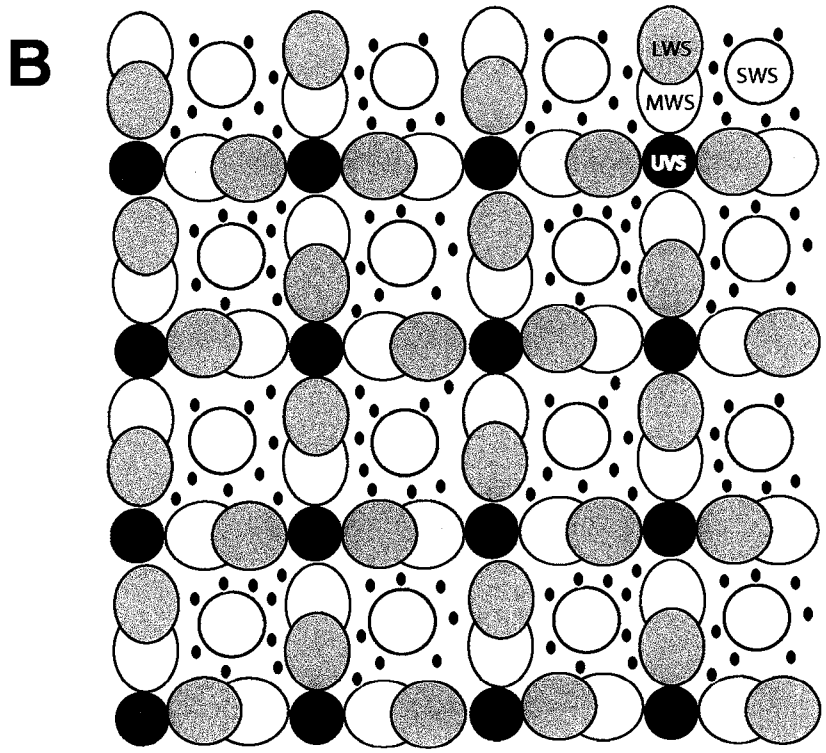
GC
PINCs
vitreal
To optic nerve...


B
LWS
MWS
SWS
UVS

visual pigment chromophore of most marine and terrestrial vertebrates is 11-cis retinal (vitamin A_1-based; "rhodopsin"), whereas many freshwater teleosts, amphibians and some reptiles can utilize 11-cis 3,4-dehydroretinal (vitamin A_2-based; "porphyropsin") (Loew, 1995). In many cases both chromophore types are present within the same photoreceptors, and the dynamic ratio of the two can change based upon environmental variables such as light regime or temperature, or during hormone manipulations (reviewed in Bridges, 1972; Levine & MacNichol, 1979; Beatty, 1984; Crescitelli, 1991; Loew, 1995). Salmonids are examples of species with just such a labile chromophore pair (Bayliss *et al.*, 1936; Wald, 1941; Kampa, 1953; Bridges, 1956; Munz, 1957; Munz & Beatty, 1965; Bridges & Yoshikami, 1970; Allen *et al.*, 1973; Allen, 1977; Allen & Munz, 1983; Alexander *et al.*, 1994; Hawryshyn & Harosi, 1994; Alexander *et al.*, 1998; Alexander *et al.*, 2001; Hawryshyn *et al.*, 2001; Hasegawa *et al.*, 2002).

The mosaic of salmonid photoreceptors in tangential view has long been recognized (Ryder, 1895; Eigenmann & Shafer, 1900; Franz, 1913; Lyall, 1957b, a; Ali, 1959) to form a regular lattice-like pattern (Fig. 2B). In the central retina, double cones form a square pattern, and the double cone partitioning membrane points towards the 'central single cone'. The remaining single cones are called the 'accessory corner cones'. The members of the double cones contain either long- or medium-wavelength-sensitive (LWS and MWS) opsins, as demonstrated by microspectrophotometry (MSP) (Bowmaker & Kunz, 1987; Kusmic *et al.*, 1993; Hawryshyn & Harosi, 1994; Kusmic & Gualtieri, 2000; Hawryshyn *et al.*, 2001). These MSP studies have also demonstrated that two populations of single

cones exist, containing either a UVS or a short-wavelength-sensitive (SWS) pigment. The single cones cannot be morphologically distinguished with certainty in MSP preparations. However, the putative identity of the SWS and UVS cones has been proposed to be the central single and accessory corner cone, respectively. This is because the number of cones containing UVS pigment (measured by MSP) and the fish's sensitivity to UV light (measured by electrophysiology or psychophysical paradigms) are both decreased in coordination with a decrease in the occurrence of the accessory corner cone (Bowmaker & Kunz, 1987; Browman & Hawryshyn, 1992; Beaudet *et al.*, 1993; Browman & Hawryshyn, 1994a; Browman & Hawryshyn, 1994b). For the sake of clarity only, in the remainder of this thesis I will use the term "UVS cone" when older literature used the term "accessory corner cone" or some similar morphological term for this cone in regard to salmonids. This assumption appears to be justified by my examination of gene expression in Chapter 2.

The photoreceptors, whose nuclei form the outer nuclear layer (ONL), pass information to the higher CNS centres through bipolar cells (Figure 2A, part of the inner nuclear layer, INL) and ganglion cells (i.e. vertical information flow). The ganglion cell axons form the optic nerve. The outputs of adjacent photoreceptors are processed (horizontal information flow) first by horizontal cells, which mediate feed-forward and feedback interactions within the photoreceptors and bipolar cells in the photoreceptor synapse. Amacrine cells also modulate horizontal information flow in the retina. A sixth class of cell in the neural retina is the glia, which serve as structural and physiological support for the neurons. Scleral to the

neural retina, the retinal pigmented epithelium (RPE) intercalates with photoreceptors. The melanin-rich RPE regenerates opsin chromophores, decreases light scatter, and plays a role in protecting the photoreceptors from damage.

Several asymmetries are known to exist across teleost retinae, presumably reflecting functional specialization based upon the different field-of-view that each different area of retina samples. Examples from salmonids will be reviewed briefly here. The embryonic fissure of salmonids leaves the optic nerve head and meets the periphery in the ventro-nasal quadrant. The density and size of photoreceptors is recognized to vary across the salmonid retina, presumably adapted to optimize photon capture and resolution, with smaller photoreceptors occurring at greater densities in the ventral hemisphere (Brett & Ali, 1958; Ali, 1959; Bathelt, 1970; Ahlbert, 1976; Beaudet *et al.*, 1997; Hawryshyn *et al.*, 2003a). These differences across the retina tend to be greater in larger fish (Ahlbert, 1976). The INL and ganglion cell layer are found to be thicker (Ali, 1959), and INL cell densities are higher (Ahlbert, 1976), in the ventral retina and this reflects higher photoreceptor densities. The arrangement of horizontal cells (nuclei are in the INL) seems to support this, as the spacing (*i.e.* density) of salmonid horizontal cells is closely matched to the spacing of either of the single cone populations (Wagner, 1972; Ahlbert, 1976). Retinal mosaics can also vary asymmetrically in large salmonids. The square mosaic of photoreceptors described above (Figure 2B) occurs in most of the central retina, but is arranged

as a "row mosaic", with double cones arranged in parallel rows in the dorsonasal retina of some large salmonids (Ahlbert, 1969; Beaudet *et al.*, 1997).

The UVS cones seem to disappear first from the ventral retina in several salmonids. The distribution of UVS cones in the central retina of rainbow trout (*O. mykiss*) are limited to the dorsotemporal retina in large (smolt) trout (Bathelt, 1970; Martens, 2000; Hawryshyn *et al.*, 2003a, see Chapter 2) and sexually mature trout (Beaudet *et al.*, 1997). This may be general to sexually mature Pacific salmonids (Beaudet *et al.*, 1997) and occurs in some other salmonid smolts such as *Salmo trutta fario* and *Salvelinus fontinalis* (Bathelt, 1970) and 'adult' *Salmo salar* (Ahlbert, 1976). Electrophysiology using functional mapping of the retina supports a dorsoventral asymmetry of UVS cones in *O. mykiss* smolts, in that sensitivity to UV light was found to be higher in the dorsal retina (Deutschlander *et al.*, 2001). A further asymmetry amongst salmonid photoreceptors appears to include their visual pigment content, as the ratio of A_1- to A_2-based chromophores can be greater in the dorsal retina of *O. mykiss* and *Salmo fario* (Muntz & Northmore, 1971; Muntz & Mouat, 1984), although work in *O. kisutch* does not support generalizing this to all salmonids at all ontogenetic stages (Munz & Beatty, 1965). Finally, the photomechanical movements of photoreceptors are less pronounced in the ventral hemisphere of young salmonids, although not in smolts (Ali, 1959).

In terms of asymmetries in growth and repair mechanisms, the dorsal retina differentiates earlier and grows faster, at least in early stages of *O. mykiss* (Mansour-Robaey & Pinganaud, 1990). Indeed, the entrance of the optic nerve

head is ventral of center in salmonids of all sizes (Ali, 1959, see also Figure 1), and this must reflect increased stretching and/or growth of the dorsal retina. Further evidence of asymmetric growth is provided by the salmonid embryonic fissure, which has an obvious curve in its path from the optic nerve head to the ventral retinal periphery; this character is associated with asymmetric retinal growth in teleosts (Easter, 1992). In *O. mykiss* retina that has been damaged experimentally using surgery, the proliferative response (described in section 1.2, below) is greatest in the dorsonasal quadrant of the retina, regardless of the location of damage (Faillace *et al.*, 2002). This seems to overlap with an asymmetry in UVS cone regeneration, because the ventral and nasal areas are where increases in UVS cone density appears to have occurred in *O. mykiss* smolts treated with TH (Hawryshyn *et al.*, 2003a).

There are also several differences along the central-to-peripheral axis of the salmonid retina. To a large degree, these differences reflect the fact that the retina continues to grow at the periphery (see section 1.2, below), adding new retina like the growth rings of a tree; thus, central retina is older than the peripheral retina. Thus, the rate of proliferation is highest in the peripheral salmonid retina (Julian *et al.*, 1998). This may be the reason that light damage has the least effect upon the retinal periphery of trout, although the amount of light reaching the retinal periphery could be limited by the iris (Allen *et al.*, 2001, see also chapter 4). The density of photoreceptors is largest and cell size smallest in the periphery (Ahlbert, 1969; Ahlbert, 1976; Beaudet *et al.*, 1997). Furthermore, the square cone mosaic is usually limited to the central retina, and

row mosaic often occurs towards the retinal periphery (Lyall, 1957a; Ali, 1959; Beaudet *et al.*, 1997; Martens, 2000). Finally, in large (smolt) salmonids, where UVS cones are absent from much of the mature central retina, UVS cones are still generated in retinal periphery, only to be immediately removed as the retina matures (Kunz *et al.*, 1994, See also Chapters 2 and 3).

1.2 Retinal Development

The neural retina, RPE and optic nerve develop as an out-pocketing of the CNS, and differentiation of cell types begins when this out-pocketing reaches the epidermis and induces lens formation (reviewed in Chuang & Raymond, 2002). The birth (i.e. terminal mitosis) of retinal cell types in embryonic development occurs in a stereotyped sequence in vertebrates. Ganglion cells and horizontal cells differentiate first, then cones and amacrine cells, and finally rods, bipolar cells and glia (reviewed in Stenkamp *et al.*, 1997). The birth of rods can occur substantially later than cones, particularly in fish. A cone-dominated retina can be established and functional in teleosts before the appearance of rods (Johns, 1977; Sandy & Blaxter, 1980; Johns & Fernald, 1981; Johns, 1982; Branchek, 1984; Branchek & Bremiller, 1984; Raymond, 1985). Generation of new rods typically occurs into adult stages of teleosts (Johns, 1977; Johns & Fernald, 1981; Fernald, 1990; Helvik *et al.*, 2001a; Helvik *et al.*, 2001b).

However, cell-type differentiation does not always mirror the sequence of their birth dates. Whereas cones are born before rods, some rods differentiate before cones, as measured by the sequence of opsin expression in goldfish and zebrafish (Raymond *et al.*, 1995; Stenkamp *et al.*, 1996; Stenkamp *et al.*, 1997;

Stenkamp *et al.*, 2001). The onset of embryonic cone opsin mRNA expression is also ordered temporally: LWS, MWS, SWS, and finally UVS (Stenkamp *et al.*, 1996).

The retina of fishes continues to grow throughout the life of the fish. This occurs through two mechanisms: 1) new retina is added in the retinal periphery, where it meets the iris; 2) the retina stretches like an expanding balloon. (Ali, 1964; Johns, 1977).

The retinal periphery, termed the circumferential germinal zone (CGZ, see Figure 1), adds new retina as the fish grows, similar to the growth rings of a tree. Thus, central retina is older than peripheral retina. The CGZ development recapitulates embryonic retinal development in most ways, facilitating studies of retinal development, including gene expression and the order of cell-type generation (Stenkamp *et al.*, 1997; Harris & Perron, 1998; Perron *et al.*, 1998; Otteson & Hitchcock, 2003). The retina of rainbow trout grows quickly compared to other teleost models, and this has been advantageous as the CGZ is expanded, increasing the space over which spatial arrangements are coordinated with temporal events (Olson *et al.*, 1999; Olson *et al.*, 2000).

During retinal stretching, inter-cone distance increases, whereas rod density stays approximately constant. Rod density is maintained through the addition of new rods into the spaces created by stretching (Fernald, 1990). The source of these new rods is proliferating cells in the central retina (Johns, 1977; Johns & Fernald, 1981; Johns, 1982). In post-embryonic fish, the proliferating cells had been thought to be limited to the ONL and are termed 'rod progenitors' (Fig 2A).

However, recent examination of the quickly-growing *O. mykiss* retina allowed identification of proliferating cells in the INL (PINCs, see Fig. 2A) for the first time in post-embryonic fish (Julian *et al.*, 1998). PINCs had previously been identified in larval teleosts (Sandy & Blaxter, 1980; Johns & Fernald, 1981; Johns, 1982), and are now known to occur in the adults of several species (Vihtelic & Hyde, 2000; Cid *et al.*, 2002; Otteson & Hitchcock, 2003), including other salmonids (Ahlbert, 1976; Candal *et al.*, 2001).

1.3 Retinal Repair

Following experimentally-induced damage, the retina of adult non-amniotic vertebrates can regenerate all retinal cell types. This is often cited as an important model of CNS development and regenerative potential.

Retinal regeneration is observed in both teleosts and urodele amphibians (salamanders and newts), although it appears that fundamentally different mechanisms are involved between these taxa. In amphibians the source of regenerating cells is a transdifferentiation of RPE cells (reviewed in Raymond & Hitchcock, 1997). This seems to mirror the regenerative potential of embryonic retina of many vertebrates, including amniotes (Raymond & Hitchcock, 1997).

In adult teleost fish however, the source of regenerated cells seems to not include transdifferentiated RPE (Raymond *et al.*, 1988b; Hitchcock *et al.*, 1992; Knight & Raymond, 1995), but is limited to proliferating cells that persist in fish retina throughout their ontogeny. Thus the regenerative potential of teleost retinae is linked to the continued growth of the retina through ontogeny: the proliferating cells that give rise only to rod progenitors during natural

development are multipotent and can produce all retinal cell types to repair retinal damage (see reviews by Raymond, 1991; Hitchcock & Raymond, 1992; Raymond & Hitchcock, 1997; Easter & Hitchcock, 2000; Raymond & Hitchcock, 2000; Stenkamp & Cameron, 2002; Otteson & Hitchcock, 2003).

Most of the early work on teleost retinal regeneration was performed on goldfish (Lombardo, 1968, 1972; Maier & Wolburg, 1979; Kastner & Wolburg, 1982; Raymond *et al.*, 1988b; Hitchcock *et al.*, 1992). However, an early study of rainbow trout retinal repair following damage induced by ouabain (Kurz-Isler & Wolburg, 1982) is most notable in the context of the current thesis topic. The authors observed undifferentiated retinal cells following damage, and speculated that they represented de-differentiated photoreceptors that had re-entered the cell cycle (Kurz-Isler & Wolburg, 1982). The demonstration that proliferating cells are prominent in established teleost retina (Johns, 1977; Sandy & Blaxter, 1980; Johns & Fernald, 1981; Johns, 1982) made this interpretation unlikely (Raymond *et al.*, 1988b). Indeed, proliferating cells are particularly abundant in the quickly growing salmonid retinae (Ahlbert, 1976; Julian *et al.*, 1998), and the source of regenerated neurons following retinal damage has been demonstrated to be the proliferating cells of the post-embryonic retina (Raymond *et al.*, 1988b). This conclusion can certainly be extended to rainbow trout (Faillace *et al.*, 2002, see also Chapter 3 & 4).

The regeneration of post-embryonic teleost retina has been demonstrated following various types of damage, including applying neurotoxic chemicals (Maier & Wolburg, 1979; Kurz-Isler & Wolburg, 1982; Raymond *et al.*, 1988b;

Negishi *et al.*, 1991; Braisted & Raymond, 1993; Braisted *et al.*, 1994; Stenkamp *et al.*, 2001), surgery (Lombardo, 1968, 1972; Hitchcock *et al.*, 1992; Hitchcock & Cirenza, 1994; Cameron & Easter, 1995), laser (Braisted *et al.*, 1994; Wu *et al.*, 2001), and light (on albino fish, Allen & Hallows, 1997; Vihtelic & Hyde, 2000, see also Chapter 4).

The mechanisms of retinal regeneration, including gene expression regulating development, typically recapitulate embryonic retinal development (reviewed in Raymond & Hitchcock, 1997; Easter & Hitchcock, 2000; Otteson & Hitchcock, 2003). The source of regenerated retinal cells was originally postulated to be the CGZ (Lombardo, 1968, 1972) and, as described above, the RPE was also considered a possibility. For at least the past decade, however, there appears to be consensus that the source of regenerated neurons is limited to the proliferating cells in the central teleost retina (Raymond *et al.*, 1988b; Hitchcock *et al.*, 1992; Knight & Raymond, 1995). Rod progenitor cells were originally considered to be the most likely source, because 1) they proliferate robustly following damage (Raymond *et al.*, 1988b; Negishi *et al.*, 1991; Braisted & Raymond, 1993; Braisted *et al.*, 1994), and 2) damage to the ONL was noted to be an obligatory component to induce a regenerative response (Negishi *et al.*, 1991; Braisted & Raymond, 1992). However, the identification of PINCs in the retina of post-embryonic rainbow trout retina (Julian *et al.*, 1998) has led to a re-examination of this hypothesis. PINCs are now thought to be the source of rod progenitors in teleosts, and their occurrence in established post-embryonic retina make them strong candidates for the source of reappearing cells (Otteson *et al.*,

2001; Wu *et al.*, 2001; Faillace *et al.*, 2002; Otteson & Hitchcock, 2003). However, it is difficult to experimentally exclude rod progenitors as a possible additional source. Finally, up-regulation of glial proliferation in damaged teleost retinae (Braisted *et al.*, 1994; Wu *et al.*, 2001), combined with the reported potential of glia to de-differentiate to stem cells in chicken retina (Fischer & Reh, 2001, 2003), have led to recent speculation that glial de-differentiation may be relevant in teleost retinal regeneration (Wu *et al.*, 2001; Faillace *et al.*, 2002; Otteson & Hitchcock, 2003).

Regardless of the exact cellular source of the reappearing cells during retinal regeneration, it seems that these cells meet all popular definitions of 'stems cells' (reviewed in Raymond & Hitchcock, 1997; Otteson & Hitchcock, 2003). There is no evidence that these cells are omnipotent 'embryonic stem cells'. However, the term 'stem cells' is often used for proliferating cells that are able to produce all cell types in particular tissues that are continually growing or self-renewing. Thus it is clear that these cells must, at least, meet definitions of 'retinal stem cell' because they: 1) persist within differentiated tissue in small numbers; 2) are self-renewing; 3) are multipotent; 4) increase proliferation in response to damage.

Spherical cells in the INL of goldfish have been proposed to be retinal stem cells (Otteson *et al.*, 2001; Otteson & Hitchcock, 2003). These cells express Pax6, similar to putative stem cells in the CGZ, and they incorporate the thymidine analogue 5-Bromo-2'-deoxyuridine (BrdU), indicating that they were proliferating. However, Faillace et al (2002) have argued that the paucity of such cells, combined with the large number of other proliferating cells that the

spherical cells would need to give rise to, make this interpretation unlikely for rainbow trout. Thus, it remains a probable hypothesis that PINCs are retinal stem cells, at least in salmonid fish.

The rapid growth rate of salmonid eyes, compared to other teleost models, has been noted by several authors (Olson *et al.*, 1999; Easter & Hitchcock, 2000; Olson *et al.*, 2000). One may argue that if continued growth is a primary feature that makes teleost retinae useful as models for understanding CNS development, then faster growth makes the salmonid especially attractive as an experimental model (e.g. see Olson *et al.*, 1999). This rapid growth could be a reason that the salmonid eye appears to have such exceptional regenerative capacity (to be identified in Chapters 2 & 3).

1.4 Thyroid hormone in retinal development

It has long been known that TH regulates both vertebrate neurodevelopment and metamorphosis of non-amniotic vertebrates. Mechanisms of TH action are typically associated with nuclear receptors mediating gene transcription as ligand-dependent transcription factors, although some apparently non-receptor mediated effects have been noted (reviewed in Forrest *et al.*, 1991; Shi *et al.*, 1998; Sachs *et al.*, 2000; Shi & Ishizuya-Oka, 2000; Forrest *et al.*, 2002). Thyroid hormone receptors (THR) have several isoforms of two gene classes, THRα and THRβ, which can act as homodimers or heterodimerize with retinoic acid receptors (Puzianowska-Kuznicka *et al.*, 1997). TH mediates both positive and

negative gene regulation, i.e. it can act to both up- and down-regulate gene expression (Forrest *et al.*, 1991; Sachs *et al.*, 2000; Forrest *et al.*, 2002).

The primary THR ligand, 3,5,3'-triiodothyronine (T_3), is about ten-times more effective at altering gene expression than its prohormone, L-thyroxine (T_4) (Galton, 1992). Deiodinases are a class of enzymes that regulate TH, activating T_4 to T_3 and deactivating both T_4 and T_3 for excretion (reviewed in Eales, 1995). The organ-specific expression of deiodinases allows the regulation of TH levels in an organ-specific manner. In teleosts, the liver has typically been found to have the highest levels of deiodinase activity (Cyr *et al.*, 1998; Adams *et al.*, 2000). Thus, it was surprising to find that the retina had deiodinase activity levels that are comparable to the liver in both *O. mykiss* and *O. nerka* (Plate *et al.*, 2002). In both salmonids examined, impressively high levels of inner ring deiodinase (IRD; TH de-activating) pathways were found in the retina, and these levels changed greatly during treatment with an analogue of gonadotropin releasing hormone (GnRH, mimicking sexual maturity) or with TH treatment (Plate *et al.*, 2002). At least in the brain and liver, deiodinase levels can change during parr-smolt transformation in salmonids (Morin *et al.*, 1993; Specker *et al.*, 2000).

The presence of deiodinases in the retina, and their regulation during development, appears to be widespread amongst vertebrates. T_4 outer ring deiodinase (ORD; TH activating) and T_4IRD activity have long been known in the rat neural retina (Ientile *et al.*, 1984). Analogous to the findings in salmonids (Plate *et al.*, 2002), levels of deiodinase activity appear to change during rat

postnatal retinal development. The peak of deiodinase's activities are temporally coordinated with thyroxine-dependent proliferation and differentiation (Ientile *et al.*, 1984), and treatment with thyroxine enhances differentiation and synaptogenesis (Macaione *et al.*, 1984). Serial analysis of gene expression shows that deiodinase II mRNA increases with age in the ONL of mice (Blackshaw *et al.*, 2001). Furthermore, TH has been found to have a role in rodent retinal development. The application of the deiodinase inhibitor propylthiouracil resulted in decreased proliferation of retinal ganglion cells (Navegantes, 1996). Recent examinations of THRβ-2 have shown that it is expressed in mouse ONL (Ng *et al.*, 2001). THRβ-2 knockout mice have shown a lack of M-cones (medium-wavelength sensitive) generated, and a concomitant increase in the number of UVS cones generated (Ng *et al.*, 2001).

The expression of a type III deiodinase (St Germain *et al.*, 1994) has been identified in the ventral retina during *Xenopus* metamorphosis (Marsh-Armstrong *et al.*, 1999). The levels of this enzyme peak at metamorphosis, and expression is localized to the dorsal retina. This asymmetrical distribution appears to explain the dorsoventral difference in proliferation and differentiation of retinal cells that begins at metamorphosis and is controlled by TH (Beach & Jacobson, 1979; Marsh-Armstrong *et al.*, 1999). It is noteworthy that these expression patterns and peaks of retinal deiodinases have been identified at times of substantive retinal development.

As reviewed above, the teleost retina continues to grow throughout life. Thus the requirement for organ-specific regulation of TH, and its effect in proliferation

and/or differentiation, may be expected to be prominent. The high levels of deiodinases found in retina of both salmonids examined may be related to a particular need to regulate the effects of TH during continued retinal growth and the variable levels of circulating TH. A T_4ORD, and minimal T_3ORD levels have also been identified in the retina of another teleost, killifish (*Fundulus heteroclitus*) (Orozco *et al.*, 2000), although information regarding their developmental profiles are not yet available. Similar to both salmonid species described above (Plate *et al.*, 2002), the levels of T_4ORD were low in the retina relative to the levels found in the liver. Evidence for a T_4ORD has been found in the retinal pigment epithelia layer of coho salmon (*O. kisutch*) (Alexander, 1998), and thus the presence of retinal deiodinases may be general to salmonids or teleosts.

The effects of thyroxine treatment on salmonid retina have long been a topic of interest in the context of understanding the tuning of the visual system to the photic environment. TH drives metamorphic changes in salmonids associated with their migration to marine waters (reviewed in Hoar, 1988). Shifts in visual pigments can be induced by TH (Bridges, 1972; Beatty, 1984) and blocked by treatment with the deiodinase inhibitor methimazole (Alexander *et al.*, 1998). TH has recently been shown to affect the visual pigments in cultured *O. kisutch* RPE (Alexander *et al.*, 2001), demonstrating that changes in gene expression relating to salmonid visual tasks occur in the retina. Of particular interest for this thesis, is the role that TH has in the ontogeny of UVS cone photoreceptors. TH has been shown to induce the loss of UVS cones and UV sensitivity in small (parr) rainbow

trout (Browman & Hawryshyn, 1992, 1994b; Deutschlander *et al.*, 2001, See Chapter 3) and their reappearance in larger (smolt) trout (Browman & Hawryshyn, 1994b; Hawryshyn *et al.*, 2003a). Preliminary data support generalizing these statements to the basal species Atlantic salmon (*Salmo salar*), and thus to all members of the genus *Oncorhynchus* (Browman *et al.*, 2001).

Many of the dorsoventral asymmetries in salmonid retinae described above (section 1.1) may be thought to be established and/or maintained by an endogenous gradient of TH across the retina. This suggestion comes from work in *Xenopus*, where a dorsoventral asymmetry exists in deiodinase enzymes that deactivate TH (Marsh-Armstrong *et al.*, 1999). This deiodinase controls a dorsoventral asymmetry of proliferation in *Xenopus* retina through metabolism of TH (Beach & Jacobson, 1979; Marsh-Armstrong *et al.*, 1999). Furthermore, the ratio of A_1- and A_2-based chromophores has a dorsoventral asymmetry in various amphibians (Reuter *et al.*, 1971; Bridges, 1975; Semple-Rowland & Goldstein, 1981; Firsov *et al.*, 1994) that can be manipulated by TH treatment (Bridges, 1972). These data have several parallels to asymmetries in salmonid retinae (Reviewed above, Section 1.1): Dorsoventral asymmetries exist in salmonid retinal proliferation (Mansour-Robaey & Pinganaud, 1990) and visual pigments (Muntz & Northmore, 1971). The latter is well-known to be controlled in salmonids by retinal TH levels (Bridges, 1972; Beatty, 1984; Alexander *et al.*, 2001). Furthermore, TH has been shown to affect retinal stretching/growth (i.e photoreceptor densities) in *O. mykiss* in a manner that is asymmetrical across the retina (Hawryshyn *et al.*, 2003a). Finally, the ontogeny of UVS cones can be

manipulated by TH (Browman & Hawryshyn, 1992, 1994b; Deutschlander *et al.*, 2001; Hawryshyn *et al.*, 2003aChapter 3) in a manner that apparently mimics their dorsoventral distribution in large salmonids. Although deiodinase enzymes have been observed to have surprisingly high activity levels in the retina of *O. mykiss* and *O. nerka* (Plate *et al.*, 2002), information regarding spatial distributions is not available.

Chapter 2:

UVS Cones: Identity and Ontogeny[1]

2.1 ABSTRACT

I have developed electroretinogram and *in situ* hybridization protocols to examine the ontogeny of photoreceptors in the retina of a land-locked salmonid, the rainbow trout (*Oncorhynchus mykiss*). I cloned cDNA fragments corresponding to the rod opsin and each of the four cone opsin gene families, which I utilized to produce riboprobes. I established the specificity of the *in situ* hybridization protocol by examining subcellular signal localization and through double labelling experiments. I confirmed the assumption that the accessory corner cones in the square mosaic are the ultraviolet wavelength-sensitive (UVS) cone photoreceptor (i.e. they expressed an SWS1 opsin) and observed UVS cones throughout the retina of small trout. Larger fish had a decrease in sensitivity to short wavelength light stimuli, and the distribution of UVS cones in the mature retina was limited to the dorsal-temporal quadrant. These larger fish also possessed differentiated UVS cones in the circumferential germinal zone (CGZ), including within areas peripheral to mature retina lacking UVS cones. These data are consistent with the loss of putative UVS cones from the CGZ of a migratory salmonid of another genus, and thus the disappearance of UVS cones appears to be general to the Family Salmonidae regardless of life history strategy. The generation, differentiation and subsequent loss of UVS cones in the

[1] The findings presented in this chapter are published in the Journal of Comparative Neurology (2003) and involved a collaboration with Stephen G. Dann, Jon Vidar Helvik, Clarissa Bradley, Heather D. Moyer, and Craig W. Hawryshyn.

smolt CGZ is a dramatic example of the supposition that the mechanisms of CGZ development recapitulate the retinal embryogenesis of that species.

2.2 Introduction

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

Salmonids, including salmon and trout, comprise a large group of closely related species with a variety of habitats, feeding strategies, and life history strategies. The variety of habitats individuals experience, often including lake, stream and marine environments, create different challenges for the growing salmonid visual system. Changes in visual function associated with changing habitat have been reported in several teleost species (e.g. Hawryshyn *et al.*, 1989; Alexander *et al.*, 1994; Shand *et al.*, 1999; Helvik *et al.*, 2001a; Shand *et al.*, 2002), representing an opportunity to observe substantial neural development

within an established CNS structure. Thus a comparative approach could be valuable to understanding control of developmental events and how these control points have evolved to optimize visual systems to various habitats. Of particular interest in this regard are species such as *Oncorhynchus mykiss* which include populations that remain land locked (rainbow trout) and others that migrate to marine environments (steelhead salmon) (Parkyn & Hawryshyn, 2000).

Salmonids have various other features that facilitate a study of retinal development, such as light-induced degeneration of the rod outer segments (Allen & Hallows, 1997; Allen *et al.*, 2001, examined in Chapter 4), and disappearance of accessory corner cones (ACC) from their square cone mosaic. Because the loss of this cone type coincides with a decrease in visual sensitivity to ultraviolet (UV) light[2], the ACC have been putatively identified as ultraviolet wavelength-sensitive[3] (UVS) cones (Bowmaker & Kunz, 1987; Kunz, 1987). This is consistent with the morphology of salmonid UVS cones detected using microspectrophotometry (Bowmaker & Kunz, 1987; Hawryshyn & Harosi, 1994; Hawryshyn *et al.*, 2001) and with the position of UVS cones in the mosaics of goldfish, zebrafish, and killifish as identified by *in situ* hybridization (Raymond *et al.*, 1993; Hisatomi *et al.*, 1996; Hisatomi *et al.*, 1997). Evidence has been provided through electron microscopy (EM) that the disappearance of UVS cones in Atlantic salmon is mediated by apoptosis (Kunz *et al.*, 1994). This has been suggested as a useful model of retinal apoptosis (Kunz *et al.*, 1994).

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[2] Ultraviolet (UV) light is defined here as radiation in the wavelength range of 300-400 nm.

[3] In teleost fishes ultraviolet-sensitive (UVS) cones express opsins of the SWS1 gene family. Short wavelength-sensitive (SWS) cones (also referred to as "blue-sensitive cones") express opsins from the SWS2 gene family. See Hunt *et al.* (2001) for further discussion of nomenclature.

Interestingly, UVS cones reappear into the retina of at least some salmonids (Beaudet *et al.*, 1997; Novales Flamarique, 2000), which represents the only known example of cone regeneration during natural development (see also Chapter 3).

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

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

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

2.3 Methods

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

To sample the retinae, fish were deeply anaesthetized in 300 mg/liter MS-222 (tricaine methanesulphonate, Crescent Research Chemicals, USA) until euthanized. Retinae were dissected during the last half of the light cycle in an attempt to maximize the amount of cone opsin mRNA present in photoreceptors. Cone opsin mRNA has been shown to be highest before dark onset in various vertebrates (Pierce *et al.*, 1993; Pierce, 1999; von Schantz *et al.*, 1999), and at least the circadian rhythm of rod opsin mRNA content (highest immediately before light onset) has been shown to match this pattern in teleosts (Korenbrot & Fernald, 1989). Retinae used to generate some samples of cDNA, and partial clones of long wavelength-sensitive (LWS) and short wavelength-sensitive (SWS) opsin cDNAs (see below), were isolated from rainbow trout obtained from Lune Fish Farm, Oster Island, Norway. Care of experimental fish and all procedures were in accordance with and approved by the University of Victoria Animal Care Committee under the auspices of the Canadian Council for Animal Care.

2.3.1 Cloning of partial opsin cDNAs and riboprobe production

Partial clones of SWS and LWS opsins were obtained by amplifying trout mRNA using degenerate primers designed to conserved regions of retinal opsins, and thus can amplify each of the cone and rod opsins (Forward 5'-AAGAAGYTCMGTCMACCTCTYAAYT; Reverse 5'-GTTCATGAAGACRTAGATDAYAGGGTTRTA; Y=T or C; M=A or C; R=G or A; D=G, A or T (Helvik *et al.*, 2001b). Retinae, including retinal pigment epithelium, were isolated from three light-adapted rainbow trout parr (mass range 10 to 35.5g, average 21g; standard length range 96 to 140mm, average 118.8 mm) obtained from Lune Fish Farm, Norway. Total RNA was isolated with TRIZOL (Gibco-BRL). mRNA was isolated using oligotex mRNA miniprep kit (Qiagen) and used to produce cDNA with the Marathon cDNA Amplification kit (Clontech). The above PCR primers were used in a PCR reaction with this cDNA (Taq from Clontech). PCR conditions were 5 minutes 94°C hotstart; 35 cycles of 45 seconds 94 °C; 50 seconds 46°C; 50 seconds 72°C; finishing with 10 minutes 72°C) and the resultant band eluted from a 1% agarose gel. This product was ligated to the plasmid pGEM-T Easy and transformed into *E. coli*. Clones containing insert, as determined by blue/white screening, were selected randomly for sequencing.

Partial clones for medium wavelength-sensitive (MWS), UVS, and rod opsins were produced with a nested reverse transcription- (RT-) PCR strategy where the reverse transcription and first round of amplification cycles are performed using the same primer set, in a one-step low stringency reaction. This

reaction product was then used as template for a second round of higher stringency PCR using an additional primer set. This strategy used primers designed to amplify conserved regions of the particular target opsin, as determined by sequence alignments of these opsins from various vertebrates. The latter included partial cDNA sequence information of chum salmon (*O. keta*) generously provided by Dr. O. Hisatomi, which had been used to predict and report amino acid sequence (Hisatomi *et al.*, 1994). One microgram of total RNA, generated from the retinae of 4 rainbow trout parr (body mass range 7-15g, average 10.2g; standard length range 65-100mm, average 97mm) obtained from Fraser Valley Hatchery, was used as template for these RT-PCR reactions. RNA was isolated using standard guanidine isothiocyanate protocols (Chomczynski & Sacchi, 1987). PCR conditions for each clone were as follows: first round consisted of a 42 °C RT reaction followed by 94 °C for 5 minutes, and 30 cycles of 45 seconds 94 °C; 45 seconds 55 °C; 1 minute 72 °C. The second round of PCR used 1 µl of the first round reaction as template under the following conditions: 94 °C for 5 minutes, and 30 cycles of 45 seconds 94 °C; 45 seconds 60 °C; 1 minute 72 °C, and finishing with a 5 minute hold at 72 °C. First round primers consisted of the following: UVS opsin partial clone forward primer 5'-GGGCTTTGTATTTTTCGTGGG-3', reverse primer 5'-TAGTCTTTGTTCTCGCTTGTTG-3'; MWS opsin partial clone forward primer 5'-AATGGCACTGAAGGAAAGAACT-3', reverse primer 5'-GCGAAGAAGGCAGGGATAGC-3'; rod opsin partial clone forward primer 5'-CCAGTTTTCTACGTCCCTATGTC-3', reverse primer 5'-

CGCGCCCTCTTCCTCCTCGA -3'. Second round, nested primers, consisted of the following: UVS opsin partial clone forward primer 5'-GACAGCCACTCAACTACATCCT-3', reverse primer 5'-GCCGTAACACAGAATGAAGGAGCAC-3'; MWS opsin partial clone forward primer 5'-ACAGGACAGGGATAGTTAGGAG-3', reverse primer CACCAGGAAGCCCATAACCAT-3'; rod opsin partial clone forward primer 5'-GGAGCCCATACGAATACCCCC-3', reverse primer 5'-CATAGGGCATCCAGCACACCA-3'. Clones containing UVS opsin fragments were ligated to pGEM-T, while rod and MWS opsin fragments were ligated to pGEM-T Easy. Clone identity was confirmed by sequencing of positive clones and comparing the results to known sequences of each opsin gene family in GenBank (NCBI; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide).

Both sense (negative control) and anti-sense riboprobe were produced from each of these clones. Riboprobe was generated using standard protocols (Barthel & Raymond, 2000). Briefly, plasmids were linearized with an appropriate restriction enzyme and riboprobes were generated using T7 RNA polymerase (Gibco-BRL) or SP6 RNA polymerase (Amersham-Pharmacia) as appropriate. Reaction mixtures for these enzymatic reactions contained either dioxigenin- (DIG-) or fluorescein- (FL-) labelled nucleotide triphosphates (Roche Biochemicals). Successful probe production was assessed by gel electrophoresis with ethidium bromide staining and compared to a standard riboprobe included on the gel. In some cases, riboprobes were also analyzed by dot blots of a

dilution series of riboprobes, with standards, which were performed as per manufacturer's protocols.

2.3.2 Wholemount in situ hybridization

Wholemount *in situ* hybridization procedures were modified from established protocols (Henrique *et al.*, 1995). Fish were maintained in dark for approximately one hour before dissection, and eyes were enucleated under deep red light. Neural retina, with a portion of the optic nerve intact, was separated from other ocular tissues. These retinae were fixed in 4% formaldehyde buffered in phosphate buffered saline pH 7.4 (PBS) overnight at 4°C. Retinae were washed several times in PBS and dehydrated through a graded series into methanol and stored at -20°C.

Retinae were rehydrated, through a graded series, into PBS containing 0.1 % Tween-20 (PTW) and dissected into pieces of appropriate size. For labelling with MWS or LWS opsin riboprobe, the tissue was treated at room temperature for 30 minutes with 10 μg/ml proteinase K (Sigma). For labelling with SWS or UVS opsin riboprobe, retinae were treated with 200 μg/ml proteinase K for 45 minutes. Tissue was then rinsed in PTW and secondarily fixed in 4% formaldehyde and 0.1% glutaraldeheyde in PBS, and prehybridized at 60°C in hybridization mix (Henrique *et al.*, 1995). Excess DIG-labelled riboprobe (approximately 1 μg/ml) was hybridized to tissue overnight at 60°C in hybridization mix. Post-hybridization washes, at 60°C, consisted of two 30 minute washes in hybridization mix and a 10 minute wash in 1:1 hybridization mix: maleic acid buffer (MAB; 100mM Maleic Acid, 150mM NaCl, pH 7.5) with 0.1%

Tween-20 (MABT). DIG-labelled probe was detected with monoclonal sheep anti-DIG conjugated to alkaline phosphatase (anti-DIG-AP) and visualized with BCIP/NBT (5'-5-bromo-4-chloro-3-indolyl-phosphate 4-toluidine salt (Gibco-BRL), plus nitroblue tetrazolium (Gibco-BRL), with levamisole (Sigma). Alternatively, colour development utilized FastRed (Roche Biochemicals) in 0.1 M Tris-HCl pH 8.2. Results were visualized and documented using a 12-bit colour Microimager II digital camera (QImaging, Burnaby, BC) mounted on a Zeiss Axioskop 2 with Atto Arc 2 HBO 100 Mercury Arc Lamp. Differential interference contrast (DIC), brightfield or multiple fluorescent images were merged and equalized using Northern Eclipse 5.0 (Empix Imaging, Inc.) and Adobe Photoshop 4.0 (Adobe Systems, Inc.).

2.3.3 In situ hybridization on sectioned material

Methods for *in situ* hybridization on sectioned material were modified from established protocols (Barthel & Raymond, 2000; Helvik *et al.*, 2001a). Light adapted-eyes, with lenses removed, were fixed in 4% paraformaldehyde in PBS overnight at 4°C and rinsed with PBS. Retinae, including retinal pigment epithelium, were dissected away from other ocular tissues, and cut into quadrants. Quadrants were kept overnight in 25% TissueTech OCT compound with 25% Sucrose in PBS. Quadrants were frozen in this solution to acquire either radial (along the long-axis of the photoreceptors) or tangential (orthoganol to the latter, through the photoreceptor mosaic) sections. A Microm HM500 (Zeiss) or CM1850 (Leica) cryostat was used to cut 10 μm sections onto SuperFrost *Plus* slides (Fisher). Tissue was rehydrated and treated at 37°C with

10 µg/ml proteinase K for various durations: 7 minutes for rod, 10 minutes for MWS or LWS opsin riboprobes, 10 minutes for SWS and 13 minutes for UVS opsin riboprobe. Tissue was secondarily fixed in 4% parafomaldehyde in PBS, acetylated, and dehydrated through a graded series of ethanol. Excess DIG- or FL-labelled riboprobe was applied in hybridization mix (Barthel & Raymond, 2000) overnight at 50°C. Post-hybridization washes were performed at 60°C in 50% formamide in 2X SSC. Probes were detected with anti-DIG-AP or monoclonal sheep anti-fluorescein conjugated to alkaline phosphatase (anti-FL-AP; Roche Biochemicals), as appropriate, and visualized with BCIP/NBT or FastRed.

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

In some cases, sections were counterstained with 200 µg/µl Hoechst 33258 (Sigma) in PBS. This stains nucleic acids and weakly stains actin

filaments allowing visualization of the nuclei, and general cell structure, using a standard DAPI filter set. Slides were mounted in 1:1 PBS:glycerol, with 6.25 µg/ml n-propylgallate for the sections developed with FastRed.

2.3.4 Immunohistochemistry

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

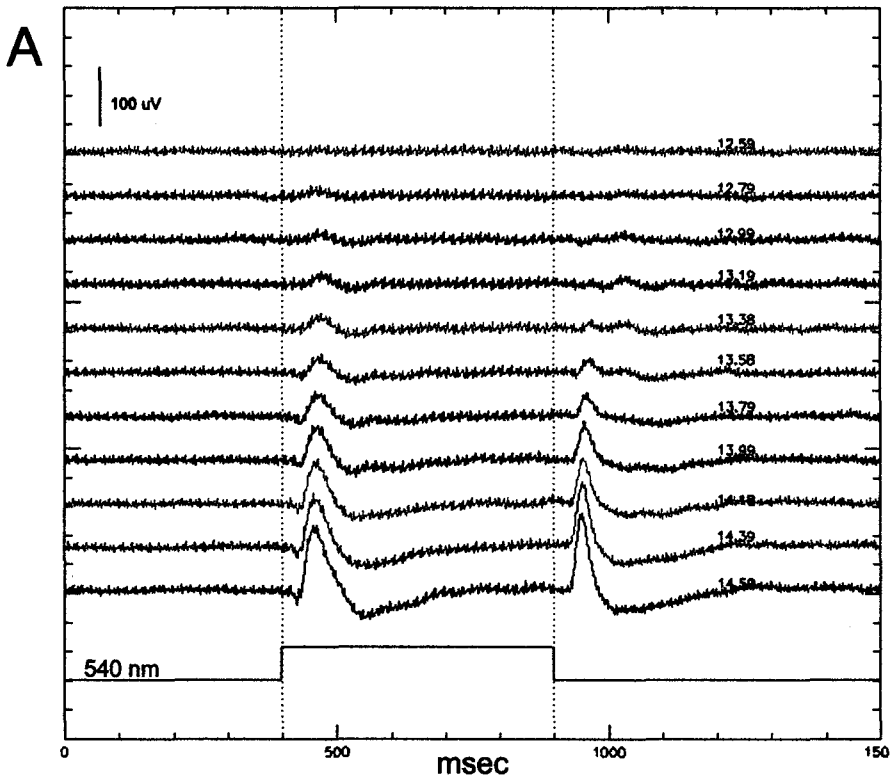
2.3.5 Electroretinograms

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

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

To determine sensitivity I measured the b-wave amplitude (Fig. 3A). Responses were amplified, filtered (3-300 Hz, Grass Instruments P-5 Preamplifier), displayed on an oscilloscope, and acquired by a data acquisition board (16-bit A/D, National Instruments). Response *versus* intensity functions were fit with a Naka-Rushton equation (Naka & Rushton, 1966a, b) using a non-linear least-squares fit. This function was then used to interpolate the threshold of light intensity required to generate a criterion response (Fig. 3B). The criterion response was determined for each fish by choosing a value above the baseline

Figure 3. Electroretinogram responses to increasing intensities of light. **A:** Responses to increasing intensities of stimulus are presented towards the bottom of the figure. Stimulus intensity, in log photons•cm^{-2}•s^{-1}•nm^{-1}, is presented near the end of each trace. The b-wave amplitude (in μV) as measured from the peak of the a-wave (first negative peak) to the peak of the b-wave (first positive peak), is plotted versus stimulus intensity (**B**) to determine the threshold stimulus required to elicit a criterion response. In this example a stimulus intensity of 13.84 log photons•cm^{-2}•s^{-1}•nm^{-1} is required to elicit the criterion of 60 μV.


A
100 uV
12.59
12.79
12.99
13.19
13.38
13.58
13.79
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14.18
14.39
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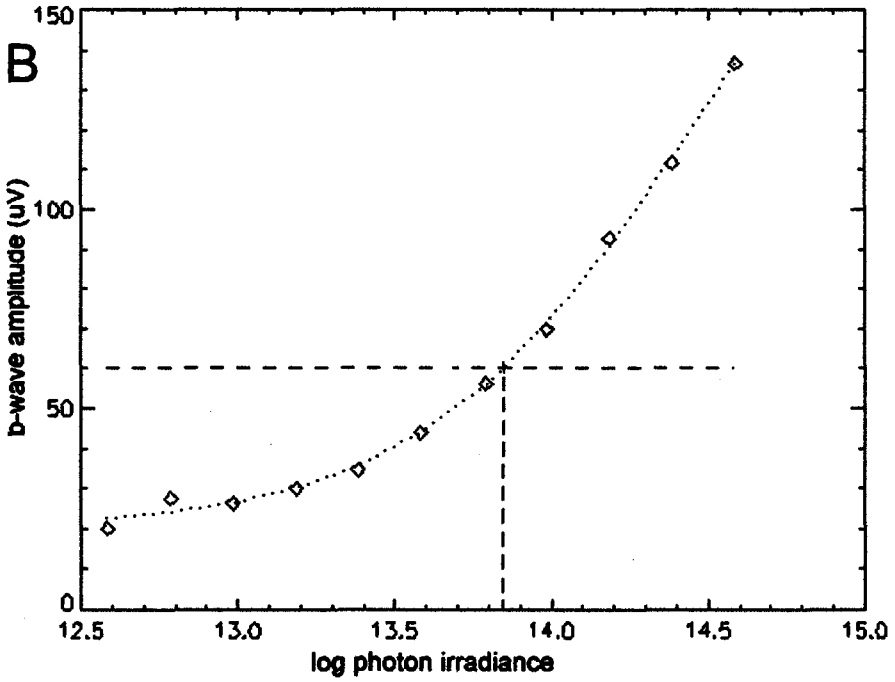
540 nm
0
500
1000
150
msec


B
150
100
50
0
b-wave amplitude (uV)
12.5
13.0
13.5
14.0
14.5
15.0
log photon irradiance

recording of responses to low-light intensities, within the linear portion of the response *versus* intensity curves. Sensitivity was the inverse of threshold intensity within various wavelengths.

Spectral sensitivity was normalized to 420 nm, averaged, and standard error was calculated. As described previously (Coughlin & Hawryshyn, 1994a; Parkyn & Hawryshyn, 2000; Deutschlander *et al.*, 2001), spectral sensitivity functions were fit using a linear-additive model of cone mechanisms. The bright yellow background allows the assumption that responses to UV stimuli do not contain contributions from the β-band of MWS and LWS cone mechanisms. The best-fit weighting (i.e. contributions) of the UVS (K_{UVS}) and SWS (K_{SWS}) cone mechanisms was then determined using a non-linear least-squares fit to the spectral sensitivity data. Normalizing the data to the 420nm response allows a comparison of the UVS cone mechanism's sensitivity relative to that of the SWS cone mechanism.

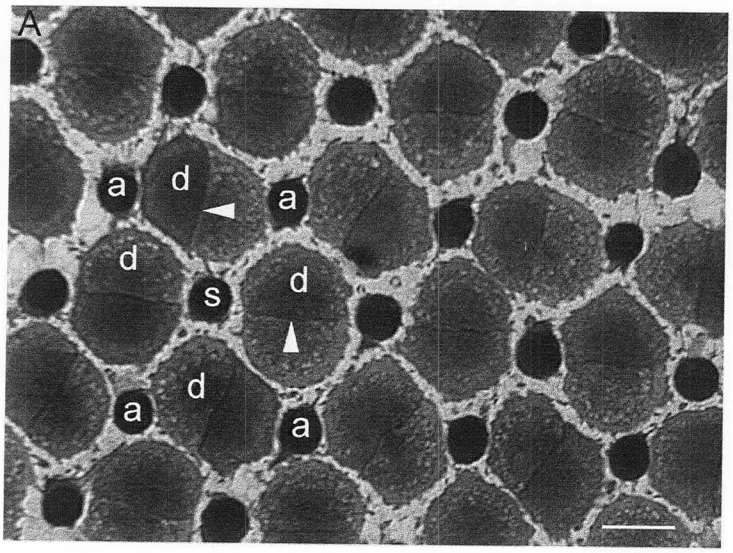
2.4 Results

Sequencing data revealed partial cDNAs with high sequence identity to each of the teleost opsin gene families as determined by BLAST search (NCBI). The five sequences utilized to produce riboprobes against UVS, SWS, MWS, LWS and rod opsins, had lengths of 596, 734, 803, 563 and 758 base pairs and are reported in GenBank (gene family/accession numbers SWS1/AF425074, SWS2/AF425075, RH2/AF425076, LWS/AF425073, and RH1/F425072, respectively). Each of these sequences is predicted to produce an amino acid sequence that would span several transmembrane domains. Full length

sequences for these cDNAs have been acquired from *O. mykiss* (data not shown; reported in GenBank under the above accession numbers), and other salmonid species (Dann *et al.*, 2004a) that further confirm their assignment to the five opsin gene families through comparisons of sequence identity.

These partial cDNA clones were used to produce riboprobes that labelled cells of expected morphology. Wholemount preparations and tangential sections reveal that the double cone partitioning membranes point towards the cones containing SWS opsin mRNA, and UVS opsin mRNA is within single cones at the corners of the square mosaic (Fig. 4). The latter, the accessory corner cone, disappears coincident with the fish's loss of sensitivity to UV light (Kunz, 1987; Browman & Hawryshyn, 1992; Beaudet *et al.*, 1993; Browman & Hawryshyn, 1994b; Novales Flamarique, 2000), and has been shown to contain a UVS pigment using microspectrophotometry (Bowmaker & Kunz, 1987; Kusmic *et al.*, 1993; Hawryshyn & Harosi, 1994; Kusmic & Gualtieri, 2000; Hawryshyn *et al.*, 2001). Thus, the assumption that accessory corner cones are the UVS cones (Bowmaker & Kunz, 1987; Browman & Hawryshyn, 1992, 1994b; Kunz *et al.*, 1994; Beaudet *et al.*, 1997; Martens, 2000; Novales Flamarique, 2000; Hawryshyn *et al.*, 2003a; Reckel *et al.*, 2003), which is central to the interpretation of data in these papers, has been confirmed. This is consistent with other species examined to date (Raymond *et al.*, 1993; Hisatomi *et al.*, 1996; Hisatomi *et al.*, 1997), and appears to be a common feature of teleosts with square mosaics. Wholemount *in situ* hybridizations revealed that MWS and LWS opsin mRNA was localized to double cones (data not shown). Using radial

Figure 4. Wholemount preparations and tangential sections reveal the specificity of ultraviolet wavelength-sensitive (UVS) and short wavelength-sensitive (SWS) opsin *in situ* hybridization. **A:** Tangential plastic section of retina, showing the square mosaic. Double cones (d) are divided by partitioning membranes (arrowheads) that point towards the central single cone (s), the putative SWS cone. The single cones in the corners of the square mosaic, the accessory corner cones (a) have been putatively identified as UVS cones. This is because they have been shown to contain a UVS pigment using microspectrophotometry (Hawryshyn et al 2001; Hawryshyn & Harosi 1994) and disappear coordinated with a decrease in sensitivity to UV light (see text). The position of the partitioning membrane allows identification of labelled cells. **B:** *In situ* hybridization labelling of SWS opsin mRNA on a tangential section, shows cells labelled with dark precipitate are in the center of the mosaic, consistent with (s). **C:** Fluorescent *in situ* hybridization labelling UVS opsin mRNA on wholemount retina, in an approximately tangential plane of focus. UVS opsin mRNA appears in the accessory corner cone consistent with (a). Scale bars = 10 μm.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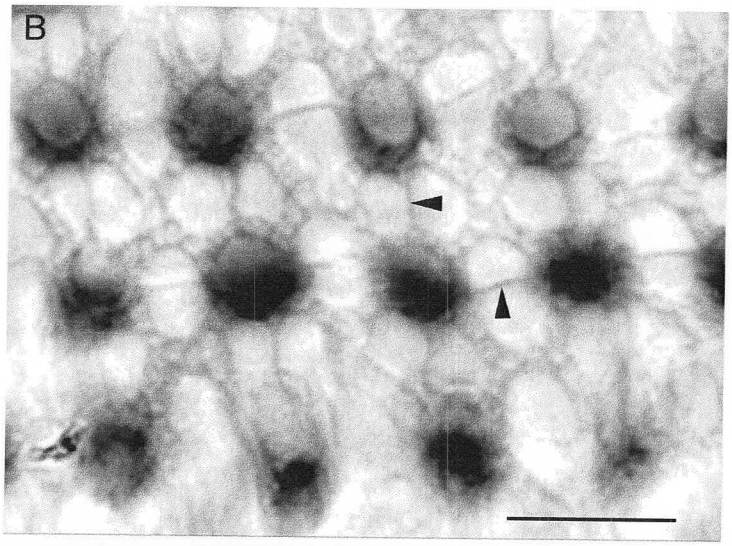
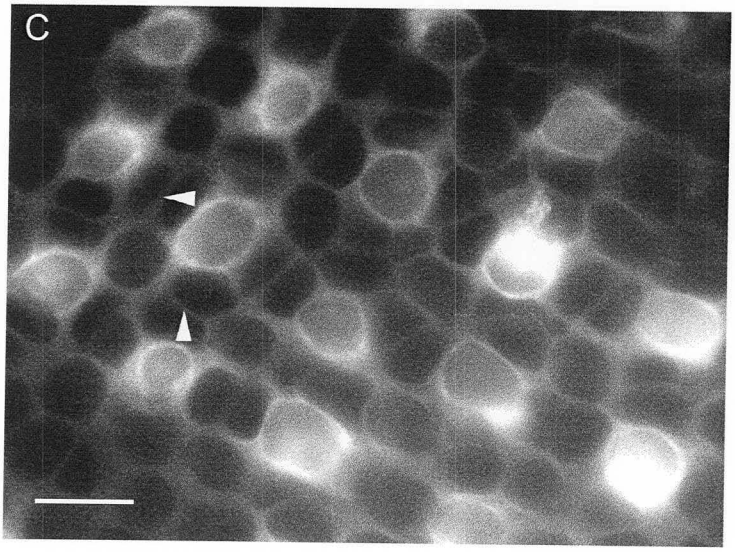
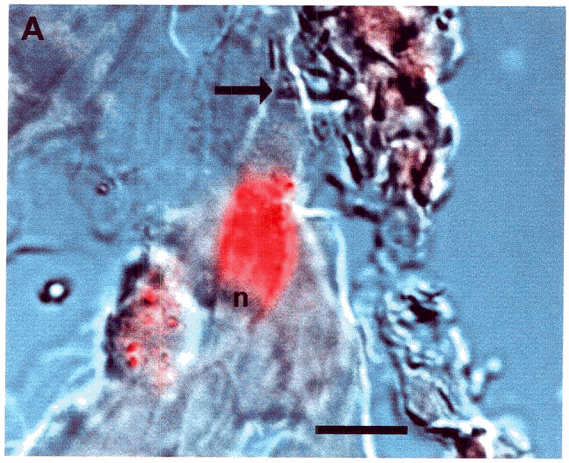

B


C

sections (Fig. 5), MWS or LWS opsin mRNA was localized to one member of double cones, while SWS or UVS opsin mRNA was localized to subpopulations of single cones. These identifications are consistent with cone morphologies of photoreceptors as identified by microspectrophotometry (Hawryshyn & Harosi, 1994; Hawryshyn *et al.*, 2001) and appear in a variety of teleosts. Consistent with other species, including other salmonids, UVS cone nuclei were vitreal to the outer limiting membrane (OLM) and found amongst rod nuclei, while SWS cone nuclei were sclerad to the OLM with other cone nuclei. Negative controls for each probe, consisting of the sense strand of riboprobe, or no probe, revealed no such labelling.

Because UVS and SWS cones are similar in morphology, and the specificity of my labelling protocol is key to interpreting results, I sought to further characterize these labels. In radial sections, it was clear that UVS opsin mRNA was localized to single cones (Fig. 5A). Double labelling of the SWS opsin mRNA protein using *in situ* hybridization and anti-SWS opsin antiserum, respectively, showed that the signals co-localized to the same photoreceptors (Fig. 5B). The mRNA was perinuclear, polarized, and consistent with the position of the endoplasmic reticulum in goldfish (Raymond, 1985) and rainbow trout (Schmitt & Kunz, 1989) cones. The antiserum has previously been shown to label SWS cones in rainbow trout (Veldhoen *et al.*, 1999), and labelled protein sclerad to the SWS opsin mRNA signal. Presumably because the *in situ* hybridization protocol includes proteinase digestions, heat and organic solvents, the antibody labelling of SWS cones was not consistent across the retina in this

Figure 5. *In* situ hybridization labelling of radial sections. **A:** Fluorescent *in situ* hybridization shows ultraviolet wavelength-sensitive (UVS) opsin mRNA (red signal) capping the nucleus (n), merged with a DIC image. This single cone is in the ventral portion of the smolt peripheral germinal zone (CGZ), and has a fully elaborated outer segment (arrow). **B:** Double label with *in situ* hybridization for short wavelength-sensitive (SWS) opsin mRNA (red label) and anti-SWS opsin antibody (green label). Hoechst 33258 stains nuclei (blue), which are capped by perinuclear, polarized SWS opsin mRNA. SWS opsin mRNA and protein are co-localized to the same single cone photoreceptors (see inset). **C:** Brightfield image of double label *in situ* hybridization demonstrating SWS opsin mRNA (red label) and UVS opsin mRNA (violet label) localize to separate populations of single cone photoreceptors with appropriate periodicity. **D:** Medium wavelength-sensitive (MWS) opsin mRNA (red label) counterstained with Hoechst 33258 reveals perinuclear localization of signal. Signal is in the "accessory" member of the double cone, as determined by cone length, and the more vitread position of nuclei associated with MWS opsin mRNA. **E:** Double label *in situ* hybridization with long wavelength-sensitive (LWS) opsin mRNA signal (dark, violet label) and MWS opsin mRNA fluorescent signal in (red label; fluorescent micrograph merged with brightfield image). Signals are localized to separate halves of the double cones. Arrow: outer limiting membrane. **F:** Double label *in situ* hybridization showing fluorescent SWS opsin mRNA signal in red, merged with DIC image of rod opsin mRNA (signal in violet). Rod opsin mRNA is in a band of outer nuclear layer nuclei, with dense localizations near the level of the SWS opsin mRNA and outer limiting membrane. Scale bar = 10 μm in **A-E**, 50 μm in **F**. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; OS, outer segment layer;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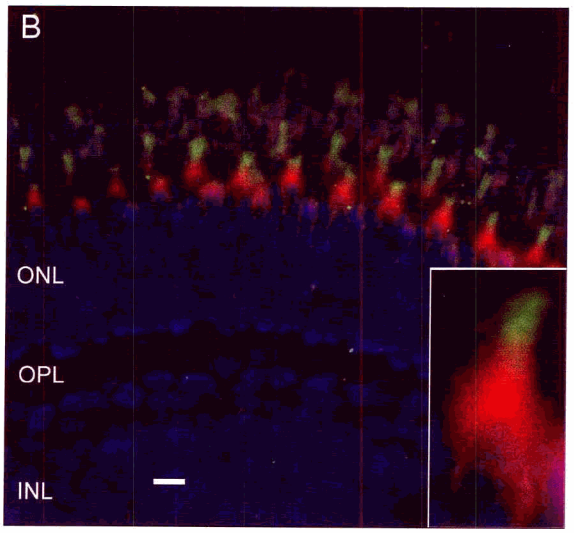
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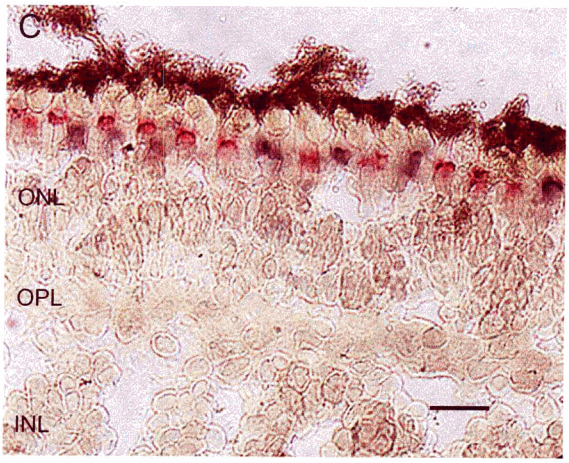
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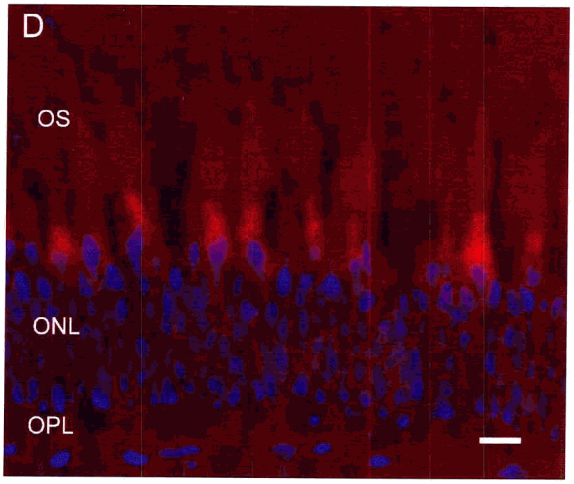
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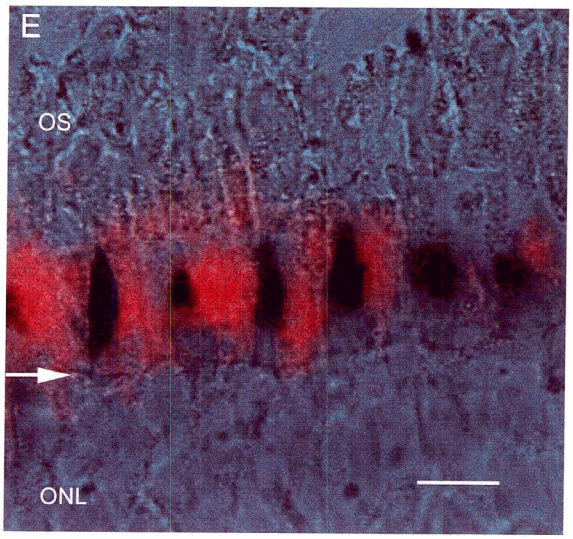
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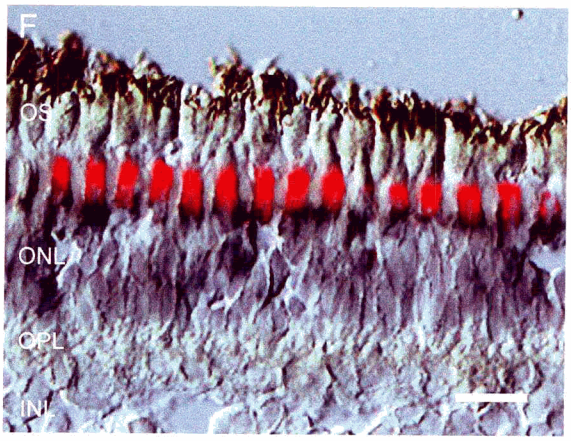
F
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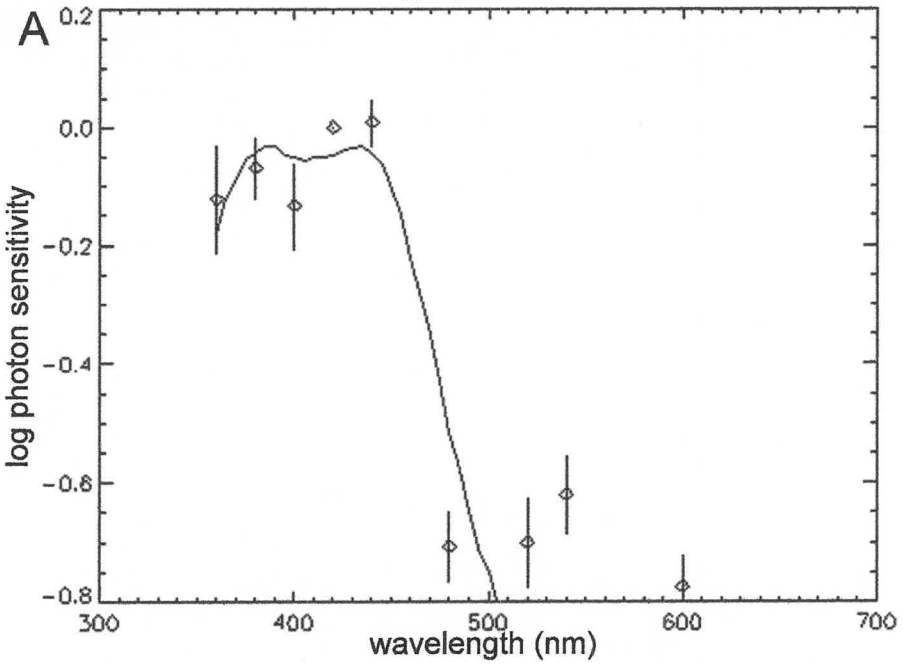
double label protocol. Where this double labelling was achieved, however, co-localization to cones containing SWS opsin mRNA was apparent. I also undertook double-label *in situ* hybridization to show that UVS and SWS opsin mRNAs localize to separate populations of single cones (Fig. 5C). These cones have appropriate morphology and occur with an appropriate periodicity. I obtained the same results regardless of the order of signal development or the type of molecule (DIG or FL) used to label the riboprobes. Using a similar approach, I focused on the specificity of the MWS and LWS opsin mRNA labels. I noted that MWS opsin mRNA is localized to the shorter "accessory" member of the double cone (Fig. 5D). I also utilized double labelling with MWS and LWS opsins and found signals localized to separate members of the double cone pair (Fig. 5E). Rod opsin mRNA was present in a band corresponding to rod nuclei in the outer nuclear layer, with more punctate labelling near the outer limiting membrane (Fig. 5F).

The loss of UVS cones during ontogeny (see below) was consistent with a decreased sensitivity to UVS light as revealed by ERGs on the same individuals examined by *in situ* hybridization (Fig. 6). This difference is apparent upon qualitative observation of the data, and is also revealed by quantifying the contributions of the UVS cone mechanism in parr (K_{UV}=0.556) *vs.* smolt (K_{UV}=0.325) fish. This decrease in sensitivity to UV light is expected based upon psychophysical data (Hawryshyn *et al.*, 1989; Browman & Hawryshyn, 1994b) and recordings from the optic nerve (Beaudet *et al.*, 1993; Parkyn & Hawryshyn, 2000; Deutschlander *et al.*, 2001). The high sensitivity to short wavelength light

Figure 6. Electroretinograms show decreasing sensitivity to ultraviolet (UV) light in parr (**A**) compared to smolt (**B**). The decrease in sensitivity to UV light in smolts is apparent upon qualitative observation, and also revealed by best-fit weighting of short wavelength-sensitive (SWS; K_{SWS}) and ultraviolet wavelength-sensitive (UVS; K_{UVS}) cone inputs (represented by line fitting the data). UVS cones were present throughout the retina of fish examined in **A**, but had a limited distribution in smolt fish reported in **B** (see cone distributions in Figure 7 & 8). Vertical bars are standard error centred on the mean. Parr n=4, smolt n=3.


A
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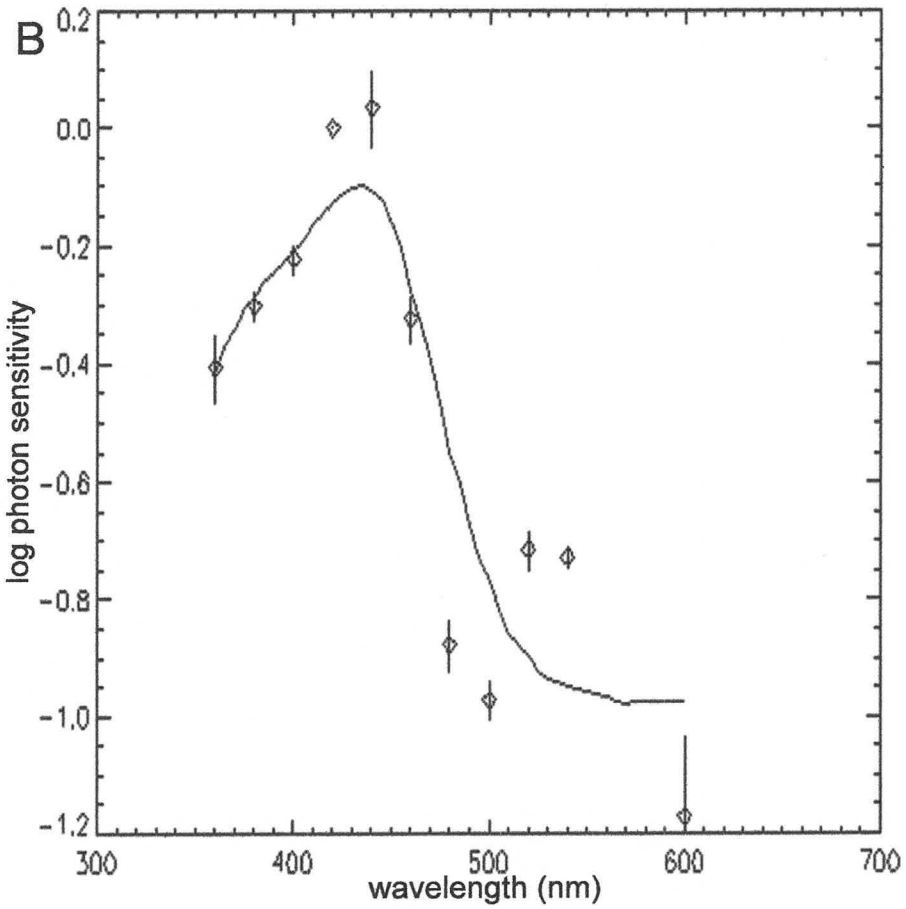
0.0
-0.2
-0.4
-0.6
-0.8
log photon sensitivity
300
400
500
600
700
wavelength (nm)

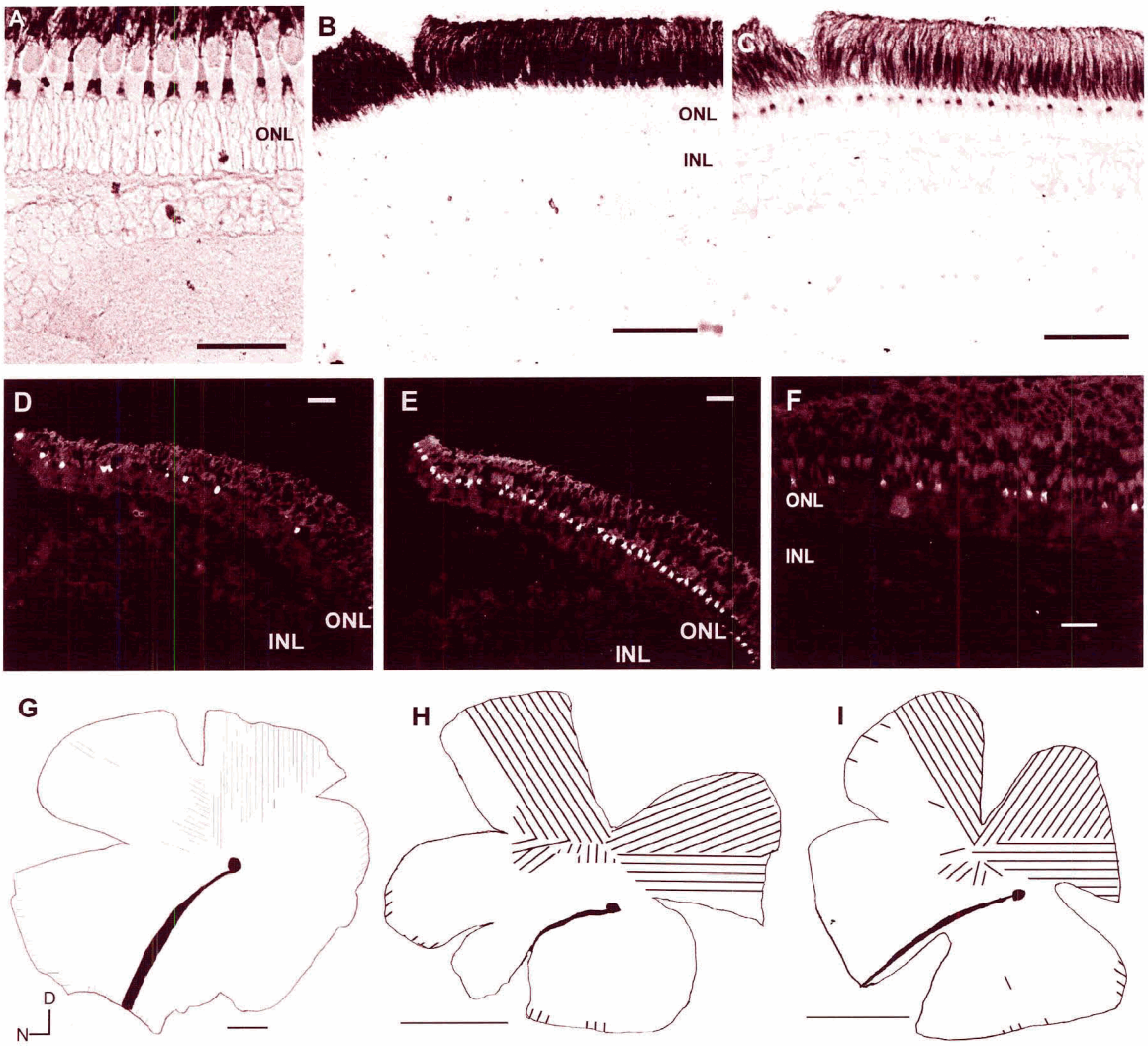

B
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
log photon sensitivity
300
400
500
600
700
wavelength (nm)

relative to longer wavelengths (Fig. 6A), under the adapting conditions utilized, demonstrates that the responses to short wavelength light are not due to β-band absorption of other cone photoreceptors. I confirmed that an independent UVS cone was measurable with ERGs by adding UV light into the background adapting light. As expected, this caused a decrease in sensitivity to UV light relative to that of other wavelengths within individual fish that had sensitivity to UV light (data not shown, see Chapter 3).

In situ hybridization revealed that UVS cones were present throughout the retina of parr trout ranging from three to seven grams. This is consistent with results using tangential sections of retina from Atlantic salmon, brown trout (Kunz, 1987; Kunz *et al.*, 1994) and sockeye salmon (Novales Flamarique, 2000). The distribution of UVS cones in the mature retina of larger (>30 g) trout was limited to the dorsal retina (Fig. 7). This is consistent with recent topographical mapping by tangential sectioning of trout retina (Martens, 2000; Hawryshyn *et al.*, 2003a). The reduced distributions of UVS cones in large fish are consistent with reduced sensitivity to UV light in these same individual fish as determined by ERGs. In areas where UVS opsin was not detected, adjacent sections were shown to contain SWS opsin (Fig. 7), so that degradation of target mRNA was deemed unlikely.

I quantified the *in situ* hybridization labelling of UVS cones and expressed it as a ratio to the number of SWS cones detected on adjacent sections (Fig. 8). I am mostly interested in the qualitative result of presence *versus* absence of UVS cones and UVS opsin mRNA. In my experience, quantification of cone

Figure 7. Distribution of ultraviolet wavelength-sensitive (UVS) cones, as revealed by *in situ* hybridization on radially sectioned retina. UVS cones were present throughout the retina of small (parr) trout, and the dorsal temporal retina of smolts (**A,** dark precipitate; **F** fluorescent signal). In smolts, UVS cones have disappeared from the ventral and nasal portions of the retina (**B**). In areas of retina where UVS cones were not detected, short wavelength-sensitive (SWS) opsin mRNA was detected on immediately adjacent sections (**C, E**), serving as a positive control for the labelling protocol. Differentiated UVS cones were present in the peripheral germinal zone (**D**), of ventral and temporal smolt retina (represented by fluorescent signal and with the periphery towards top left of panel), immediately adjacent to areas of mature retina that lack UVS cones. Compared to parr that had UVS cones throughout their retina, smolts had a decreased distribution of UVS cones (**G, H, I**). This is coordinated with a decrease in sensitivity to UV light (Fig.4). These results were similar for all smolt retinae examined, and a detailed map was created for retina from three individuals (**G, H, I**) where the lines indicate the detection of UVS opsin mRNA. The orientation of these lines is an artifact of tissue orientation during sectioning. The retina was traced using an overhead projector during dissection into pie-shaped pieces, allowing the location of sections to be documented. These pie-shaped pieces were each carefully mounted, sectioned radially, processed, and examined for the presence of UVS and short wavelength sensitive opsin mRNA. D, dorsal; N, nasal. Scale bar = 50 μm in **A, D, E, F**; 100 μm in **B** and **C**; 2 mm in **G**; 5 mm in **H** and **I**.


A
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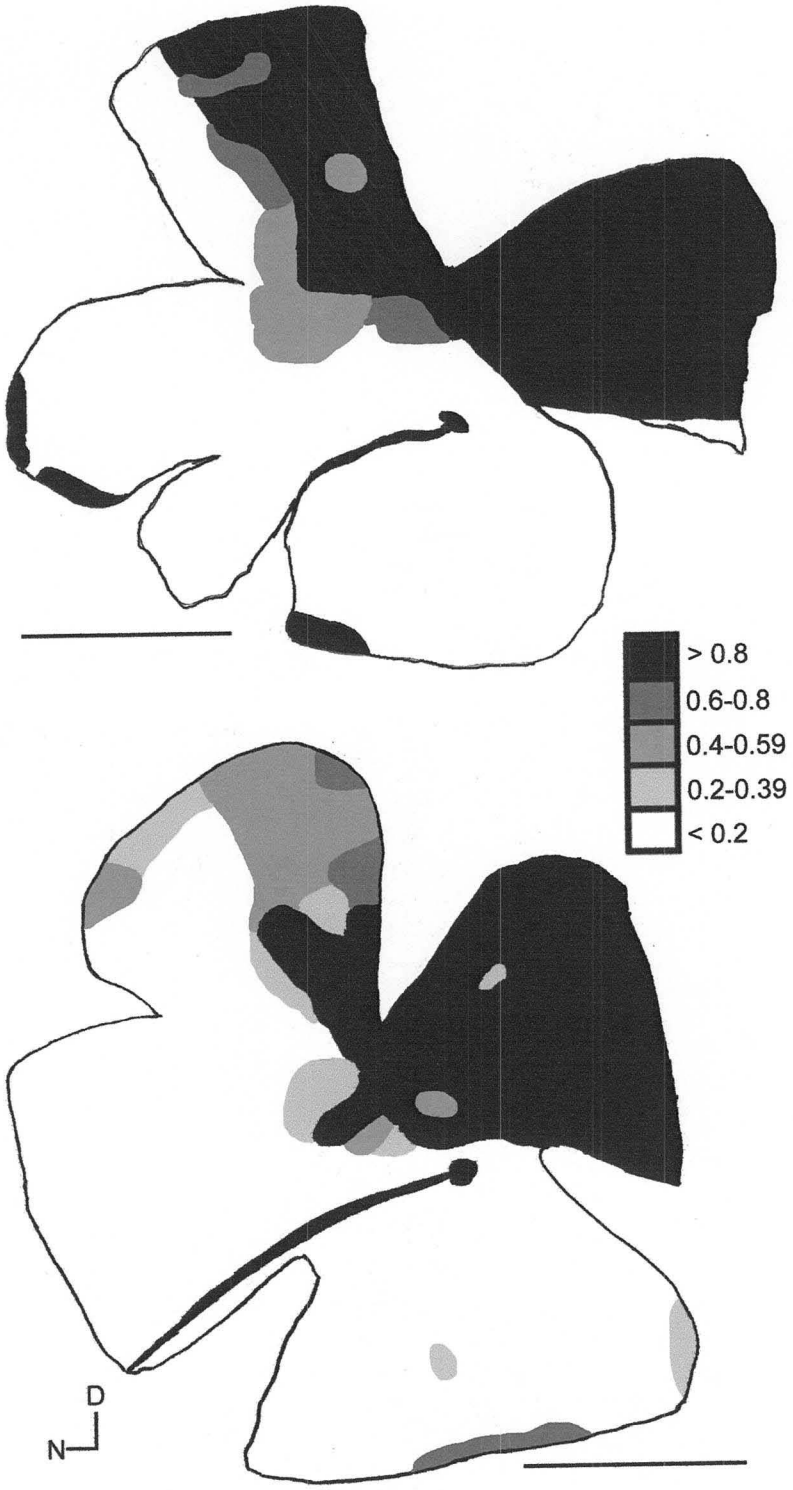
I

Figure 8. Density of ultraviolet wavelength-sensitive (UVS) cones smolt retina from two individuals, represented as a ratio to the number of short wavelength sensitive (SWS) cones detected in immediately adjacent radial sections. Location within the retina was determined as in Figure 4G-I. Number of UVS cones within a field of view were counted, and expressed as a ratio to the number of short wavelength-sensitive cones in a similar field of view (determined by utilizing landmarks in the tissue) in an immediately adjacent section. Fields examined along a section were separated by approximately 400 μm. Scale bars are 5 mm. D, dorsal, N, Nasal.


> 0.8
0.6-0.8
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0.2-0.39
< 0.2
D
N

photoreceptor densities is more accurate using plastic sections (e.g. Beaudet *et al.*, 1997; Martens, 2000; Hawryshyn *et al.*, 2003a) or immunohistochemistry (see Chapter 3). Thus Figure 8 is presented to highlight the robust nature of my protocol, including the use of an effective positive control for areas where UVS cones were not detected. Within the context of these qualifiers, I note that the nasal border of the UVS cone distribution seems to gradually decline in density, and thus could represent an area where UVS cones are being removed from the retina.

UVS cones were detected in the CGZ of smolts, in areas peripheral to mature retina that lack UVS cones (Figures 7 & 8). UVS cones were detected robustly in the CGZ but were absent from adjacent mature retina in the same section (Fig. 7D, E). Acting as a positive control, adjacent sections showed SWS cones throughout the retina (Figures 7 & 8), indicating that the state of the tissue did not play a role in the lack of UVS cone labelling in mature retina. Several UVS cones in the smolt CGZ had outer segments (Fig. 5A), implying full differentiation. Expression of opsin mRNA can also be considered an indication of differentiation. This is consistent with the presence of ACC in the CGZ of smolt retina in sockeye (Novales Flamarique, 2000), Atlantic salmon and brown trout (Kunz, 1987; Kunz *et al.*, 1994). This implies that the UVS cones either transdifferentiate (i.e. transmute) into some other cell type (e.g. rods), become dormant, or die via programmed cell death (See Chapter 3). Adjacent to the differentiated UVS cones, more centrally, I consistently detected degenerating UVS cones. These cells, in the appropriate position and containing UVS opsin

mRNA, lacked clear cone morphology. This is consistent with EM evidence from Atlantic salmon, which suggests that differentiated ACC disappear via PCD (Kunz *et al.*, 1994).

2.5 Discussion

Salmonids are an attractive model for studying retinal development. Subsequent to embryogenesis, substantial developmental changes occur within the salmonid retina. This further retinal development is characterized by a loss of UVS cones and visual sensitivity to UV light (Kunz, 1987; Hawryshyn *et al.*, 1989; Novales Flamarique, 2000; Deutschlander *et al.*, 2001), and shifting visual pigments (i.e. chromophore A_1/A_2 ratio) (Alexander *et al.*, 1998), allowing the study of how visual systems change to accommodate changing photic environments. Differences exist in life history strategies (e.g. the timing of migrations) between species or between populations within the same species. These differences can be interpreted in a framework of a well-established phylogeny to examine both visual ecology and how retinal development has evolved (Parkyn & Hawryshyn, 2000). Importantly, UVS cones reappear at sexual maturity, when the fish return to their natal streams (Beaudet *et al.*, 1997; Novales Flamarique, 2000). This represents the only known regeneration of cone photoreceptors during the natural life history of a vertebrate. Thyroid hormone treatment can act to induce events of interest (e.g. UVS cone disappearance and regeneration) (Browman & Hawryshyn, 1992, 1994b; Alexander *et al.*, 1998), providing the important advantage of a controlled onset time (Reme *et al.*, 1998). The investigation of neural development in salmonids can utilize tools of

transgenesis (Devlin *et al.*, 2001), and genome sequencing is underway in a variety of salmonid species (reviewed in Phillips & Rab, 2001; Milchert *et al.*, 2002; Thorgaard *et al.*, 2002). I sought to facilitate these models by developing tools to readily examine visual sensitivity to UV light via ERGs and to label each of the rod and cone photoreceptor types via *in situ* hybridization.

To date the examination of sensitivity to UV light in salmonids has been limited to psychophysical techniques or recordings from higher order retinal neurons, optic tectum, or the torus semicircularis (Hawryshyn *et al.*, 1989; Beaudet *et al.*, 1993; Coughlin & Hawryshyn, 1994b; Deutschlander *et al.*, 2001). ERGs have long been used to assess visual function in salmonids (Millodot, 1967; Ali & Kobayashi, 1968; Allen & Munz, 1983), but the study of sensitivity to UV light is particularly difficult, having been achieved in only a few teleosts (Whitmore & Bowmaker, 1989; Chen & Stark, 1994; Hughes *et al.*, 1998). ERGs are simpler and more reliable than other protocols, and do not require surgery. The latter point facilitates developmental studies because individual fish can be recorded from multiple times allowing one to examine within-individual differences between developmental stage or before/after treatment. The utility of ERGs has also eased studies of polarization vision, including the detection of the most complex polarization sensitivity for a vertebrate, and the detection that polarization sensitivity must be, at least in part, mediated by HCs (Hawryshyn *et al.*, 2002; Hawryshyn *et al.*, 2003b). Currently our laboratory is using the ERG methodology to examine questions regarding salmonid polarization sensitivity. Furthermore, the ERG is a more stable preparation than past experiments, such

as compound action potential recordings, allowing automation of stimulus delivery and response acquisition.

Previous examinations of retinal development in salmonids have relied upon identification of cone types by their morphology. Examinations of UVS cone ontogeny have relied on the tentative assignment of the accessory corner cone, in square mosaic, as being maximally sensitive to UV light. This histological method has limited interpretive power within other retinal mosaic types, which often occur in areas of interest adjacent to the CGZ (Beaudet *et al.*, 1997; Martens, 2000; Hawryshyn *et al.*, 2003a). This methodology is also limited to tangential sections, however radial sections are often required to visualize nuclear labelling in cell fate determination protocols (Julian *et al.*, 1998). Thus I developed a label for UVS cones and confirm that, consistent with other fish species (Raymond *et al.*, 1993; Hisatomi *et al.*, 1996; Hisatomi *et al.*, 1997), accessory corner cones express UVS opsin mRNA.

I utilized these tools to examine the changing distribution of UVS cones as the rainbow trout goes through metamorphosis, often referred to as smoltification[4]. I found that UVS cones were present throughout the retina of young (parr) rainbow trout. This result is similar to migratory sockeye salmon (*O. nerka*) (Novales Flamarique, 2000) as well as salmonids from the genus *Salmo* (e.g. Atlantic salmon) (Kunz, 1987; Kunz *et al.*, 1994). I found that in larger, post-metamorphic rainbow trout, the distribution of UVS cones in the mature retina is

[4] Smoltification is a term often reserved for ocean-going salmonids, which alter their physiology to osmoregulate during migration to marine environments. The developmental changes that occur in rainbow trout may not meet some definitions of smoltification, however they do mimic many of the same physiological changes. Importantly, visual system changes at this time of development appear to be similar to those of ocean-going populations.

limited to the dorsal temporal retina. In areas where UVS cones were not detected, robustly labelled SWS cones were detected on adjacent retinal sections. Coordinated with this decrease in UVS cone distribution, I show that the sensitivity to UV light is reduced in post-metamorphic fish. Thus the decrease in UV sensitivity is coordinated with the UVS cone distribution as revealed by *in situ* hybridization. The results are also consistent with a recent histological examination of accessory corner cones in large rainbow trout (Martens, 2000; Hawryshyn *et al.*, 2003a). The results are also supported by optic nerve recordings of rainbow trout undergoing thyroid hormone treatment (mimicking metamorphosis) and steelhead smolts where the remaining sensitivity to UV light stimuli is localized to the dorsal retina (Deutschlander *et al.*, 2001). It appears that the loss of UVS cones in rainbow trout, during natural development, is not as substantial as that of sockeye salmon (Novales Flamarique, 2000). More data is needed before one can assess if this represents a difference in experimental method, species, developmental timeline, and/or a difference between anadromous (ocean migrating) vs. non-anadromous (landlocked) populations.

Changes in the visual system which occur at metamorphosis (e.g. smoltification), when fish are preparing to move to new environments (e.g. marine), provide opportunities to study the control of neural development. Examining changes in visual function at metamorphosis has been fruitful in other species, such as *Xenopus* (Marsh-Armstrong *et al.*, 1999; Mann & Holt, 2001). It has been established that thyroid hormone, which peaks during smoltification, plays a key role in salmonid visual development (Browman & Hawryshyn, 1992,

1994b; Martens, 2000; Deutschlander *et al.*, 2001; Hawryshyn *et al.*, 2003a, see chapter 3) and that the retina of rainbow trout and sockeye have substantial levels of deiodinase enzymes (Plate *et al.*, 2002). These deiodinases provide the ability to regulate the amount of activated thyroid hormone that reaches the retina, independent of serum hormone levels. Deiodinase expression in *Xenopus* retina peaks at metamorphosis, controls cell proliferation and differentiation, and is asymmetrical along the dorsoventral axis (Marsh-Armstrong *et al.*, 1999). We are continuing to examine retinal deiodinase expression, particularly within the context of the presently described dorsoventral asymmetry of UVS cone disappearance during natural development. We also noted that retinal deiodinase activity levels differ between rainbow trout and sockeye, such that deactivation of TH is higher in rainbow trout (Plate *et al.*, 2002). These differences coincide with the apparent species differences in post-metamorphic UVS cone distributions. It is noteworthy that TH-treatment in rainbow trout leads to a disappearance of UVS cones from the entire mature retina (Chapter 3), which is identical to the natural ontogeny reported for sockeye and Atlantic salmon (Kunz, 1987; Kunz *et al.*, 1994; Novales Flamarique, 2000). It is possible that my TH-treatment mimics higher levels of activated TH in the retina of naturally developing sockeye, as would be suggested by the differences in retinal deiodinase levels between these species.

In the following chapter, I differentiate between hypotheses regarding the fate of the disappearing UVS cones. The disappearing UVS cones have been suggested to transmute (transdifferentiate) into another cell type (Furst, 1904;

Lyall, 1957b). Alternatively, they may become dormant, similar to the proposed fate of rainbow trout photoreceptors following damage by light (Allen & Hallows, 1997) or ouabain (Kurz-Isler & Wolburg, 1982). The balance of current evidence supports the contention that UVS cones are dying by programmed cell death, as suggested by EM evidence from an anadromous species of another salmonid genus (Kunz *et al.*, 1994).

2.5.1 UVS cones in the smolt peripheral germinal zone

I observed that the CGZ of smolt retina contains UVS cones. My *in situ* hybridization labelling of these cells confirms the identity of these disappearing cells, as was suggested by histological methods in Atlantic salmon (Kunz *et al.*, 1994). This is especially interesting in areas generating mature retina that will lack UVS cones. These short-lived UVS cones express opsin mRNA (Figures 7 & 8) and have fully elaborated outer segments (Fig. 5A), and these data support the contention that they are fully differentiated. The generation, differentiation and subsequent loss of UVS cones in the smolt CGZ is a dramatic example of the supposition that the mechanisms of CGZ development recapitulate the retinal embryogenesis of that species (Harris & Perron, 1998; Perron *et al.*, 1998; Olson *et al.*, 1999).

I am curious if these short-lived UVS cones are generated in the smolt CGZ for some particular function. Photoreceptors are metabolically expensive to produce, and yet my data support a wide phylogenetic distribution of UVS cone generation and disappearance in the CGZ of the Family Salmonidae. A similar distribution of UVS cones in the CGZ of halibut has been detected by *in situ*

hybridization in Atlantic halibut (*Hippoglossus hippoglossus*; J.V. Helvik, unpublished data). Ancestral teleosts are believed to have possessed four cone types (Bowmaker, 1998), and many extant teleosts have only three cone types (Engstom, 1963). Thus one can conclude that mechanisms of retinal development, other than apoptosis of a fourth cone type, have readily evolved to generate the three-cone mosaic observed in the salmonid smolt. This view is supported by several examples where the presence and absence of four cone types occurs between species of the same family (Engstom, 1963). Taken together, this argues in favor of the UVS cone serving some function in the smolt CGZ; if this were not true, other mechanisms of retinal development leading to a mosaic with three cone types would be favorable.

While all available markers suggest that the UVS cones in the smolt CGZ are fully differentiated (see also immunohistochemistry for UVS opsin protein in Chapter 3), it is unlikely that they serve any visual function due to their position behind the iris, which would considerably limit the amount of light reaching them (See Figure 1). It was suggested previously that the disappearing UVS cones may make space for rods (Kunz *et al.*, 1994). It is also possible that UVS cones are generated to affect the differentiation of other adjacent cell types. A population of horizontal cells, H3, receive input from UVS cones, and other cone types in some teleosts (Kamermans & Spekreijse, 1995). These horizontal cells could require the formation of contacts with UVS cones for their appropriate differentiation, maybe of their late forming (at least during embryogenesis) gap junctions (Schmitt & Kunz, 1989) that contribute to receptive field properties.

Additionally, it has been suggested that either the SWS or UVS cones in the CGZ of fishes act as the "founder cell", (equivalent to photoreceptor R8 in *Drosophila*) in that they differentiate first, create the spacing for the photoreceptor mosaic, and induce surrounding cells to the appropriate fate (Raymond, 1995). If it is true that UVS cones are generated in the smolt CGZ solely to affect the differentiation of adjacent cells, then this has broader implications for interpreting the overproduction and subsequent pruning of neurons during neural development. The neurotrophic theory speculates that the overproduction of neurons (in some developing structures 50% of neurons are eliminated) allows for the selection of neurons that have formed proper connections and are appropriately positioned (Raff *et al.*, 1993). Thus the hypothesized roles of neuronal death is to control cell number and eliminate neurons that have formed inappropriate connections (Clarke *et al.*, 1998). However, it is apparent that neuronal death plays other important, as yet unknown, roles in nervous system morphogenesis (de la Rosa & de Pablo, 2000; Kuan *et al.*, 2000). The current example supports the contention that some of the excess neurons function to direct the differentiation of surviving neurons. This hypothesis is similar to that of the role of generating vestigial organs during development. This role for dying neurons during development may be widespread, as it would typically be difficult to detect or describe. Indeed, the current example of disappearing UVS cones in the smolt CGZ is a clear example solely because the degenerating neuron is readily identifiable.

Chapter 3:

Mechanisms of UVS Cone Disappearance and Reappearance[5]

3.1 Abstract

Stem cell treatment holds promise for ameliorating deficiencies in retinal degenerations, however little is known about how stem cells generate cone photoreceptors or how regenerated cones are functionally integrated. Here, I establish a powerful animal model for such studies, rainbow trout (*Oncorhynchus mykiss*), and make the first report of cone photoreceptors regenerating into an established retina during natural development. I demonstrate that thyroid hormone manipulations drive both the disappearance and reappearance of the UVS cone, the same cone class as the human blue-sensitive cone, in a manner similar to its natural ontogeny. I find that only UVS cones, and not other cones, incorporate BrdU during UVS cone reappearance. Thus, ultraviolet-sensitive cones regenerate from stem cells, known to reside in established *O. mykiss* retina. The regenerated cones successfully transmit visual information to higher order retinal neurons. My findings represent a novel mechanism for the visual system to refine its sensitivity to suit its environment, and a novel opportunity to study how stem cells generate cone photoreceptors.

[5] The findings presented in this chapter have been submitted for publication and involved a collaboration with Stephen G. Dann, Kathy M. Veldhoen and Craig W. Hawryshyn.

3.2 Introduction

Electrophysiology and histology on the retina of salmonid fishes has indicated a remarkable ontogeny of photoreceptors, whereby the UVS cones disappear from the retina (Furst, 1904; Lyall, 1957b; Bowmaker & Kunz, 1987; Hawryshyn *et al.*, 1989; Beaudet *et al.*, 1993; Novales Flamarique & Hawryshyn, 1996; Novales Flamarique, 2000; Deutschlander *et al.*, 2001, see Chapter 2) and subsequently reappear at a later life-history stage (Beaudet *et al.*, 1997; Novales Flamarique, 2000). In Pacific Salmon (*Oncorhynchus*), disappearance of UVS cones has been associated with smoltification (Hawryshyn *et al.*, 1989; Beaudet *et al.*, 1993; Novales Flamarique, 2000; Deutschlander *et al.*, 2001, see Chapter 2), a metamorphic transition that prepares migratory salmonids for the marine environment. Reappearance of UVS cones has been linked to sexual maturation and the return migration of the fish to freshwater spawning grounds (Beaudet *et al.*, 1997; Novales Flamarique, 2000). The disappearance of UVS cones was demonstrated to be an apoptotic event based upon electron microscopic evidence (Kunz *et al.*, 1994). The mechanism whereby this cone class can reappear into an established retina has remained unknown, despite its potential relevance to both visual ecology and the developmental mechanisms of retinal regeneration. In this context, it is noteworthy that salmonid UVS cones express SWS1 opsin (Dann *et al.*, 2004a, See Chapter 2), and thus may be homologous to mammalian "blue-sensitive" or "S-cone" photoreceptors at the cellular level.

Thyroid hormone (TH) manipulations can affect the disappearance (Browman & Hawryshyn, 1992, 1994b; Deutschlander *et al.*, 2001) and reappearance

(Browman & Hawryshyn, 1994b; Hawryshyn *et al.*, 2003a) of UVS cones. Similar to its role in tadpole metamorphosis, TH is both necessary and sufficient for smoltification in salmonids (Hoar, 1988). Indeed, TH treatment mimics many diverse aspects of smoltification including changes in muscle physiology, osmotic regulation, visual pigment shifting, colouration and behaviour (Hoar, 1988; Coughlin *et al.*, 2001). Here, I used TH treatment to induce smoltification of *O. mykiss,* producing a controlled onset time for the events of interest, and allowing a dissection of developmental mechanisms in the UVS cone ontogeny.

The disappearance of the UVS cone in salmonid retinae has now been recognized for a century (Furst, 1904). Although the sensitivity of the UVS cone was unknown (as was the existence of UV vision), the cones were identified by morphology, and thus UVS cones were termed '*Zwischenzapfen* ' (in-between cones). On page 20, Furst (1904) notes[6]:

> "...a tangential cross section at the level of the outer inner members of the cones is shown. Such cross sections of adult fish retinas have already been presented in earlier studies, especially in Schafers (1900) communications. Theodor Beer (1898) has even studied the arrangements ophthalmologically. Comparing my figure (Fig. 29) to those I have generated from the young salmon, especially Fig 23 from a 125 day old salmon, I notice that the cones are larger and more closely packed, but that the quadrilateral arrangement is partially kept. The four cone pairs still form a quadrilateral with a center cone in the center. The UVS cones were no longer encountered in the large salmon. The rods are visible only as very fine thread-like cross sections around the center cone and at the location of the UVS cone."

The fate of the disappearing UVS cone was thought to be a transdifferentiation into rod photoreceptors. On page 36 Furst writes:

> "The UVS cones are no longer encountered as a cone in the adult salmon. Therefore I suggest that their development has taken a different direction.

[6] These passages were kindly translated from German by Theodore Haimberger, 2004.

> Their morphology has changed and they can now possibly be found amongst the rods. The large rods are also being changed. Together with the surely later developing rods, that belong to the cells with the nuclei in the inner row of the vision cell layer, they have not yet grown at the age of my salmon. They change into the Schwalbe rods. To these belong the fine thread-like inner members, which have come about by the tremendous development of the cones - pair and center cones - and by the resulting pressure at their level on the other terminal organs.
>
> In the young salmon (from 125 to 150 days) I find large paired cones, the double cones, less developed single cones, the center and UVS cones, and strong rods. The adult salmon have extremely large double cones, slightly smaller center cones and the Schwalbe rods. The rods are located around the center cones and in the space were the UVS cones had been."

The work of Furst seems to have influenced Lyall (1957a & b), who also noted the disappearance of UVS cones in trout. Lyall also proposed that UVS cones transdifferentiate into rods, a process that she termed 'transmutation'. Transmutation describes the derivation of rods from cone photoreceptors over evolutionary (not ontogenetic) time. The popularity of transmutation as an evolutionary topic at the time, being described by Walls in two widely-cited publications (Walls, 1934, 1942), may have also influenced Lyall's conclusions. Transmutation remains a viable evolutionary hypothesis today (Kojima *et al.*, 1992; Taniguchi *et al.*, 1999), but ontogeny's recapitulation of phylogeny can no longer be considered as a causal link for developmental (or evolutionary) mechanisms. Whereas the latter argues against the usage of the term 'transmutation', and several authors have dismissed the concept because of fundamental differences between rods and cones (Engstom, 1960; Ahlbert, 1976; Kunz *et al.*, 1994), I believe the evidence supporting a transdifferentiation was accurate. I also believe, however, that the interpretation of the data was not correct. The observations of Lyall and Furst can be addressed in the context of

new data from the past two decades, and the hypothesis of transdifferentiation can be refuted.

The hypothesis that UVS cones transdifferentiate into rods was based upon two types of observations: i) an increase in the number of rods concomitant with UVS cone loss, and ii) the morphological aspects of the UVS cone are intermediate between that of rods and cones (Furst, 1904; Lyall, 1957a). Although the observations were accurate, they are not evidence of transdifferentiation. The source of rods is now known to be rod progenitors (Johns & Fernald, 1981; Johns, 1982; Fernald, 1990; Julian *et al.*, 1998; Easter & Hitchcock, 2000; Otteson & Hitchcock, 2003). Further, the intermediate aspects of the UVS cone morphology (a rounded nucleus that is located between the rod nuclei and the nuclei of the other cone types), are observed in all *O. mykiss* UVS cones, including those that do not disappear from the dorsal-temporal retina (see Chapter 2). This morphology of the UVS cone has been described in other teleosts, including examples such as zebrafish (Engstom, 1960; Raymond *et al.*, 1993) where UVS cones do not disappear (Temple *et al.*, 2003; Allison *et al.*, *In Preparation*).

The goal of the present work was to discriminate between two remaining hypothetical mechanisms of UVS cone disappearance and reappearance in salmonid fishes: i) Cell dormancy; ii) Cell death and regeneration. The first hypothesis has emerged from observations that were interpreted as "dormancy" of salmonid photoreceptors following two different types of experimentally-induced retinal damage (Kurz-Isler & Wolburg, 1982; Allen & Hallows, 1997, See

Chapter 4). Indeed, dormant UVS cones, lacking opsin expression and outer segments, would not have been recognized in previous analyses. This idea proposes that UVS cones reappear through a resumption of opsin expression and an elaboration of new outer segments. Although no such mechanism has been documented during natural development, this idea could be conceptually related to examples of differential opsin expression during teleost ontogeny (Carleton & Kocher, 2001).

The second hypothesis, that UVS cones reappear from proliferating retinal stem cells, is derived from a substantial body of evidence demonstrating regeneration of all cell types when teleost retinae are damaged experimentally (reviewed in Chapter 1). Such regenerative potential is linked to the mechanisms of retinal growth. Retinal growth occurs throughout the life of fish by retinal stretching and through a population of proliferating cells at the periphery of the retina (Lyall, 1957b; Ali, 1964; Johns, 1977), the circumferential germinal zone (CGZ). Importantly, proliferating cells are also present throughout the mature central retina. During natural development these progenitor cells of the central retina are thought to give rise exclusively to rod photoreceptors as the retina stretches, such that the density of rods remains approximately uniform through post-embryonic life (Johns & Fernald, 1981; Otteson & Hitchcock, 2003). In *O. mykiss* the progenitor cells of the central retina proliferate at a high rate, and this has allowed for the first identification of progenitor cells in the inner nuclear layer (PINCs) of adult fish (Julian *et al.*, 1998). PINCs had previously been identified in larval teleosts (Sandy & Blaxter, 1980; Johns, 1982), and are now known to

occur in the adults of several species (Vihtelic & Hyde, 2000; Cid *et al.*, 2002; Otteson & Hitchcock, 2003), including other salmonids (Ahlbert, 1976; Candal *et al.*, 2001). Proliferation of PINCs is up-regulated after experimentally induced damage (Vihtelic & Hyde, 2000; Candal *et al.*, 2001; Faillace *et al.*, 2002), and they are thought to be the source of regenerating cones that appear after damage of goldfish retina (Wu *et al.*, 2001).

3.3 Methods

3.3.1 Experimental animals

O. mykiss occurs in populations that typically remain landlocked (rainbow trout) and those that are migratory (steelhead salmon). For experiments reported here, I utilized rainbow trout from the Vancouver Island Trout Hatchery, Duncan, British Columbia, Canada. The fish were maintained in 350 litre flow-through tanks at the University of Victoria, with a mean water temperature of 15±1°C under a 12L:12D photoperiod. Fish were maintained in these conditions for a minimum of two months prior to sampling. Thyroid hormone (TH) treatment was performed under the same conditions, except that fish (including non-treated control fish) were transferred to standing water in 30 litre aquaria; TH treatment was completed by adding 300 µg/litre L-thyroxine (Sigma, St. Louis, MO) in 1.5 mL of 0.1 N NaOH to the water, which was changed daily. Fish maintained as controls for TH treatment were held in identical conditions with only vehicle (NaOH) added to the water. In regards to the actions of thyroid hormone on the distributions of UVS cones, I have observed indistinguishable results on a second brood-stock of trout from this hatchery, and another from Fraser Valley

Trout Hatchery, Abbotsford, British Columbia, Canada. To examine fish after cessation of TH treatment, fish that had received 6 weeks of TH were put onto flow-through water. Six weeks of TH treatment was chosen because I have found it to be sufficient to induce the loss of UV cones and UV sensitivity in a variety of experiments (although I can also observe the effects in a much shorter time-period). To examine the effects of caspase inhibitor, fish that had received two days of TH treatment were given an intraocular injection of zVAD-fmk (Calbiochem, San Diego, CA) dissolved in 0.1% DMSO in PBS, to produce a final concentration of approximately 300 μM. The caspase inhibitor zVAD-fmk blocks caspase enzymes, which are highly-conserved mediators of programmed cell death. This inhibitor has been used at similar concentrations to block cell death in zebrafish (Williams *et al.*, 2000; Williams & Holder, 2000; Dong *et al.*, 2002; Sanders & Whitlock, 2003), Atlantic salmon (Fladmark *et al.*, 1998), and rainbow trout (Moyes *et al.*, 2002). Individuals receiving caspase inhibitor, along with sham-injected negative controls, were maintained on TH treatment and sacrificed two days later. Control and treated fish also received sham injection in the contralateral eye. BrdU treatments in preliminary experiments were completed by intraocular injection of 10 μl of 0.1 M 5-Bromo-2'-deoxyuridine (BrdU; Sigma). Alternatively, fish were maintained in one litre of water with 10 μM BrdU and clinoptilolite (a mineral used in aquaculture to absorb ammonia) for 48 hours. To sample the retinae, fish were deeply anaesthetized in 300 mg/litre MS-222 (tricaine methanesulphonate, Crescent Research Chemicals, USA) until euthanized, and retinae were fixed in 4% paraformaldehyde. Electroretinograms

were performed according to protocols in Chapter 2. Additional details regarding the UV adaptation experiments in my ERG experiments have also been presented elsewhere (Hawryshyn *et al.*, 2003b). Care of the fish and all procedures were in accordance with and approved by the University of Victoria Animal Care Committee under the auspices of the Canadian Council for Animal Care.

3.3.2 Immunohistochemistry & in situ hybridization

To label UVS cones, I produced polyclonal antisera against the twenty N-terminal amino acids predicted from my cDNA sequence (Chapter 2) of *O. mykiss* UVS opsin (*i.e.* SWS1 opsin, accession number AF425074). The predicted amino acids are: MGKDFHLYENISKVSPFEGPC. A cysteine residue, which is not predicted to appear at this position of UVS opsin, was included at the carboxyl terminus of the synthesized peptide (synthesized by UVic-Genome BC Proteomics Centre, Victoria, BC, Canada) and allowed conjugation to sulfhydryl-reactive imject maleimide-activated mariculture keyhole limpet hemocyanin (Pierce, Rockford, IL, USA). This conjugate was purified by dialysis, and periodically injected with Freund's adjuvant (Gibco-BRL, Gaithersburg, MD) into four BALB/c mice. I used the same antigen to produce polyclonal antiserum in a female Fisher 344 rat. These antisera were prepared by Immuno-Precise Antibodies Ltd. (Victoria, BC, Canada).

Western blots were carried out on retinal homogenates using 1:1000 rat polyclonal anti-UVS sera and 1:3000 goat-anti-rat secondary antibody conjugated to alkaline phosphatase (Vector Labs, Burlingame, CA), diluted in 3%

non fat milk (Becton Dickson, Sparks, MD) using standard protocols. Retinae were homogenized in ice-cold buffer containing 30mM Tris-Cl pH 7.5, 10mM EGTA, 5mM EDTA, 250 mM sucrose, 1% octyl β-D-glucopyranoside (Sigma) and 1 mM PMSF. Homogenate was diluted in Red Loading Buffer (New England Biolabs Inc.) and resolved on a 12.5% SDS-polyacrylamide gel. Proteins were electroblotted onto BioTrace™ NT Membrane (Pall Gelman Laboratory) at 100 volts for 1 hour.

Immunohistochemistry was carried out using standard protocols on retinal wholemounts or cryosections (10 μm), and all antibodies were diluted in phosphate buffer solution with 0.1% tween (PTW) and 2% heat-inactivated horse serum (Sigma). Anti-UVS sera raised in mouse and rat were diluted 1:5000 and 1:100, respectively. Anti-SWS2 polyclonal sera, previously shown to label *O. mykiss* SWS cones (Veldhoen *et al.*, 1999, see also Chapter 2), was diluted 1:500. Double cones were labelled with a mouse monoclonal antibody zpr-1 (Zebrafish International Resource Center, Eugene, OR) diluted 1:250 (previously called 'FRet 43', see Larison & Bremiller, 1990). Secondary antibodies, used at a dilution of 1:1000, were goat-anti-rabbit-fluorescein, goat-anti-mouse-Texas Red (Vector Labs) or goat-anti-mouse Alexa Fluor 594 and chicken anti-rat-Alexa Fluor 488 (Molecular Probes, Eugene, OR). BrdU immunohistochemistry utilized a rat monoclonal antibody against BrdU (Clone BU1/75, Harlan Sera-Lab, Loughborough, England). For BrdU immunohistochemistry, slides were incubated for 30 minutes in 2N HCl, washed in PTW, fixed in 4% paraformaldehyde, and incubated with proteinase K for 10 minutes at 37ºC (see

Chapter 2 for proteinase K protocol), and washed again in PTW before primary antibody was applied. The secondary antibody used in BrdU labeling was goat-anti-rat-Texas Red for double labelling with the anti-UVS mouse serum; For double labelling of BrdU with *in situ* hybridizations chicken anti-rat Alexa Fluor 488 was typically used in combination with FastRed development of mRNA localization, and BrdU immunohistochemistry began after visualization of the *in situ* hybridization signal. Slides were sometimes counterstained with 200 µg/µl Hoechst 33258 (Sigma) which stains nuclei with a fluorescence detectable with standard DAPI filter sets. Results were visualized and documented using a 12-bit colour Microimager II digital camera (QImaging, Burnaby, BC) mounted on a Zeiss Axioskop 2 with Atto Arc 2 HBO 100 Mercury Arc Lamp. Images were merged and equalized using Northern Eclipse 5.0 (Empix Imaging, Inc.) and Adobe Photoshop 4.0 (Adobe Systems, Inc.).

In situ hybridization protocols to label opsin mRNA on retinal sections were performed as previously described (Chapter 2), wherein label specificity was established. For double labelling with BrdU immunohistochemistry, *in situ* hybridization signals were visualized prior to beginning immunohistochemistry, typically with FastRed (Roche Biochemicals, Nutley, NJ) which produces a red product that is visible using either light or fluorescent microscopy.

I quantified the distributions of UVS cones using both *in situ* hybridizations and immunohistochemistry. For the former, I counted the number of UVS cones and SWS cones labelled on adjacent radial sections, as previously described (Chapter 2). Thus the SWS cone labelling served as a positive control for UVS

cone labelling. I report the results as ratio of UVS: SWS cones for a given location (topographic maps drawn in Adobe Photoshop), wherein a full complement of UVS cones in the retinal mosaic represented by a ratio of 1. I applied a similar strategy in my immunohistochemistry, by counting the number of UVS cones (labelled with rat anti-UVS serum) and number of double cones (labelled with zpr-1) in a given location of wholemount retina. I used Alexa Fluor secondary antibodies for this analysis. The area for counting included at least 40 double cones, and 56 sites were examined per retina. For this quantification, a full complement of UVS cones was represented by the ratio 0.5 (UVS cones : double cones). Both of these quantification methods include very effective controls for the integrity of the labelling and any distortion of the tissue that may occur during processing. The methods are more than accurate enough to report what I view as a qualitative result: *i.e.* my interests and interpretations are focused upon UVS cone presence *vs.* absence.

3.3.3 Apoptosis in CGZ of smolt

To confirm the occurrence of programmed cell death in the CGZ of *O. mykiss* smolts, I took advantage of my observation that in these large fish UVS cones are lost near the CGZ of the ventral retina, but are maintained in the dorsotemporal retina (Chapter 2). I performed terminal deoxyuridine triphosphate nick-end labelling (TUNEL) as per manufacture's protocols (Roche Biochemicals) on radial sections, and counted labelled cone nuclei (i.e. the most scleral nuclei of the ONL) in the 200 μm of retina adjacent to the CGZ. I compared the number of TUNEL labels amongst cone nuclei on an equal number of sections (8-10)

from dorsotemporal and ventrotemporal retina for each of three individuals. I used a paired t-test (with α=0.05) to test the null hypothesis that the number of TUNEL labels was equal between the dorsotemporal and ventrotemporal retinal sections. I made a similar qualitative comparison of wholemount retina adjacent to the CGZ, double labelled with anti-UVS rat serum and Annexin-V (an early marker of PCD, conjugated to enhanced green fluorescent protein; Clonetech, Franklin Lakes, NJ).

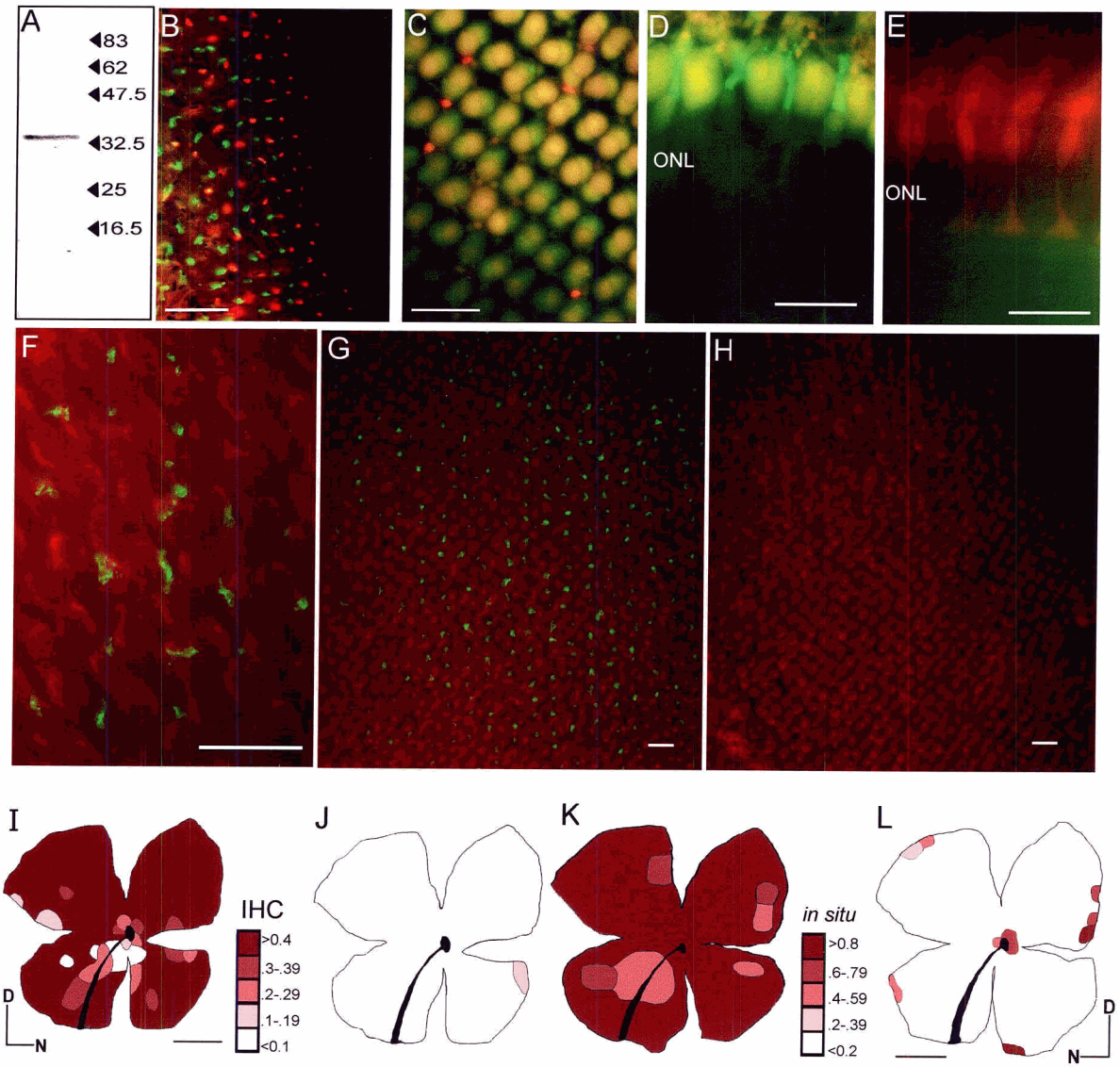
3.4 Results

3.4.1 UVS cone programmed cell death

I developed antibodies against the *O. mykiss* UVS opsin N-terminus to label UVS cones in the *O. mykiss* retina (See Figure 9A-D for assessment of antibody specificity). The antibody appears to label UVS cones in other teleosts as well, including zebrafish (Temple *et al.*, 2003; Allison *et al.*, *In Preparation*), damselfish (Hawryshyn *et al.*, 2002), goldfish and coho salmon (data not shown). My mapping of UVS cone distributions using these antibodies showed that UVS cones disappeared from the retina when trout were treated with TH (Figure 9E-L). I confirmed this result using my previously developed (Chapter 2) *in situ* hybridization labelling of UVS opsin with careful positive controls. These data are consistent with previous findings (Browman & Hawryshyn, 1992, 1994b; Deutschlander *et al.*, 2001) and my electroretinogram (ERG) results (below). TH treatment of *O. mykiss* reduced the distribution of UVS cones in a manner very similar to natural development (Chapter 2). This resulted in a complete loss of

Figure 9. Thyroid Hormone (TH) induces the disappearance of ultraviolet-sensitive (UVS) cone photoreceptors in a manner very similar to natural development. **A.** Western blot of *O. mykiss* retinal protein reacted with rat-anti-UVS polyclonal antibody. Position of markers noted by arrowheads and marker size (kDa). The single band near 34 kDa is close to the predicted size of the UVS opsin and demonstrates the specificity of our rat-anti-UVS serum. This result has been replicated on retinal homogenates from multiple individuals, and negative controls showed no such band. **B.** Double-labeling with mouse-anti-UVS-antiserum (red fluorescent signal) and anti-SWS2-antiserum(Veldhoen *et al.*, 1999) (green fluorescent signal) on wholemount trout retina showed that separate populations of single cones are labelled. The two classes of single cones appear in the expected alternating periodicity based upon previous morphological (Bowmaker & Kunz, 1987; Browman & Hawryshyn, 1992; Beaudet *et al.*, 1993; Browman & Hawryshyn, 1994b) and *in situ* hybridization(Allison *et al.*, 2003) evidence. **C.** Labeling with rat anti-UVS-Ab (red fluorescent signal) on tangential section of retina. As expected, not all the UVS cone outer segments were in the same plane of section (as revealed in adjacent sections). The labels that were present are revealed to be accessory corner cones, as expected, based upon the position of the double cone partitioning membrane (revealed by the green auto-fluorescence in the fluorescein filter channel). **D.** Labeling with mouse anti-UVS-Ab (green fluorescent signal) on radial section of retina. We have obtained very similar results with both mouse and rat polyclonal sera as those presented in panels B-D. **E.** Immunohistochemistry with zpr-1 antibody (red

fluorescent signal) labels double cones in rainbow trout. This wholemount retina was processed in the same manner as that used for quantifications of UVS cone distributions, however the retina was serendipitously positioned to view a radial plane. **F & G.** Wholemount retina from untreated *O. mykiss* used for quantifying UVS cone distributions by calculating the ratio of UVS cones (green fluorescent signal) to double cones (red fluorescent signal). **H.** as per G, but this retina comes from a fish treated with TH. **I & J.** Maps of UVS cone density in control (K) & TH-treated (L) *O. mykiss* determined using immunohistochemistry against UVS opsin and zpr-1 (as per panels F-H). A full complement of UVS cones in a given area is represented by a ratio of 0.5; higher ratios are represented by darker colors in the map. The embryonic fissure (represented in black) extends from the optic nerve head to the ventro-nasal peripheral retina. D, dorsal; N, nasal. Scale bar = 0.5 cm. **K & L.** Maps of UVS cone density in control (I) & TH-treated (J) *O. mykiss* determined using *in situ* hybridization on radial sections. Careful positive controls were employed, such that the number of UVS cones to SWS cones was determined on adjacent radial sections and reported as a ratio, as described previously, wherein the specificity of these labels was defined (Allison *et al.*, 2003). A full complement of UVS cones for K& L in a given area is represented by a ratio of 1.0. In the TH treated fish, UVS cones continue to be generated in the growing peripheral retina. Scale bar = 0.5 cm; In panels B-H the scale bar = 25 μm.

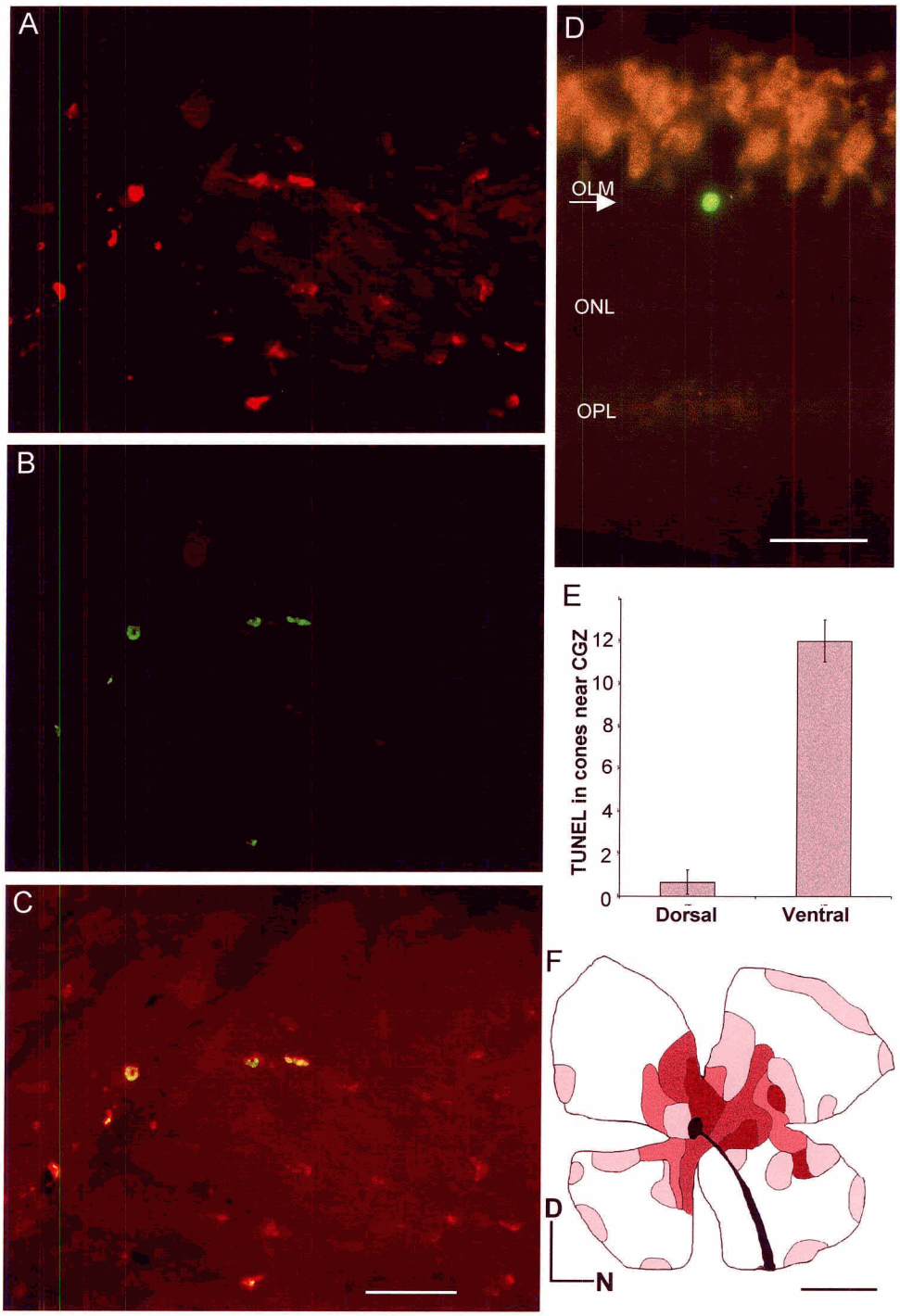

A
83
62
47.5
32.5
25
16.5
B
C
D
ONL
E
ONL
F
G
H
I
IHC
>0.4
.3-.39
.2-.29
.1-.19
<0.1
D
N
J
K
in situ
>0.8
.6-.79
.4-.59
.2-.39
<0.2
L
D
N

UVS cones from the central retina, similar to other salmonid species sockeye salmon (Novales Flamarique, 2000) and Atlantic salmon (Kunz *et al.*, 1994).

Previous data, derived by electron-microscopic examination of Atlantic salmon (*Salmo salar*), indicated that UVS cones were generated in the CGZ of large fish and were immediately removed from the retina by apoptosis (Kunz *et al.*, 1994). Where this process occurred it created mature retina that lacked UVS cones. Our previous data confirmed that similar populations of UVS cones were generated and immediately removed from most of the peripheral retina of *O. mykiss* but not from the dorsotemporal smolt retina (Chapter 2). I exploited this regional difference to examine if *O. mykiss* UVS cones disappear by programmed cell death (PCD) similar to *S. salar*. Double-labelling in five individual *O. mykiss* using my anti-UVS antibody and Annexin V, an early marker of PCD, showed co-localization in photoreceptors of the ventral retina only (Fig. 10A-C). Similarly, TUNEL labelling on radial sections showed significantly ($p<0.02$, $t=9.815$, $df=2$) more TUNEL-labelled photoreceptors (in the scleral ONL where cones reside) near the ventrotemporal CGZ (Fig. 10D & E; where UVS cones disappear) than the dorsotemporal CGZ (where UVS cones remain).

Further, I utilized the controlled onset time of TH-induced UVS cone disappearance (above), and blocked PCD using caspase inhibitor. Mapping the distribution of UVS cones using *in situ* hybridization demonstrated that fewer UVS cones disappeared when TH and caspase inhibitor were applied together, as compared to when TH was applied alone (compare Fig. 10F to Fig. 9L). This experiment represents within-fish data, and was repeated in three individuals. In

Figure 10. Ultraviolet-sensitive (UVS) cone photoreceptors disappear by programmed cell death (PCD). **A.** Immunohistochemical detection of UVS cones in the periphery of the smolt *O. mykiss* ventral retina. Central retina is towards the top of the panel. **B.** Same field as in panel A, with detection of Annexin V conjugated to enhanced green fluorescent protein, an early marker of PCD. **C.** Merged image from panels A & B shows co-localization of Annexin V and UVS opsin in several individual cells. No such fluorescence was observed in the dorsotemporal retina where UVS cones are not disappearing from the retina. **D.** Radial section of smolt *O. mykiss* ventral retina labelled for DNA strand breaks using the TUNEL method. Arrow indicates a labelled photoreceptor in the scleral ONL (green signal, red auto-fluorescence provided for spatial reference). Scale bars for A-D = 25 μm. ONL, outer nuclear layer; INL, inner nuclear layer; IPL inner plexiform layer; OPL, outer plexiform layer; OLM, outer limiting membrane. **E.** The occurrence of TUNEL labels amongst the photoreceptor nuclei was significantly ($p<0.02$, $t=9.815$, $df=2$) greater in the ventral retina, where UVS cones are disappearing. Error bars represent 1 standard deviation. **F.** Map of UVS cone density determined using *in situ* hybridization on radial sections, as per Figure 9K & 1L. This is the contralateral eye to that presented in Fig 9L, the fish receiving thyroid hormone. This eye was treated with caspase inhibitor, and substantially fewer UVS cones disappeared (compare 3F with 1L). These results are representative of data from three individuals. Thus caspase inhibitor, which blocks PCD, blocks the disappearance of UVS cones; therefore UVS cones disappear by PCD. Scale bar = 0.5 cm.


A
B
C
D
OLM
ONL
OPL
E
TUNEL in cones near CGZ
0
2
4
6
8
10
12
Dorsal
Ventral
F
D
N

all three cases, TH eliminated UVS cones from the established retina (Fig. 9L), whereas the disappearance of UVS cones was blocked by caspase inhibitor, injected in the contralateral eye (Fig. 10F). Negative controls for this experiment included sham injection (i.e. no caspase inhibitor, physiological saline, and DMSO; see methods) in the contralateral eye, and other individuals where both eyes received sham injection. In all cases, these negative controls were indistinguishable from the results reported in Figure 9L, where UVS cones were absent from the central retina due to the effects of TH.

3.4.2 Stem cells are the source of UVS cones

Examination of UVS cone distribution in trout several weeks after cessation of TH treatment revealed that UVS cones had reappeared into much of the retina, often at densities similar to untreated fish (Fig. 11). Because there was an almost complete loss of UVS cones during TH treatment, I concluded that these UVS cones were not residual but reappeared into the retina after TH treatment ceased.

In several fish, BrdU was applied during the days following the termination of TH treatment. I completed double-labelling for UVS opsin and BrdU on retinal sections (Fig. 12). I demonstrated that proliferating stem cells in the central retina differentiate into cones that express UVS (i.e. SWS1) opsin mRNA and protein. Thus the source of many of the reappearing UVS cones was stem cells that were proliferating following my application of BrdU. Fourteen cells have been identified with UVS opsin mRNA co-localized with BrdU, amongst the central retinae of five individual fish. No such co-localization was ever observed with SWS2 opsin

Figure 11. Regeneration of ultraviolet-sensitive (UVS) cone photoreceptors into the retina following removal of thyroid hormone (TH). These trout were treated with TH and thus we can conclude they had lost their UVS cones (Fig. 9); here, the UVS cones have reappeared subsequent to removal of the TH treatment. **A.** *In situ* hybridization detects UVS opsin mRNA (purple precipitate) on a radial section of retina. Arrow located at retinal periphery indicates the level of the *in situ* hybridization signal. Scale bar = 100 μm. **B & C** Immunohistochemistry against UVS opsin detects regenerated UVS cones (green fluorescent signal) amongst the double cones (red fluorescent signal) in dorsal wholemount retina. Scale bars = 25 μm. **D.** Representative topographic map of the ratio of UVS : SWS (short wavelength-sensitive) cones, determined by *in situ* hybridization on radial sections, as per Figure 9K & 9L. A full complement of UVS cones in D is represented by a ratio of 1.0. The green line approximates the position held by the circumferential germinal zone (CGZ) when TH treatment was stopped, as determined by an abundance of BrdU labelled cells (BrdU was applied at the cessation of TH treatment). The distance of the regenerated UVS cones of the central retina from the CGZ indicates the source of the cones was proliferating cells in the central retina (see p. 90). **E.** Topographic map of regenerated UVS cones as determined by immunohistochemistry, reported as a ratio to the number of double cones, as per Figure 9I & 9J. A full complement of UVS cones in E is represented by a ratio of 0.5.

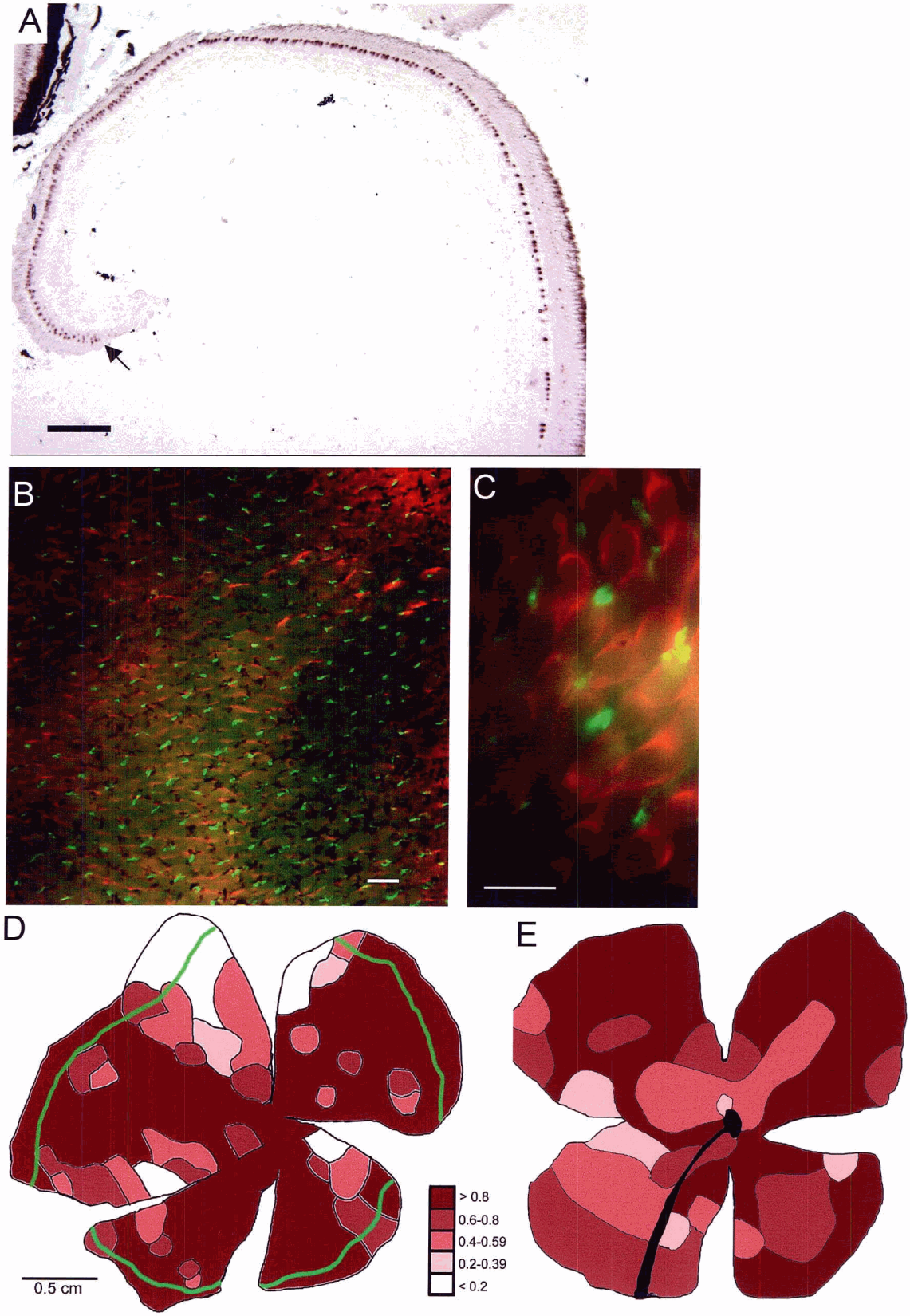
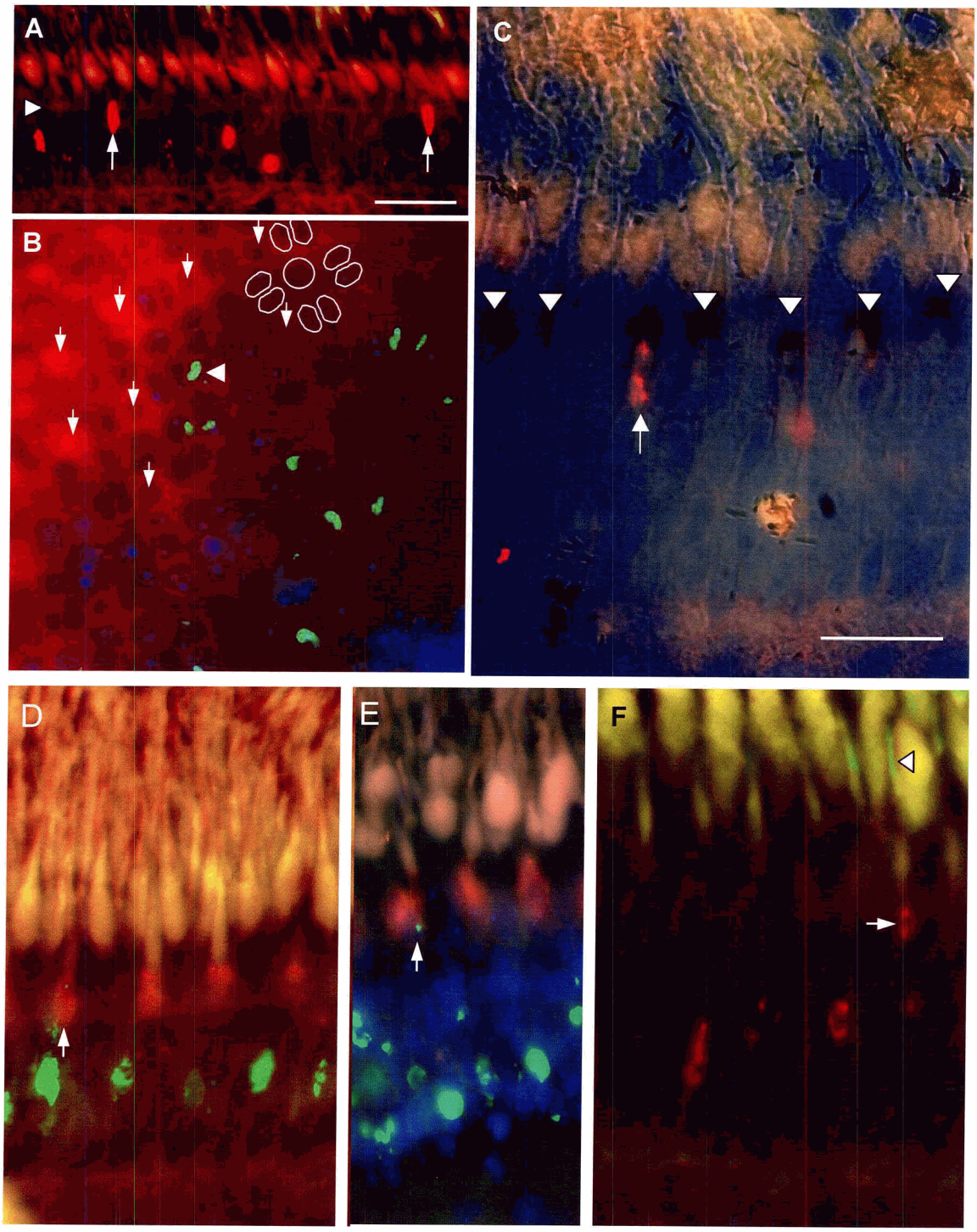

A
B
C
D
E
0.5 cm
> 0.8
0.6-0.8
0.4-0.59
0.2-0.39
< 0.2

Figure 12. The source of reappearing ultraviolet-sensitive (UVS) cones is the proliferating stem cells in the central retina. BrdU was incorporated into the nuclei of UVS cones, but not other cones. Each of these panels is from fish where UVS cones have reappeared into the retina following cessation of TH treatment. Fish were treated with BrdU in the days following the cessation of TH treatment, and it was incorporated into UVS cones. **A.** radial section processed for BrdU (red fluorescence) show two labelled UVS cone nuclei (arrows), as defined by their morphology: protruding through the outer limiting membrane (arrow head) **B.** Approximately tangential section, where the pattern of nuclei (DAPI stained, Blue fluorescence) clearly define the position of UVS cone nuclei. Four pairs of double cone nuclei are outlined, and their partitioning membrane points to the SWS cone, which is also outlined (circle). The UVS cones appear in the corners of this repeating pattern (arrows), as confirmed by the *in situ* hybridization localization of UVS opsin mRNA (red). One of these UVS cone nuclei contains BrdU (green fluorescence, arrowhead). Other BrdU signals are below the cone nuclei, and represent rod nuclei and/or progenitor cells that are prominent at the bottom right in this plane of section. **C.** BrdU (red fluorescence, arrow) is co-localized with UVS opsin mRNA (BCIP/NBT product, resulting in a dark precipitate, arrowheads) detected with brightfield microscopy. Fish sacrificed 5 months after cessation of TH treatment. **D.** Similar to panel C, with BrdU in green fluorescent signal, and UVS opsin mRNA as red fluorescent signal. Arrow indicates UVS cone containing BrdU. **E.** As per panel D, with the addition of DAPI stained nuclei demonstrating that the only nucleus near this diluted BrdU signal is capped with UVS opsin mRNA. **F.** BrdU (red fluorescence, arrow) is contained in a cell that is immunoreactive for UVS opsin (arrowhead). Panel F is the sole result we report where BrdU was applied via intraocular injection (others results obtained by adding BrdU to the fish's water). The BrdU was delivered 11 days after, and the fish was sacrificed five months after, cessation of TH treatment. Scale bars = 25 µm. Scale bar in panel C representative for panels B-F.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mRNA, nor was the BrdU ever found in the nuclei of double cones. Thus I observed that 1) BrdU appears exclusively in UVS cones, not other cones; 2) BrdU incorporates in UVS cones during periods when UVS cones are reappearing. These data demonstrate that proliferating cells are producing UVS cones.

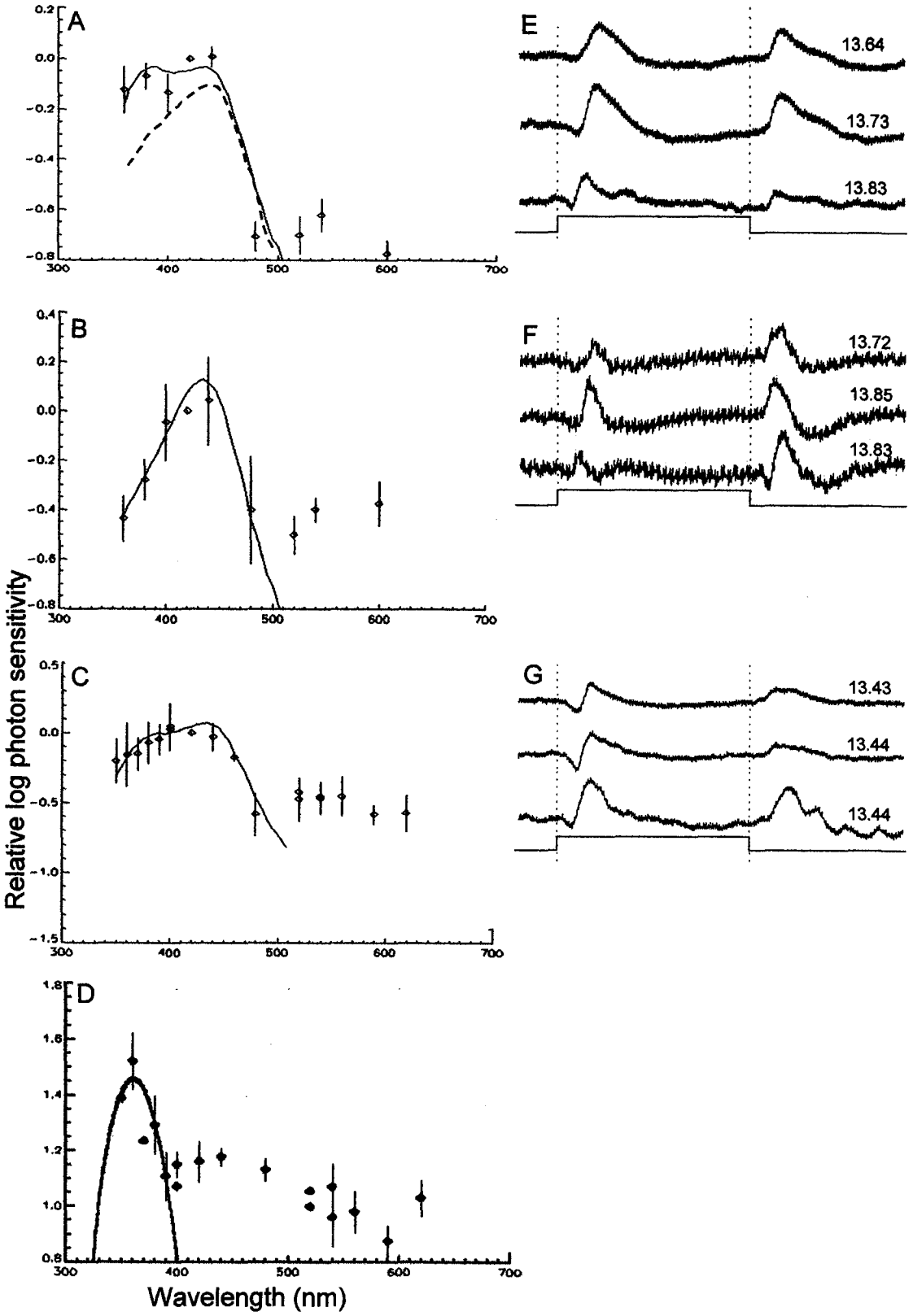
After TH removal, UVS cones reappeared in the central retina, at considerable distances from the CGZ. The position that had been occupied by the CGZ at the time when TH treatment was terminated was approximated by the abundance of cells labelled by BrdU, in the vicinity of the new CGZ. This position was noted in each section and integrated into my maps of UVS cone distribution (green line in Figure 11D). There was a sizable distance (e.g. 1 cm) between UVS cones that had reappeared in the central retina and the CGZ. This indicates that the proliferating cells of the central retina are the most likely source of the reappearing UVS cones.

3.4.3 Functional integration of UVS cones

Electroretinogram (ERG) analysis demonstrated that sensitivity to UV light disappeared during TH-treatment (Figure 13), and the resulting spectral sensitivity was very similar to that measured in smolts (Chapter 2). This finding was consistent with previous analyses of sensitivity to UV light during TH treatment (Browman & Hawryshyn, 1992, 1994b; Deutschlander *et al.*, 2001). Several weeks after the cessation of TH treatment, the sensitivity to UV light had regenerated (Figure 13C), to levels similar to that observed in untreated fish. This is consistent with UVS distribution maps above, and with previous

Figure 13. Electroretinograms demonstrate that the fish's sensitivity to UV light disappears and regenerates in coordination with the disappearance and reappearance of ultraviolet-sensitive (UVS) cone photoreceptors. **A.** Spectral sensitivity of small trout (parr, data points represented by diamonds) has sensitivity to UV light (360-380 nm) similar to the sensitivity to light at 420 nm. The solid line represents the best-fit weighting of UVS and SWS cone mechanisms. During natural development this sensitivity to UV light decreases in larger fish (smolts, dashed line). This panel is modified from a previous presentation(Allison *et al.*, 2003). The coefficients of the cone mechanisms in the best-fit weighting of the parr were K_{UVS}=0.556, and K_{SWS}=0.963. **B.** Spectral sensitivity of small parr treated with thyroid hormone (TH) for two weeks, shows a lack of UV sensitivity, very similar to the sensitivity of larger fish during natural development (compare to the dashed line in A). n=3, K_{UVS}=0.237, and K_{SWS}=1.125. **C.** Spectral sensitivity of fish treated with TH and subsequently maintained without TH treatment for 10 weeks. The sensitivity to UV light has regenerated. n= 6, K_{UVS}=0.522, and K_{SWS}=1.066. **D.** This difference spectrum confirms that the regenerated sensitivity to UV light was mediated by an independent UVS cone mechanism. The spectral sensitivity of three of the fish that were examined in panel C was determined again with UV light included in the background light, which specifically adapted the sensitivity to UV light. The difference of the two spectral sensitivities is shown, and the solid curve represents a template of the UVS cone sensitivity (λ_{max}=360 nm) fit by hand (see Methods in Parkyn & Hawryshyn, 2001). Panels **E-G** show the waveform of

responses to a 500 msec monochromatic stimulus from each of the spectral sensitivity results in A-C, respectively. The responses to 380, 420, and 540 nm (top to bottom) stimuli with similar intensities (in log photons·cm^{-2}·s^{-1}·nm^{-1}; intensity appears at the end of each trace) are all presented in each individual panel. Spectral sensitivity was determined with the fish adapted to a bright yellow background field (500 nm long-pass filter), allowing a comparison of the UVS and short wavelength-sensitive (SWS) mechanisms by adapting (decreasing) the sensitivity of the other cone mechanisms. The adaptation conditions also ensure the responses to UV light are not mediated by the β-band of the other cone mechanisms.


A
B
C
D
E
F
G
13.64
13.73
13.83
13.72
13.85
13.83
13.43
13.44
13.44
Relative log photon sensitivity
Wavelength (nm)

psychophysical experiments (Browman & Hawryshyn, 1994b). I confirmed that the regenerated sensitivity to UV light represented an independent cone mechanism by repeating ERGs under different photic background conditions that used UV light to specifically light adapt (decrease the sensitivity of) the UVS cone mechanism (Figure 13D).

3.5 Discussion

The various lines of evidence presented above support the hypothesis that the remarkable ontogeny of salmonid UVS cones occurs through cell death and subsequent regeneration from proliferating stem cells. The UVS cone death appears to be associated with salmonid migration from shallow freshwater to deeper water, e.g. marine environments. Its regeneration may be preparatory for the return migration to, or behavioural requirements upon, the freshwater spawning grounds.

I have demonstrated that programmed cell death (PCD) of UVS cones occurs in naturally developing retina, particularly in the peripheral retina of *O. mykiss* smolts. This proliferating peripheral tissue gives rise to UVS cones which are removed from the ventral retina immediately after they differentiate (Chapter 2), in a "conveyer belt fashion" (Kunz *et al.*, 1994). This contrasts with dorsotemporal areas of *O. mykiss* retina, where UVS cones are not removed (Chapter 2). I used TUNEL labelling and anti-UVS opsin antibody / Annexin-V double-labelling to compare the areas. The differences in labelling between these regions demonstrated that UVS cones disappear by PCD. My data confirm a detailed electron micrographic study that examined peripheral retina of an Atlantic

salmonid from a more basal genus, *Salmo salar* (Kunz *et al.*, 1994), and this conclusion may be general to the Pacific salmonids (genus *Oncorhynchus*).

The sequence of CGZ development in adult teleosts closely recapitulates embryonic retinal development (Otteson & Hitchcock, 2003). The differentiation and disappearance of UVS cones is an example of this, because the process occurs both in the developing central retina and the peripheral retina of larger fish (Chapter 2). My claim that UVS cones differentiate before their death (Chapter 2) was confirmed in the current work by immunohistochemical detection of the UVS opsin protein in peripheral photoreceptors (e.g. Fig 10A). Not surprisingly, my current data also demonstrated that the *mechanism* of UVS cone disappearance, PCD, is recapitulated in the central and peripheral retina. Whereas TH treatment led to the loss of UVS cones from the entire central retina, caspase inhibitor blocked programmed cell death in the contralateral eye, and drastically limited UVS cone disappearance. These data provide evidence to exclude dormancy as a primary mechanism, and that PCD is the mechanism of UVS cone disappearance.

The TH treatment used in these experiments induced pathways very similar to natural development, where surges in TH blood levels are associated with the metamorphic events (Hoar, 1988). The decreased sensitivity to UV light I observed during TH treatment was not discernible from natural ontogeny, similar to previous findings (Browman & Hawryshyn, 1992, 1994b; Deutschlander *et al.*, 2001). Deiodinases, enzymes that activate and deactivate TH in an organ-

specific manner, have been observed to have high activity levels in *O. mykiss* retina (Plate *et al.*, 2002).

3.5.1 Regeneration of UVS Cones

When TH treatment was removed, the distribution of UVS cones returned to a state similar to that of untreated fish. This provided us with a controlled onset time for the UVS cone reappearance, which I considered prerequisite to any study of the mechanisms. For example, if I had limited my studies to natural development only, the appropriate timing of BrdU application would have been difficult to estimate, and impractical to replicate, due to expected variation in the growth trajectories of salmonids. Most importantly, a negative result using BrdU in such experiments would have been impossible to interpret with certainty.

When BrdU was applied shortly after the cessation of TH treatment, and fish were allowed to survive for several more weeks, I noted that BrdU was contained in some single cone photoreceptors, but not in double cones. When BrdU was co-localized with mRNA for UVS (i.e. SWS1) or SWS2 opsin, it was found that only UVS cones contained BrdU. My *in situ* hybridization and immunohistochemical labels were specific for different domains of the UVS opsin, congruent with the transmembrane domains and the extracellular N-terminus, respectively. The differentiation of stem cells to UVS cones was thus demonstrated at the levels of opsin mRNA and opsin protein expression. BrdU was not observed in cones of the central retina if it was applied to control fish or during the first weeks of TH treatment, indicating that no substantial BrdU incorporation was occurring through DNA repair mechanisms. The time between

the termination of TH treatment and the examination of regenerated cones was sufficient for stem cells to differentiate into cones, which can occur in less than 20 days in goldfish (Wu *et al.*, 2001). BrdU was incorporated into several UVS cones, but never into other cones, and only if it was applied during the period when UVS cones reappeared. Together, these data demonstrated that the reappearing UVS cones were generated from a source of proliferating cells.

Experiments utilizing localized damage have established that the source of new cells in the regenerating teleost retina is not the CGZ, but it is the centrally located proliferating cells in the mature retina (Raymond *et al.*, 1988b; Raymond & Hitchcock, 1997; Wu *et al.*, 2001; Otteson & Hitchcock, 2003). In all vertebrate retinae, proliferating cells move radially to their final location, within a few cell diameters of their origin. The restricted lateral migration exhibited by progenitor cells, combined with the sizable lateral distance (up to 1 cm) between the periphery and regenerated UVS cones, makes it unlikely that the CGZ was the source of new UVS cones. I conclude that the proliferating stem cells known to occur in the central, mature salmonid retina (Ahlbert, 1976; Julian *et al.*, 1998; Candal *et al.*, 2001; Faillace *et al.*, 2002) are the source of these regenerating cones.

It should be noted that my experimental design does not formally eliminate the possibility that some UVS cones are becoming dormant, although my results clearly establish that PCD and regeneration are major mechanisms of the UVS cone ontogeny.

I feel it is reasonable to extend these conclusions regarding the source of reappearing UVS cones to unperturbed salmonid ontogeny; I assert that regeneration represents the major source of reappearing UVS cones during natural development of salmonid retinae. In various *Oncorhynchus* species, evidence exists that the UVS cones return near the time of sexual maturity (Beaudet *et al.*, 1997; Novales Flamarique, 2000). Observations in *Salmo salar* are also consistent with this (Ahlbert, 1976). Changes in TH levels occur during sexual maturation and migration of salmonids (reviewed in Cyr & Eales, 1996), and application of GnRH analogue (mimicking sexual maturation) significantly affects plasma TH levels and retinal TH metabolism (Plate *et al.*, 2002). In regards to the alternate hypothesis (i.e. dormancy) I note that during natural development of this species, and one from a more basal salmonid genera, the disappearing UVS cones undergo apoptosis (Kunz *et al.*, 1994, current study). Therefore, occurrence of dormant UVS cones is negligible. Stem cell regeneration is the only alternative mechanism to the dormancy hypothesis known at this time. My data showing BrdU incorporation in reappearing UVS cones confirms that stem cells can give rise to UVS cones that integrate appropriately in the retinal mosaic, intercalating between existing photoreceptors and RPE cells. Further, the population of stem cells proliferate at an impressive rate in the quickly-growing trout retina; This facilitated the use of *O. mykiss* for the first identification of PINCs in a post-embryonic fish (Julian *et al.*, 1998). The PINCs have been proposed to be the source of regenerating cones in damaged goldfish retina (Wu *et al.*, 2001). Indeed, the area of *O. mykiss* retina where

these stem cells are most responsive to experimentally-induced damage (Faillace *et al.*, 2002), regardless of the location of retinal damage, overlaps with the area where UVS cones reappear during natural development (Beaudet *et al.*, 1997; Novales Flamarique, 2000).

Ultimately, the most important aspect of retinal regeneration is the restoration of visual function (Raymond & Hitchcock, 1997). I examined spectral sensitivity using ERGs and demonstrated that regenerated UVS cones are functional. This is consistent with psychophysical measurements in similar experiments treating *O. mykiss* with TH (Browman & Hawryshyn, 1994b). My measurements using adaptation with UV light allowed me to convincingly conclude that the regenerated sensitivity represents an independent UVS cone mechanism. Importantly, these ERG results focused on the b-wave of the evoked response and primarily represent bipolar cell activity, with possible small contributions from third order neurons (Stockton & Slaughter, 1989; Tian & Slaughter, 1995; Awatramani *et al.*, 2001). Therefore the regenerated UVS cones had made functional connections such that they are able to transmit information to higher order neurons.

My conclusions refine previous interpretations on the role of proliferating cells in the central teleost retina. The pluripotent character of these cells has been recognized from experiments inflicting retinal damage. It has been widely held, however, that during natural development these cells give rise solely to rod photoreceptors as the retina stretches, thus maintaining rod cell densities. My results strongly implicate a broader potential for these cells during natural

development, suggesting that they can give rise to UVS cones during normal ontogeny in salmonid fishes and possibly in other species. I have not determined which population(s) of proliferating cells in the central retina are the source of UVS cones. Recent data support the proliferating cells of the inner nuclear layer (PINCs) as the source of cones regenerating in damaged goldfish retina (Wu *et al.*, 2001). However, I am not aware of data that formally exclude the "rod progenitors" of the outer nuclear layer from generating non-rod cell types during normal development. Thus, rod progenitors are another potential source for reappearing UVS cones, suggesting that future consideration may be given to revision of progenitor cell terminology. It is also possible that the progenitor populations are replenished by the de-differentiation of glia (reviewed in Raymond & Hitchcock, 1997; Faillace *et al.*, 2002; Otteson & Hitchcock, 2003). This is supported by studies of chick retina which demonstrated that in response to damage proliferating cells express glial markers and could differentiate into neurons (Fischer & Reh, 2001).

3.5.2 Signalling Mechanisms in UVS Cone Ontogeny

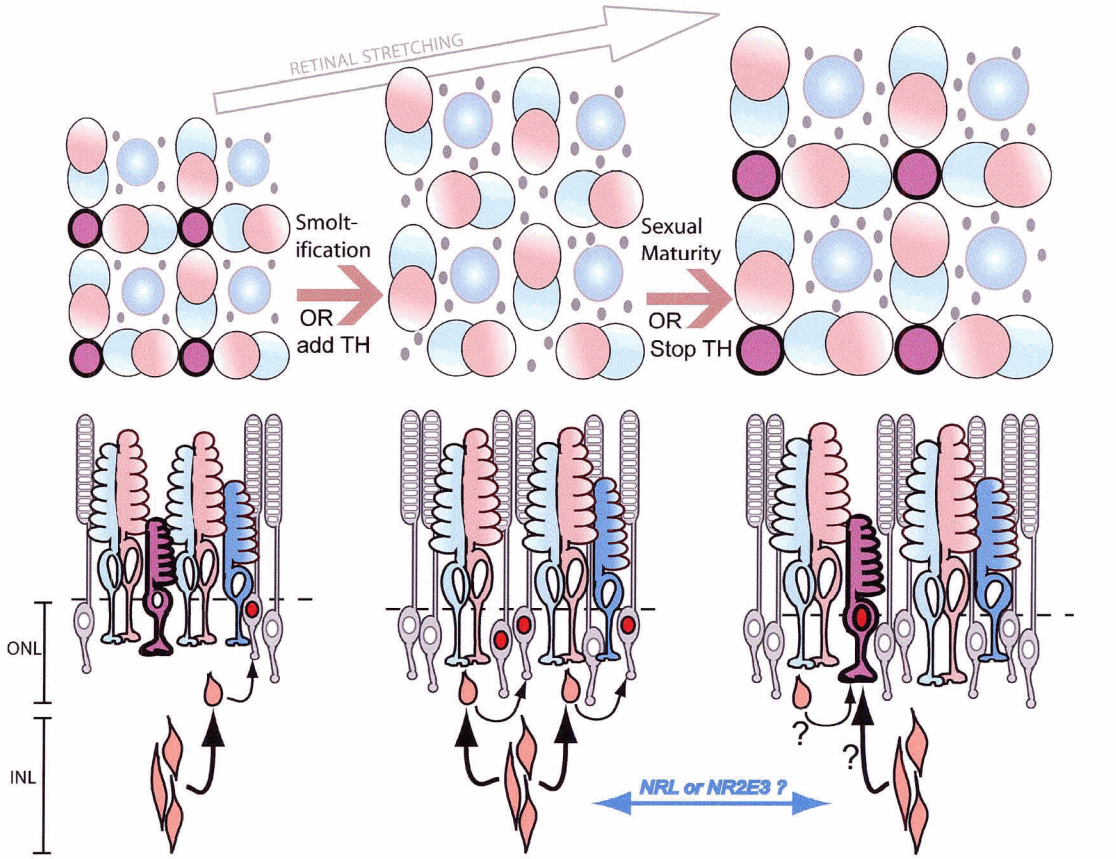
My ongoing efforts now consider the signals and gene expression that instruct or permit the retinal progenitors to produce UVS cones. One might speculate that the increased growth rate of the salmonid eye compared to other fish creates, or allows, greater stretching of the retina. This would change the spatial cues that differentiating cells experience, and may affect their fate. Indeed, stretching plays a substantive role in salmonid retinal growth (Lyall, 1957b; Ali, 1964) and it has been shown that TH can affect the stretching of *O. mykiss* retina (Hawryshyn *et*

al., 2003a). Spatial cues are of importance in generating the photoreceptor mosaic and determining cone cell fate during embryonic development (Stenkamp *et al.*, 1997; Easter & Hitchcock, 2000; Stenkamp & Cameron, 2002). Furthermore, stretching of the retina has been proposed as an important cue for cone regeneration subsequent to surgical damage (Cameron & Easter, 1995; Easter & Hitchcock, 2000). It is noteworthy in this regard that UVS cones are the last cones to differentiate during embryonic and peripheral retinal development of cyprinids, and this can probably be extrapolated to other fishes with similar cone mosaics. Thus the regenerating UVS cone might experience a spatial environment similar to the cellular environment during embryonic UVS cone generation, wherein the other cone classes are differentiated and positioned in a geometrical mosaic.

I have summarized my current understanding and working hypotheses of salmonid UVS cone ontogeny in Figure 14. Near the time of salmonid sexual maturation, progenitor cells, previously thought to be restricted to rod cell fate during natural development (Johns & Fernald, 1981; Johns, 1982; Raymond & Hitchcock, 1997; Faillace *et al.*, 2002; Otteson & Hitchcock, 2003), give rise to UVS cones. Here I note a striking parallel in *O. mykiss* retinal stem cell differentiation to some pathways elaborated in mammalian embryonic retinal development. In particular, phenotypes of mutations in the gene *NR2E3* (or *PNR*; resulting in human enhanced S-cone syndrome), and its upstream modulator *NRL*, include an increase in S-cones (homologue of the *O. mykiss* UVS cone) being generated from retinal progenitors, at the expense of generating rod

Figure 14. Summary and working hypotheses regarding the ontogeny of ultraviolet-sensitive (UVS) cones (represented by violet circles & small violet cones) in *O. mykiss*. The top row represents a tangential section of retina, revealing the square mosaic of double cones (DC; represented by green/pink circles), short wavelength sensitive (SWS) cones (blue circles) and numbers of rods (small dots amongst cones). The lower panels represent radial sections. **A.** Small trout (parr) possess a mosaic with a full complement of UVS cones (UVS:SWS ratio=1; UVS:DC ratio=0.5). Rod precursors and proliferating inner nuclear layer cells (PINCS) of the outer and inner nuclear layers, respectively, are known to be present in the retina of teleosts, including salmonids, and generate rods at a basal rate. This increases the number of rods per mosaic unit as the retina stretches, such that the rods stay at an approximately equal density throughout ontogeny. **B.** During metamorphosis into larger fish, which prepares migratory populations for the marine environment, or during thyroid hormone (TH) treatment of small fish, UVS cones disappear by programmed cell death. This may have adaptive value. For example, decreasing detection of scattered UV light may be important to larger fish with increasing distances to visual targets. Also, more rods seem to be added to the retina at this time (Ahlbert, 1976; Kunz *et al.*, 1994) , possibly via an up-regulation of the progenitor cell proliferation when fish move to deeper water (see Chapter 5). **C.** Near sexual maturity, or after cessation of the above TH treatment, UVS cones reappear into the retina at their original location and at similar densities to untreated fish. The cones are functional, and communicate effectively with higher order neurons.

The incorporation of BrdU into these reappearing UVS cones show that they are generated from the progenitor cells. *This is the only known example of cones regenerating during natural development.* The data re-define the teleost retinal progenitor cells (at least for *O. mykiss*) which were widely held to only generate rods during natural development. We have not yet discovered if it is the progenitors of the ONL or INL that are giving rise to the UVS cones. Regardless, there is a switch in the fate of the progenitor cells from producing rods to producing UVS cones. Noting the impressive growth rate of the salmonid eye, we hypothesize that retinal stretching may permit and/or instruct the progenitor cells to differentiate into UVS cones. Further, the switch in fate is strikingly similar to that of mammalian retinal progenitors during embryonic development when the function of *NRL* or *NR2E3* genes are disrupted. Mutation of *NR2E3* in humans, for example, produces enhanced S-cone syndrome, characterized by an increase in S-cones (homologues of the UVS cone) being produced at the expense of rods, which are absent in this syndrome.



A. **Small trout (parr)**
- *has UVS cones*
- *basal level of rod produciton*

B. **Larger trout (smolt) or TH-treated parr**
- *UVS cones lost by PCD*
- *new rods produced from INL & ONL progenitors*

C. **Sexually mature trout or cessation of TH-treatment**
- *UVS cones reappear*
- *Source of UVS cones could be INL &/or ONL progenitors*

photoreceptors (Mears *et al.*, 2001). Thus *NR2E3* appears to be involved in switching retinal progenitor differentiation from a rod to S-cone (UVS cone) cell fate. Unfortunately, I am not aware of homologues of *NR2E3* or *NRL* being identified in teleosts. Similar to the genes above, mutation of a subclass of thyroid hormone receptor, *THRβ-2*, results in an increase in UVS cones, at the expense of M-cones, in mice (Ng *et al.*, 2001). Thus TH has been implicated as having a role in the differentiation of the UVS cone cell fate in both salmonids and mice. One level of potential interaction between *NRL* and *THRβ-2* has been investigated in mice, with evidence indicating they have distinct functions in retinal cell differentiation (Yanagi *et al.*, 2002).

Many details of the signaling pathways leading to UVS cone PCD remain to be elucidated. It is clear however, that TH treatment can mimic the cell loss (Browman & Hawryshyn, 1992, 1994b; Browman *et al.*, 2001Current Data) and the PCD mechanism. Retinoic acid can also induce UVS cone loss (Browman & Hawryshyn, 1994a), and this may involve heterodimerization of retinoic acid receptors with TH receptors (Puzianowska-Kuznicka *et al.*, 1997). Hawryshyn and colleagues have recently demonstrated that two transcription factors, NF-KB and AP-1 (*i.e.* c-jun), bind to the UVS opsin promoter, but not to the other opsin promoters in rainbow trout (Dann *et al.*, 2002; Dann *et al.*, 2003a; Dann *et al.*, 2004b). These transcription factors are of interest because they are known to interact with TH signaling mechanisms (Perez *et al.*, 1993; Liden *et al.*, 1997; Lee *et al.*, 2000), and to be modulated during photoreceptor PCD (Hafezi *et al.*, 1997; Hafezi *et al.*, 1999; Krishnamoorthy *et al.*, 1999; Wenzel *et al.*, 2002). Thus it

may be that the UVS opsin is being down-regulated by these transcription factors and this leads to PCD, similar to the PCD of rod photoreceptors when rod opsin is not expressed properly. Although this may not be the primary signaling mechanism of TH action (e.g. TH induces PCD in many cell types in a variety of tissues), it remains unanswered why a photoreceptor would regulate its opsin while in the midst of PCD. Finally, it may be noteworthy that a novel protein has been isolated that appears to be regulated in trout retina, but not other tissues examined, during smoltification and this protein could be playing a role in retinal developmental events (Dann *et al.*, 2003b).

The effect of TH on UVS cone ontogeny appears to depend on the time when it is delivered. In small rainbow trout TH induces UVS cone loss (Browman & Hawryshyn, 1992; 1994b, see also Chapter 3), whereas in larger rainbow trout it induces UVS cone regeneration (Browman & Hawryshyn, 1994b; Hawryshyn *et al.*, 2003a). This observation appears to have been extended to Atlantic salmon (Browman *et al.*, 2001). Similar paradoxical effects of TH have been observed in chromophore shifting in coho salmon (Alexander *et al.*, 2001). These paradoxical effects are difficult to explain with our current knowledge. Likely explanations involve differences in the retina itself when TH is applied. Ontogenetic changes in deiodinase enzymes or TH receptors could mediate different effects of the hormone (reviewed in Chapter 1). Furthermore, differences in the amount of retinal stretching (*i.e.* in retinal cell spacing) could have important impacts on the types of cells generated. Another hypothetical mechanism is that TH might induce a dramatic increase in UVS opsin expression within particular retinal cells;

this could produce an excitotoxicity in the UVS cones on small fish, and result in the differentiation of retinal stem cells into UVS cones in large fish. There is little support for the idea that over-expression of opsins leads to cell death, however knockout of *GRK*, a gene that deactivates opsin after it is excited by light, can lead to light-induced photoreceptor apoptosis (Chen *et al.*, 1999a). This hypothesis could begin to be tested by examining UVS opsin mRNA and protein levels in the retina in the hours immediately following onset of TH treatment.

3.5.2 Further implications

One of the initial places where stem cell therapy is expected to be practical in a clinical setting is the repair of retinal damage. The primary goal of retinal regeneration must be two-fold: using stem cells to generate photoreceptors and, second, directing these photoreceptors to integrate into existing neural pathways, thus restoring visual ability. The primary concern in a clinical setting is the restoration of photopic vision, and thus animal models with cone-rich retinae are desirable.

Studies of retinal regeneration in teleosts have been extremely valuable in understanding of retinal repair mechanisms. These studies have invariably used experimentally-induced damage to initiate a regenerative process. The current model has a critical advantage: It allows examination of regenerative events without the confounding effects of damage. To understand the complex mechanisms whereby stem cells differentiate, one must be able to examine both the intrinsic cell machinery as well as the cellular environment that the stem cell experiences; both are known to affect cell fate. Damage induces an inflammatory

response and generates an inconsistent assortment of retinal and immunological cell types. These confounds make an exploration of gene expression somewhat impractical. The current data introduces the only known example of cone photoreceptors regenerating into a retina during natural development. Furthermore, an endocrine manipulation (i.e. TH treatment) mimics the natural development of the organism, and allows the convenience and power of a distinct onset time for study of the regenerative events. The isolation of progenitor cells that are generating cone photoreceptors, combined with differential analysis of the transcriptome (Dann *et al.*, 2003b) and proteome (Veldhoen *et al.*, 2003), should be fruitful in attempts to understand the pathways required to turn a proliferating stem cell into a functionally integrated cone photoreceptor. The model provides the rare opportunity to examine the regeneration and integration of cones into existing neural retina.

Chapter 4:

Mechanisms underlying apparent survival of rod photoreceptors during photic damage of albino trout[7]

4.1 Abstract

Studies of light-induced damage in albino vertebrates have served as effective models of retinal degeneration. Previous data show that light damage in albino rainbow trout (*Oncorhynchus mykiss*) leads to an expected loss of rod outer segments (ROS), but in contrast to other albino vertebrates the population of rod nuclei in the outer nuclear layer (ONL) does not change. The aim of this study was to differentiate between two hypotheses that could account for these surprising results: (1) rod nuclei remain intact during light damage, or (2) rod nuclei are dying and being replaced by cell proliferation. A further aim was to examine if photic history modulates retinal damage, as in rodents, where it has been shown that pre-exposure to moderate light is protective. Albino and normally pigmented trout with different photic histories were transferred into a regimen of full exposure to daylight, or remained protected from direct sunlight. Histology, TUNEL method, and immunohistochemical assay of proliferating cell nuclear antigen (PCNA) and rod opsin expression were applied to samples collected after 0,2,5,10 and 20 days of exposure. ROS were maintained in pigmented fish regardless of light treatment, and in albinos protected from sunlight. Albinos in full daylight lost ROS in their central retinas, and by day 20, some individuals had lost rod nuclei in a portion of the central retina. In other

[7] The findings presented in this chapter are in preparation for publication and involved a collaboration with Ted E. Hallows, Trudi D. Johnson, Craig W. Hawryshyn, and Donald M. Allen.

areas of the retina, the number of rod nuclei did not change in spite of ROS damage. Rods nearer to the peripheral retina maintained ROS regardless of light damage. Cones remained intact in all retinal samples, including those in which rod nuclei had degenerated.

In light-damaged albinos, pyknotic and TUNEL-labelled cell nuclei were localized to the vitreal half of the ONL in the central retina: thus, areas of cell death were correlated with loss of ROS. I observed an increase in TUNEL labelling of rod nuclei during light damage in each of the 20 fish I examined. This included significantly more TUNEL label after 10 days of damage when compared to undamaged albinos. Increased proliferation (PCNA labelling of nuclei) occurred in the central retina with light damage, and subsequently decreased. This increase apparently replaced lost rods, although this mechanism was not always sufficient and some rods were not replaced. I conclude that apoptotic demise of rods, but not cones, occurs in light-damaged albino trout. This death can be balanced by increased proliferation of rod precursor and/or retinal stem cells (hypothesis #2 above).

Photic history affected cell death and proliferative mechanisms induced by light damage, and the light-induced ectopic localization of rod opsin. This is the first such observation in a non-mammalian organism.

4.2 Introduction

Previous research on albino rodents has been valuable in understanding the toxicology of light damage and photoreceptor programmed cell death (Shahinfar *et al.*, 1991; Li *et al.*, 1996). Damage can be caused by constant illumination with moderate intensity or by cyclical illumination of higher intensity (Organisciak & Winkler, 1994). Factors affecting the susceptibility of photoreceptors to light damage have been shown to include diet, circadian factors, ocular pigmentation, and the history of light exposure. Such studies have also begun to elucidate the molecular pathways leading to photoreceptor programmed cell death (Hao *et al.*, 2002; Reme *et al.*, 2003). Thus, despite their nocturnal habit and rod-dominated retinae, the data from light-damaged rodent retinae have contributed greatly to our understanding of the mechanisms of photoreceptor cell death and retinal degenerations in humans.

Surface-dwelling diurnal fishes can be an attractive complement to the study of nocturnal rodents for several reasons. First, their cone-rich duplex retina, evolved to operate in a bright-light environment, may be considered more representative of the human fovea. Furthermore, the retina continues to grow throughout the life of the fish, and this provides a regenerative potential. Retinal growth occurs through two primary mechanisms (Lyall, 1957b; Ali, 1964; Johns, 1977): 1) addition of new retina by the circumferential germinal zone (CGZ) at the retinal periphery, and 2) stretching of the retina, with progenitor cells producing new rods to maintain their density (Johns & Fernald, 1981; Fernald, 1990). Importantly, continuous growth also gives the teleost retina a regenerative

capacity that could facilitate understanding of repair mechanisms (reviewed in Raymond & Hitchcock, 1997; Easter & Hitchcock, 2000; Raymond & Hitchcock, 2000; Stenkamp *et al.*, 2001; Stenkamp & Cameron, 2002; Otteson & Hitchcock, 2003).

The source of regenerating cells following experimentally-induced retinal damage in teleosts is the progenitor cells of the central retina, as demonstrated by the kinetics of cell division following localized damage (Raymond *et al.*, 1988b; Easter & Hitchcock, 2000; Otteson & Hitchcock, 2003). Two populations of progenitor cells are present in the central retina of fish, defined morphologically as being in the outer nuclear layer (often called rod progenitors) and the proliferating inner nuclear layer cells (PINCs). The latter replenish the former during natural development (Julian *et al.*, 1998). These progenitor cells may be replenished by glia de-differentiating to proliferating cells (reviewed in Fischer & Reh, 2003). These progenitor cells meet every definition of stem cell, including their ability to regenerate every cell type following retinal damage (Wu *et al.*, 2001; Faillace *et al.*, 2002; Otteson & Hitchcock, 2003). Thus, in addition to being effective models of photoreceptor degeneration, light-challenged teleost retina might also contribute to understanding how stem cells can be signalled to replace lost photoreceptor cells.

Rainbow trout, *O. mykiss,* provide an opportune model for study of light damage and regeneration in the retina. The cone photoreceptors, belonging to four cone classes (Hawryshyn & Harosi, 1994; Hawryshyn *et al.*, 2001, see Chapter 2 and 3), are patterned in a mosaic (Browman & Hawryshyn, 1992;

Beaudet *et al.*, 1993; Beaudet *et al.*, 1997, see Chapter 2) that can facilitate an understanding of mechanisms of cone differentiation (reviewed in Stenkamp & Cameron, 2002). The eye and retina of rainbow trout grow quickly compared to other commonly used teleost models (Olson *et al.*, 1999; Easter & Hitchcock, 2000). This has led to the discovery of PINCs in adult retina (Julian *et al.*, 1998), previously identified only in embryonic retinal development (Johns, 1982). The PINCs in rainbow trout increase their rate of proliferation in response to surgical injury (Faillace *et al.*, 2002). Additionally, the potential to regenerate photoreceptors in salmonids (including rainbow trout) appears to be substantial, as indicated by the reappearance of UVS cones late in the fish's natural life-history (Beaudet *et al.*, 1997; Hawryshyn *et al.*, 2003a, see Chapter 3).

Most importantly, raising albino rainbow trout in full sunlight or exposing them to 3000 lux constant incandescent light leads to loss of rod outer segments (ROS) (Allen & Hallows, 1997; Allen *et al.*, 1999). Remarkably, the number of nuclei in the outer nuclear layer (ONL), dominated by rod nuclei, did not decrease (Allen & Hallows, 1997). This was a surprising contrast to observations on light-damage in rodents and other fish where the number of photoreceptor cells is reduced (Allen *et al.*, 1999; Vihtelic & Hyde, 2000). When albino trout were subsequently transferred to dim light conditions, ROS reappeared (Allen & Hallows, 1997). These data led to the formation of two hypotheses (Allen & Hallows, 1997), which are not mutually exclusive: 1) rods were surviving light damage, i.e. ROS were pruned from rod nuclei, which produced new ROS when fish were transferred to dim-light conditions, and 2) Rods were dying and being

replaced immediately by generation of new rods from progenitor cells. The primary objective of this work was to discriminate between these two hypotheses.

The occurrence of cell death and regeneration of photoreceptors has recently been demonstrated in light-damaged albino zebrafish (Vihtelic & Hyde, 2000). However, both of the above hypotheses remain viable for rainbow trout because of important differences between these species in their response to damaging light. Unlike rainbow trout, the ONL cell population of zebrafish was immediately reduced upon light treatment (Vihtelic & Hyde, 2000). Furthermore, while a regenerative potential was clearly established in zebrafish, it occurred after cessation of light treatment (Vihtelic & Hyde, 2000) and thus is not strictly relevant to the hypotheses above. Another distinction is that in albino zebrafish the light treatment led to cone cell death (Vihtelic & Hyde, 2000), whereas in albino trout it did not (Allen & Hallows, 1997). This implicates the existence of a protective mechanism in rainbow trout, although this interpretation could be due to methodological differences (e.g. light intensity).

It has been demonstrated that the loss of ROS in rainbow trout is localized to the central, mature retina (Allen *et al.*, 2001). Rods in the peripheral part of the retina, near the developing CGZ, apparently are not damaged. The protective mechanism may be a combination of reduced light reaching the photoreceptors due to their position behind the iris, and/or an exposure to growth factors due to the proximity of the rods to the CGZ (reviewed in Allen *et al.*, 2001).

Examination of cell death and proliferation in the current work support the hypothesis that rods of albino trout die and the ONL population is maintained by

proliferation. An unexpected decrease in the number of ONL nuclei was observed in localized areas of the central retina of affected albinos, which had not been observed previously in trout. I hypothesized that this was due to the photic conditions used to maintain the fish prior to experimentation, which had included reduced light intensity as compared to previous experiments. This result was verified under a repeated protocol using pre-treatments that varied the photic history of the fish prior to their exposure to intense light.

4.3 Methods

4.3.1 Fish Handling & Dissections

Fish were maintained at Kamas State Fish Hatchery in Summit County, UT. The albino trout (*Oncorhynchus mykiss*) used in this study have an autosomal recessive mutation in the tyrosinase gene (Bridges & von Limbach, 1972).

Two experiments, *Series 1* and *Series 2*, treated normal and albino fish with high-intensity damaging light. *Series 1* was designed to differentiate between the primary hypotheses presented above by testing if increased cell death and proliferation occur during exposure of albino trout to damaging light. Fish were kept in shaded light or moved to direct sunlight to induce damage. During *Series 1*, an unexpected decrease in the number of rod nuclei occurred in localized areas of the retina. This had not been observed before in albino trout. I hypothesized that this was due to the different light regime that fish were maintained in prior to treatment with damaging light. The latter hypothesis was

tested in *Series 2*, which repeated *Series 1* except that fish were maintained in two different light regimes prior to damaging light treatment. The two photic regimes consisted of exposure to moderate light intensities (pretreated) and dim light intensities (not-pretreated) for ten days immediately prior to the treatment.

Series 1. On July 22, 2001, albino and normally pigmented rainbow trout were transferred from a covered raceway (where they had been raised completely protected from direct sunlight) into an open raceway with little opportunity for behavioral avoidance of sunlight. This methodology was differed from previous experiments where trout were raised in outdoor raceways and sampled at various times of the year (Allen & Hallows, 1997; Allen et al., 1999; Allen et al., 2001). Fish were transferred at times in order that eyes could be sampled after periods of 0,2,5,10 and 20 days. Normal fish ranged from 6.5 to 9.0 cm in standard length, albinos ranged from 9 to 13 cm and were 2 months older than normally pigmented fish.

Series 2. On July 26, 2002 albino and normally pigmented rainbow trout which had been kept under indoor cyclic fluorescent light for 1 month remained in place (not pre-treated) or were placed outdoors in shaded daylight for 10 days (pre-treated). Fish from both groups were identified by fin-clip and then placed in full sunlight Aug 5, Aug 10 and Aug 13 and all groups were sampled on August 15. This provided samples of pretreated and not-pretreated fish after 0, 2,5 and 10 days. The 10-day duration was selected because this represented the time-point with the most dramatic change in PCNA and TUNEL labeling during the previous experiment. Several of the albino fish assigned to the pretreated 0 day

treatment group were lost to snake predation, leaving fewer individuals per time point for examination. In Series 2, albino and normally pigmented trout were aged-matched and ranged from 9 to 12 cm at time of sampling.

Light levels for the locations of the fish were measured by Dr. Allen at the water surface. Indoor fish were on a 12h : 12h light : dark cycle under fluorescent lights, whereas fish outdoors were exposed to 13.7h : 10.3h during treatment. The light levels for outdoor exposed fish were about 60,000 – 100,000 lux. The area under the wavelength vs. intensity curve the spectrometer (400-700 nm) reported led to estimates that the trout in the covered raceway (pretreated) received an order of magnitude more light than the indoor (not-pretreated) trout. The light intensity for the exposed fish (receiving damaging light) were estimated to be at least an order of magnitude greater than in the covered raceway. These measurements of light intensities are between 400-700 nm at mid-day. These light levels should be analyzed using a spectroradiometer that can measure into the UV range, which was not available to Dr. Allen. Regardless, the absolute number of quanta and spectral character of light the fish are exposed to in this facility would remain estimates, with light levels changing based on several factors including cloud cover, time-of-day, water depth, and behavioural avoidance of the light.

Enucleated eyes were placed immediately into chilled fixative as appropriate to each method (below).

4.3.2 Histology & Labelling

Plastic sectioning was performed using standard methods as previously described (Allen & Hallows, 1997; Allen *et al.*, 1999; Allen *et al.*, 2001) on eyes fixed in glutaraldehyde & paraformaldehyde. The sclera of these eye was cut in a manner to define orientation of the eye.

Other eyes from each treatment were processed for TUNEL labelling. They were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) and held on ice. These eyes were shipped overnight to Victoria BC. They were rinsed in PBS and dissected into four quadrants. The embryonic fissure, which reaches the peripheral retina at the ventral-nasal aspect of the eye, was used as a landmark. Eyes were cryo-preserved, frozen, and sectioned (10 µm thickness) as described previously (Chapter 2). Each of the four quadrants was frozen such that radial sections were examined from a known location in the retina. After re-hydration, sections were treated with 10 µg/ml proteinase K at 37°C and post-fixed in 4% paraformaldehyde, using nuclease-free solutions. TUNEL labelling and negative controls were performed as per manufacturer's protocols (Roche Biochemicals, Nutley, NJ). Signal produced by TUNEL labelling is a green fluorescent product, which was visualized and documented as described previously (Chapter 2).

Other eyes from each treatment were processed for detection of proliferating cell nuclear antigen (PCNA), which is expressed during S-phase of the cell cycle. Eyes were placed in cold fixative composed of 38% formaldehyde diluted in 9 parts ethanol. These eyes were also shipped to Victoria. Eyes were hemisected along the nasal-temporal axis and cryo-preserved as above.

Sectioning produced radial sections (10 μm) along the nasal-temporal axis. Sections were processed for PCNA immunohistochemistry using standard protocols as described previously (Chapter 2). Primary antibody was mouse-anti-PCNA (PC-10; Santa Cruz Biotechnology, Santa Cruz, CA) and secondary antibody was goat-anti-mouse conjugated to alkaline phosphatase (Vector Labs, Burlingame, CA). Both antibodies were diluted 1:1000 in PBS with 1:50 heat-inactivated horse serum (Sigma, St. Louis, MO). Signal was visualized using BCIP/NBT as per manufacturer's protocol (Gibco/BRL, Gaithersburg, MD), which produces a purple precipitate.

Other immunohistochemistry was performed on sections adjacent to those used for TUNEL labelling. Primary antibodies were mouse-anti-bovine rhodopsin (K16-155C, provided by Dr. Hargrave, University of Florida, Gainesville (Adamus *et al.*, 1991)), previously shown to label rods and one member of the double cones in rainbow trout (Veldhoen, 1996). Presumably, the double cone labelling represents the MWS cones that express a sub-class of the rhodopsin gene, Rh2 (Chapter 2). I also employed mouse monoclonal antibodies zpr-1 and zpr-3 (Zebrafish International Resource Center, Eugene, OR) raised against zebrafish retinal antigens, that label double cones and rod inner-segments of zebrafish, respectively. These were diluted 1:250 and visualized with 1:1000 goat-anti-mouse conjugated to Alexa-Fluor-594 (red fluorescent signal; Molecular Probes, Eugene, OR).

Labelled cells were counted in each section and the length of section measured using image analysis software (Northern Eclipse, Empix Imaging,

Inc.). For PCNA quantifications the optic nerve was defined as the division between nasal and temporal retina, and the labelled cells of the CGZ were not included in the counts. To assess proliferation in the central retina, I also counted the number of PCNA labels in a single field of view (approximately 300 μm) of nasal retina that was one field of view removed from the optic nerve. I examined eyes from three individuals per treatment for the Series 1 TUNEL measurements, and four individuals for all other analyses. I compared values amongst treatments in SPSS software using analysis of variance with a post-hoc Tukey test (α=0.05).

4.4 Results

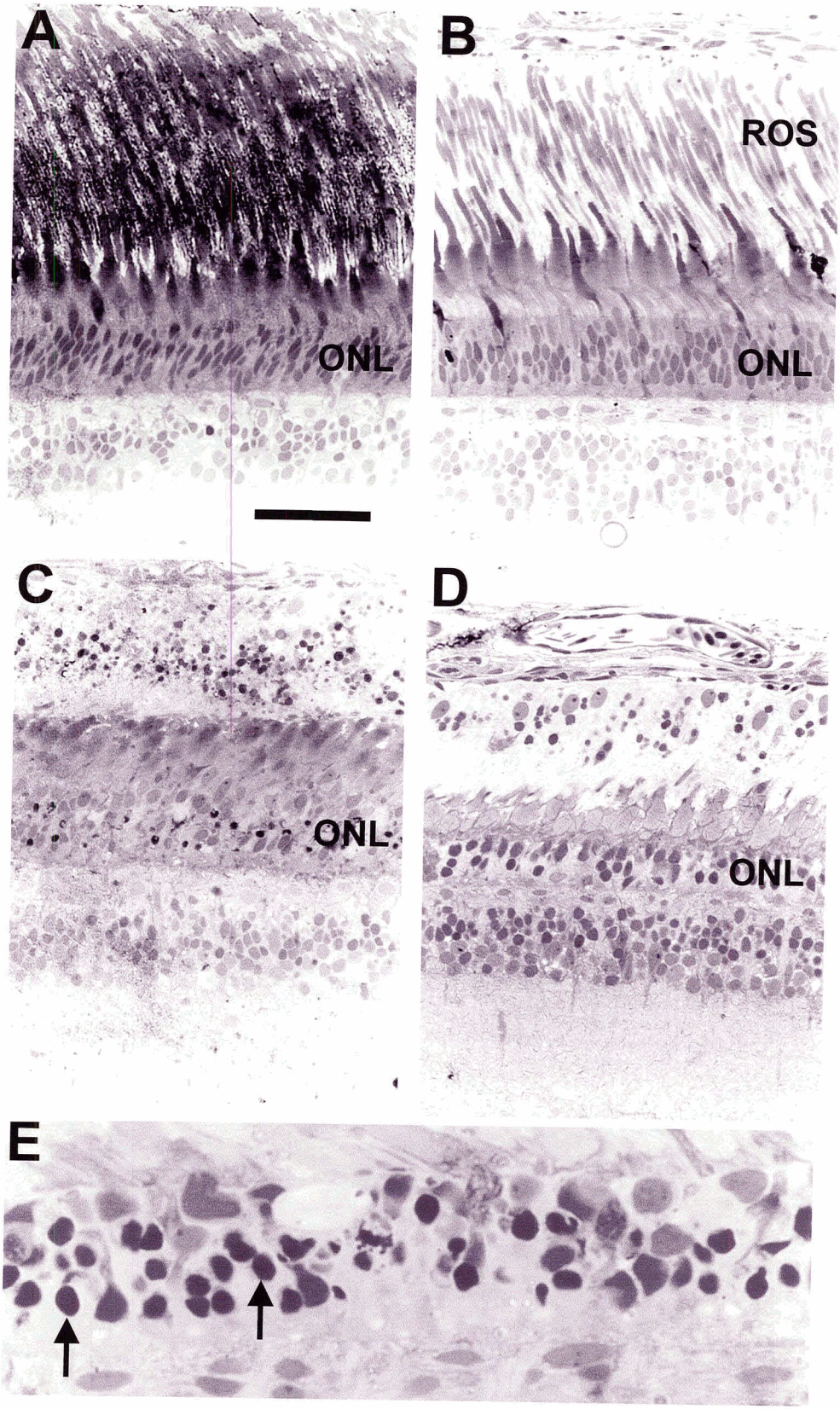
4.4.1 Histology

Plastic sections in radial plane demonstrated that rod outer segments (ROS) and normal retinal structure were maintained in pigmented fish regardless of light treatment (Figure 15A), and in albinos protected from sunlight (Figure 15B) in both years. This was consistent with previous light-damage paradigms on these fish (Allen & Hallows, 1997; Allen *et al.*, 1999; Allen *et al.*, 2001). Albino trout in full daylight lost ROS in their central retinas (Figure 15C), as expected. By day 20 of intense light exposure, some individuals had lost rod nuclei in a portion of the central retina (Figure 15D). This had not been observed in albino trout during previous experiments (Allen & Hallows, 1997; Allen *et al.*, 1999; Allen *et al.*, 2001) where fish were maintained under higher light intensity prior to exposure to damaging light. In other areas of the 20 day treated retina, rod nuclei were maintained despite ROS elimination. In all cases, rods nearer to the peripheral margins maintained ROS, as observed previously (Allen *et al.*, 2001). Cones remained intact in all retinal samples, including those in which rod nuclei had degenerated (Figure 15D). In all light-damaged albinos, pyknotic cell nuclei were observed, and they were localized to the vitreal half of the ONL in the central retina (Figure 15E).

4.4.2 Series 1 TUNEL

TUNEL labelling was used to detect cell death, and determine if there was an increase in rod cell death during high-intensity light treatment of albino trout.

Figure 15. Histology of retina from albino and normally pigmented trout during light damage. **A.** Plastic sections reveal that there is no alteration to retinal structure after 20 days of light treatment on normally pigmented trout. **B.** Albino trout protected from intense light (0 day controls) also show normal retinal structure, except a lack of melanin. **C.** Light damage leads to loss of rod outer segments (ROS) but no change in the number of outer nuclear layer (ONL) nuclei in albino trout treated with light for ten days, which led to the concept that ROS were 'pruned' from the rod nuclei (Allen, 1995). **D.** After 20 days of light damage in albino trout some areas of retina were damaged to an unexpected degree, in that rod nuclei had been lost from the ONL. Further, the area previously occupied by ROS now contains macrophages and degenerated ROS. **E.** Pyknotic rod nuclei were observed in the ONL. E is a magnified view of Panel C. Scale bar is representative for panels A-D and represents 50 μm.


A
ONL
B
ROS
ONL
C
ONL
D
ONL
E

TUNEL labelling on light-damaged albino trout from Series 1 demonstrated unequivocally that many rod photoreceptors were dying during light treatment of albino fish (Figure 16A,B). TUNEL-positive nuclei were localized to the vitreal half of the ONL, and thus were rod nuclei. This was also the same location where pyknotic[8] nuclei were observed in semi-thin plastic sections (Figure 15E). I observed no difference in TUNEL labelling in pigmented fish despite light treatment. Untreated albinos had low levels of TUNEL labelling similar to pigmented fish, however each of the twelve individuals treated with damaging light had elevated TUNEL-positive nuclei in some location of the retina. There was a qualitative increase in cell death at the earliest duration of treatment I examined (2 days) in all but the dorsal retina.

Quantification of TUNEL labelling revealed a significant increase in labelling (total number of labels per individual) in albino trout treated for 10 days as compared to the 0 days (Figure 17A, $p=0.030$). There was considerable variability between individuals, such that different locations exhibited the highest mean levels of labelling at different durations of treatment (Figure 17B). Thus, significant differences were not observed for individual retinal locations in the small sample size I examined (I considered individual fish as the sampling unit). Of the times examined the mean labelling for cell death was highest after 10 days, except in the ventral retina. It is noteworthy that the increases in TUNEL labelling were observable earlier than the disappearance of rod nuclei.

[8] Pyknotic nuclei are rounded, darkly-stained nuclei that are often associated with cells undertaking apoptotic programmed cell death.

Figure 16. Immunohistochemistry and TUNEL labelling during light damage. **A & B.** TUNEL labelling (green fluorescent signal) was localized to the central retina. and the outer nuclear layer in light damaged albino trout (these examples from Series 1, 10 days treatment). **C.** Rod opsin immunohistochemistry on retina from normally pigmented fish and undamaged albinos revealed the expected localization of the label (red fluorescent signal) to the inner and outer segments of rods and one member of the double cones (bar = 25 μm, representative of panels C-F). **D.** Rod opsin expression was reduced from the rod outer segments, and mis-localized in outer and inner nuclear layers (ONL and INL) of light damaged albinos. **E.** Similar mis-localization was apparent for zpr-3 immunoreactivity (red fluorescent signal) that labels rod inner segments (and is not normally in the ONL). **F.** no such mis-localization was apparent for zpr-1 immunoreactivity (red fluorescent signal) that labels double cones inner segments and cone pedicles (compare to Figure 11E). Panels D through F, with signal localization in red, have been merged with an image of autofluorescence (green) as a reference for location.

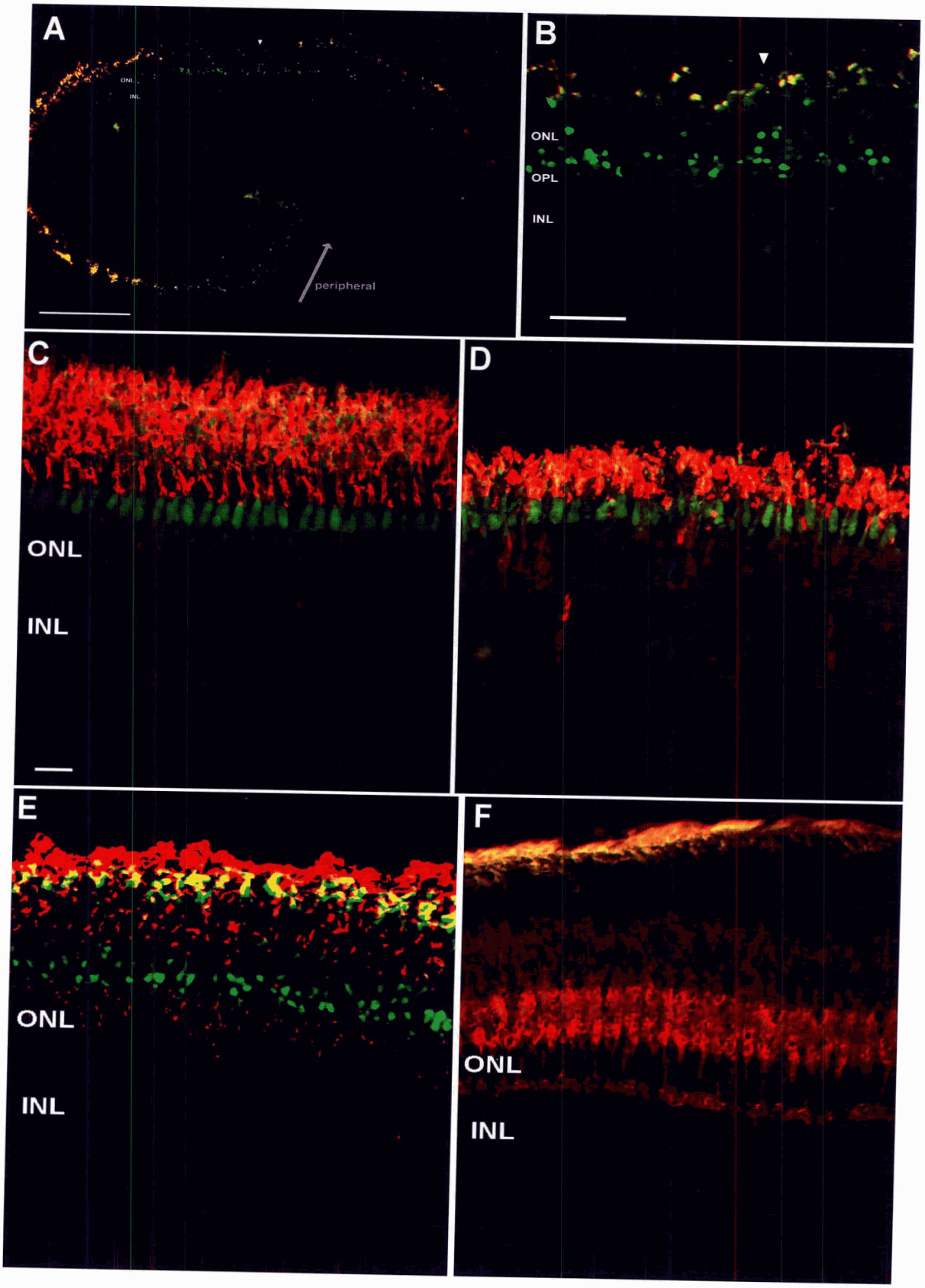
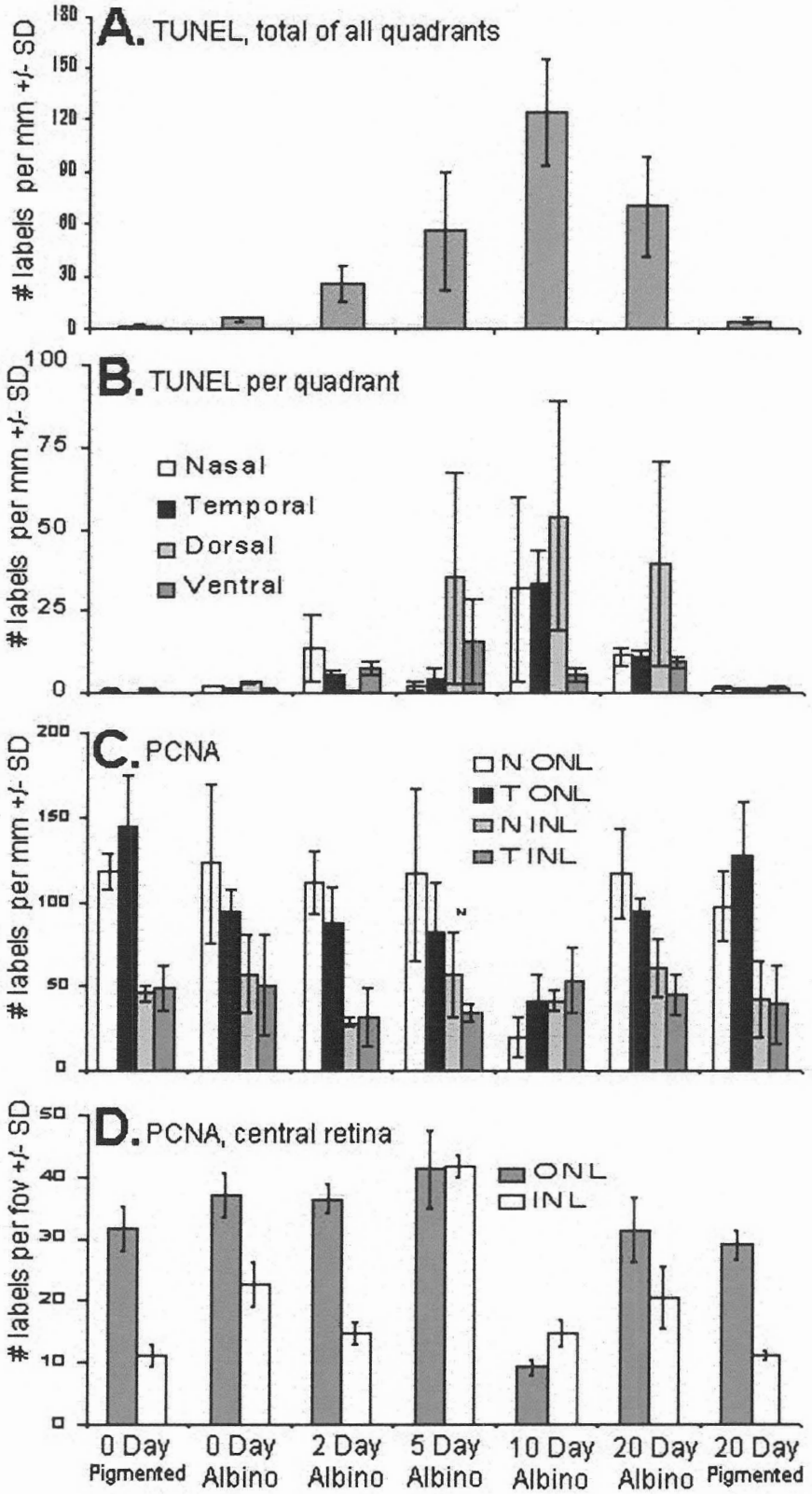

A
ONL
INL
peripheral
B
ONL
OPL
INL
C
ONL
INL
D
E
ONL
INL
F
ONL
INL

Figure 17. Quantifications of TUNEL labelling from Series 1 albino and normally pigmented trout treated with light for various durations. Light modulates cell death and proliferation in albino trout. (Experiment Series 1). TUNEL and proliferating cell nuclear antigen (PCNA) labelling, indicating cell death and proliferation, respectively, quantified in various areas of albino and normally pigmented rainbow trout treated with various durations of light. **A.** TUNEL labelling of rod nuclei in four retinal locations. There was a significant difference between 0 and 10 days of light damage in albino trout when each location was summed. **B.** No significant differences in TUNEL labelling were measured for a given location considered on its own. Normally pigmented trout showed no such increase regardless of light treatment or quantification strategy. **C.** PCNA labelling in the outer and inner nuclear layers (ONL and INL) of the entire nasal or temporal retina (excluding the CGZ). In albino trout, PCNA labelling of the nasal ONL significant decreased at 10 days compared to all other time-points. **D.** PCNA labels in ONL & INL of a field of view (FOV, approximately 300 μm) in the central retina, measured on the nasal retina at a distance of one FOV from the optic nerve. Results are similar to those in panel B, except that amongst albino trout the number of labels significantly increased in the INL when 5 days treatment was compared to earlier time-points.


A. TUNEL, total of all quadrants
labels per mm +/- SD
B. TUNEL per quadrant
Nasal
Temporal
Dorsal
Ventral
C. PCNA
N ONL
T ONL
N INL
T INL
D. PCNA, central retina
ONL
INL
labels per fov +/- SD
0 Day Pigmented
0 Day Albino
2 Day Albino
5 Day Albino
10 Day Albino
20 Day Albino
20 Day Pigmented

4.4.3 Series 1 PCNA

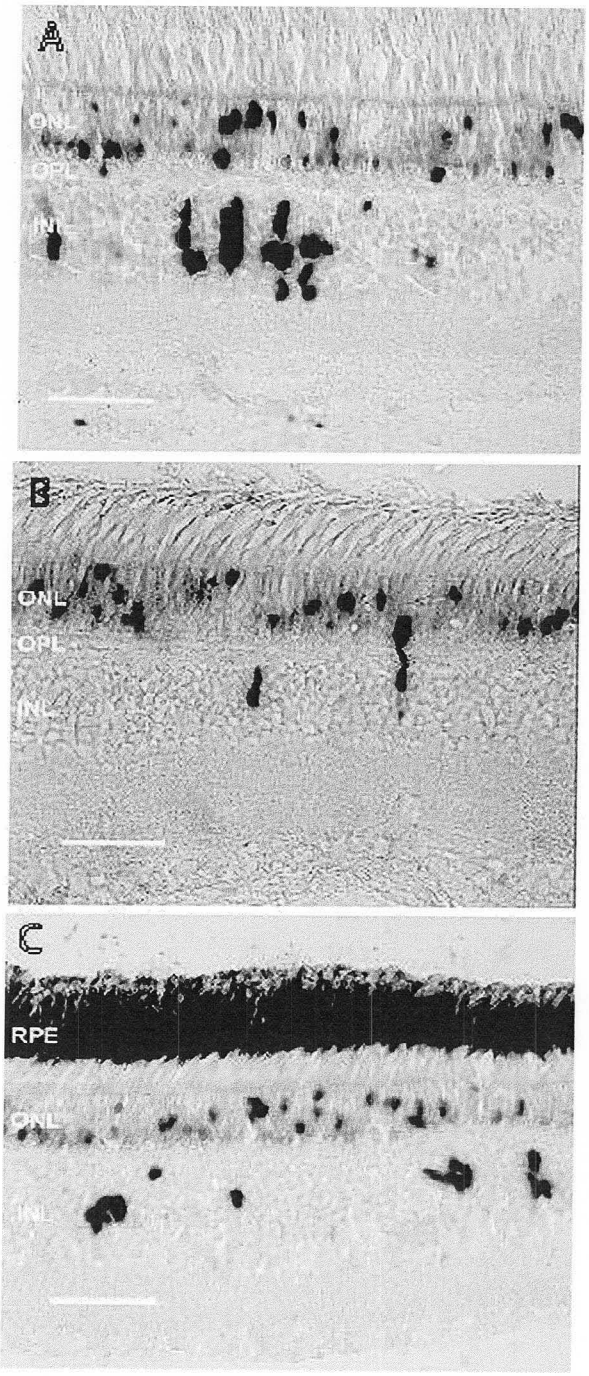
PCNA immunohistochemistry was used to detect levels of proliferation in the retina, and address whether proliferation was increased during damaging light treatment. I quantified PCNA labelling across the entire retina, and subsequently quantified the labelling in an area limited to the central retina (see Methods).

PCNA labelling revealed the expected populations of dividing cells (Figure 18) based on previous examinations of rainbow trout (Julian *et al.*, 1998), in that the peripheral retina (CGZ) was strongly labelled and clusters of dividing cells were apparent in both the outer and inner nuclear layers (ONL and INL) of the central retina.

In Series 1 there was no significant change in PCNA labelling pigmented trout regardless of light treatment (Figure 17C). Untreated albinos had similar levels of PCNA labelling as pigmented trout.

During light treatment of albino trout, PCNA labelling was modulated by light in all areas of the retina examined, such that there was a decrease and subsequent increase in labelling (Figure 17C). Within the time-points tested, the effect of light on PCNA labelling was most apparent at 10 days, where the number of labelled nuclei in the nasal ONL was significantly lower than the 5 day treatment ($p=0.010$). The number of labelled nuclei in the nasal ONL significantly increased between 10 and 20 days of light treatment ($p=0.010$). This is co-incident with the peak of cell death as measured by TUNEL method in the 10 day treatment. These quantifications of PCNA labelling were taken from the entire

Figure 18. PCNA labelling of retina from albino and normally pigment trout. Immunohistochemical label was visualized with a dark precipitate. **A.** Retina from an albino trout from Series 1 that had received 5 days of light treatment. **B.** Retina from an albino trout from Series 1 that had received 20 days of light treatment. One cluster of PCNA positive cells appears to be migrating from the Inner nuclear layer (INL) to the outer nuclear layer (ONL). **C.** Retina from a normally pigmented trout from Series 1 that had received 20 days of light treatment. The retinal pigment epithelium is dark in panel C. OPL, outer plexiform layer. Scale bar is 50 μm.


A
ONL
OPL
INL
B
ONL
OPL
INL
C
RPE
ONL
INL

length of retina, excluding the CGZ.

Because histological assessments of light-induced damage reveal that the damage is greatest in the central retina, I also quantified the number of nuclei labelled with PCNA in a central area of retina (Figure 17D). This revealed a pattern of light damage very similar to that described above (compare Figures 17C & 17D), except that I also found a significant increase in PCNA labelling in the INL of albinos receiving When I repeated the ANOVA on this data without the 10 and 21 day data, the significant difference remained between 5 days of light treatment as compared to untreated albinos ($p=0.001$) or albinos exposed to 2 days of light ($p<0.001$).

4.4.4 Series 2 TUNEL

The observation that light caused a decrease in the number of ONL nuclei after 20 days of exposure to damaging light during Series 1 was unexpected. This led to the speculation that the different light regime the trout were raised in was the source of the difference as compared to past years when trout were reared outdoors in open raceways. Thus in Series 2, fish were maintained in two separate light regimes for ten days prior to treatment. The two regimes left the fish in dim light (not-pretreated) or exposed them to stronger, shaded outdoor daylight (pretreated). These two groups of fish were then exposed to high-intensity damaging light (similar to Series 1) for ten days and sacrificed. I focussed on the 10 day damaging light treatment time-point, as I had observed the most dramatic effect of light at 10 days in the experiments from Series 1.

My TUNEL labelling experiment on retinae in Series 2 confirmed the Series 1 results with an obvious qualitative increase in TUNEL positive cells, which were localized to the vitreal portion of the ONL (i.e. they were rod nuclei). This result of increased TUNEL labelling during light damage (Figure 19A) was statistically significant for all quadrants of the retina for not-pretreated albino trout ($p<0.002$ for each quadrant) and for pretreated retina when all locations were summed for a given individual ($p=0.002$). In all four quadrants the pretreatment seemed to have a protective effect, as there was significantly fewer TUNEL-positive nuclei compared to the not-pretreated fish ($p<0.005$ for each quadrant). Thus photic history had an effect on TUNEL labelling.

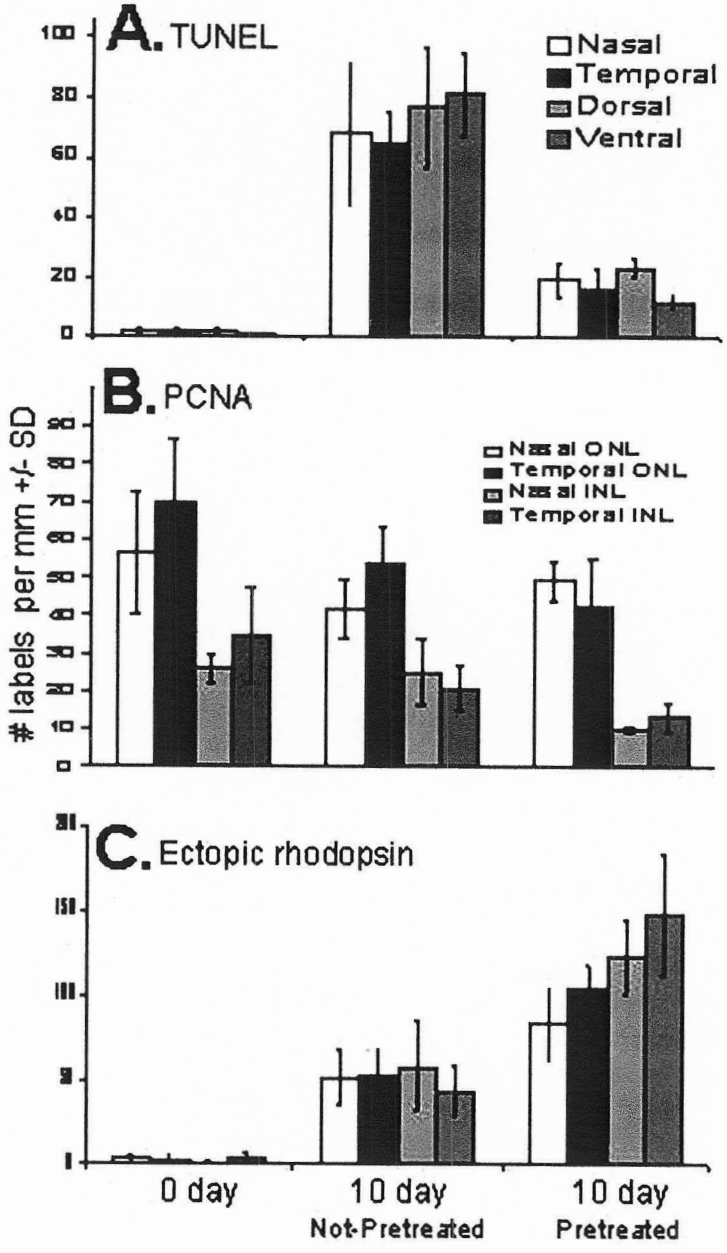
4.4.5 Series 2 PCNA

In Series 2, I observed a significant decrease in the number of PCNA-positive nuclei (Figure 19B) in the nasal INL after ten days of treatment ($p=0.007$), very similar to Series 1, however this was limited to pretreated retina. The number of PCNA-positive nuclei at this location was significantly less in pretreated as compared to not-pretreated fish ($p=0.010$). Thus, photic history had an effect on proliferation.

4.4.6 Series 2 Ectopic Rod Opsin

I also examined immunohistochemical localization of rod opsin in retinae from Series 2. This was not completed on fish from Series 1 due to availability of materials. In normally pigmented fish, my immunohistochemistry revealed the expected localization of label to inner and outer segments of rods and one member of the double cones (Figure 16C). I observed rod opsin expression to be

Figure 19. Photic history modulates the effects of damaging light in albino trout (Experiment Series 2). Details similar to Figure 17, but fish were either maintained in dim light (not-pretreated) or exposed to moderately intense light (pretreated) for ten days prior to exposure to high-intensity damaging light. **A.** TUNEL labelling indicates rod cell death was significantly increased after ten days of damaging light in each location of albino trout that were not-pretreated as compared to fish not exposed to damaging light (0 days) and compared to albinos that were pretreated with moderate light. **B.** Proliferating cell nuclear antigen (PCNA) labelling, indicating proliferation, in outer and inner nuclear layers (ONL and INL) of albino trout retina. After exposure to 10 days of damaging light, proliferation was significantly decreased in the INL of pretreated albino trout as compared to unexposed (0 day) albino trout and compared to albino trout that were not-pretreated. **C.** Immunohistochemical detection of rod opsin expression in light damaged albino trout revealed ectopic, disorganized expression amongst the rod nuclei (see Figure 2). The occurrence of this was significantly higher in all areas of the retina from all fish treated after 10 days of damaging light as compared to unexposed (0 day) fish. The number of observed localizations of ectopic rod opsin was modulated by photic history, and was significantly different amongst pretreated and not-pretreated albinos in all but the ventral retina. Legend as per panel A.


A. TUNEL
Nasal
Temporal
Dorsal
Ventral
B. PCNA
labels per mm +/- SD
Nasal ONL
Temporal ONL
Nasal INL
Temporal INL
C. Ectopic rhodopsin
0 day
10 day
Not-Pretreated
10 day
Pretreated

mis-localized in ONL of light damaged albinos (Figure 16D). This appears to represent mis-localized expression in rods, not MWS cones, (i.e. RH1, not RH2 immunoreactivity) because I noted similar mis-localization of zpr-3 immunoreactivity (Figure 16E) that labels rod inner segments, and no mis-localization of zpr-1 immunoreactivity (Figure 16F) that labels double cones. The mis-localization of rod opsin (Figure 19C) was significantly higher in the light damaged central retinae of pretreated ($p<0.002$ for all quadrants) and not-pretreated fish ($p<0.015$ for all quadrants except ventral) when compared to controls. There was negligible mis-localization in the peripheral retina. The mis-localization of rod opsin in each quadrant of the retina was significantly higher in the pretreated albinos than in not-pretreated albinos ($p<0.007$ for all quadrants except nasal). A parallel to this was the observation of several rod opsin immunoreactive cells in the INL of the pretreated albinos, whereas no such INL mis-localization was observed in the not-pretreated group. Thus photic history had an effect on ectopic rod expression.

4.4.7 Results Summary

Exposure to full sunlight did not measurably affect cell death or proliferation in normally pigmented trout. In albino trout, however, light exposure caused a dramatic increase in TUNEL and PCNA label, indicating increased cell death and proliferation. The loss of rod nuclei from some portions of the retina was a further demonstration that light damage leads to rod death. Significant increases in rod cell death, as detected by TUNEL, preceded observable decreases in the number of rods. Together with modulations of proliferation, these data support

the hypothesis that rod nuclei are dying and being replaced in light-damaged albino trout retinae.

The loss of rod nuclei was unexpected based on previous experiments. I hypothesized that the principal difference compared to previous experiments was due to the photic history of the fish. Comparing fish pretreated with moderate intensity light to those that were not-pretreated supported this hypothesis: pre-treatment affected cell death, proliferation, and ectopic expression of rod opsin.

4.5 Discussion

Previous investigations of light damage in albino rainbow trout retinae demonstrated that despite rod outer segment (ROS) degradation, there was no decrease in the number of ONL nuclei (Allen & Hallows, 1997; Allen *et al.*, 1999; Allen *et al.*, 2001). This remains a surprising result, because it distinctly contrasts results of other teleost models and rodent models where similar insults that induce ROS loss also produces a decrease in the rod and cone photoreceptor (ONL) nuclei (Marotte *et al.*, 1979; Penn, 1985; Raymond *et al.*, 1988a; Allen *et al.*, 1999) through apoptosis (Shahinfar *et al.*, 1991; Li *et al.*, 1996; Vihtelic & Hyde, 2000). Two mechanisms for these observations on albino trout were contemplated (Allen & Hallows, 1997): 1) that the rod nuclei were protected from death or 2) rods were dying and being replaced by proliferating cells.

Subsequent to the formation of these hypotheses, several reports have indicated that both proliferation and photoreceptor regeneration may be prevalent in rainbow trout retina. The potential for UVS cones to reappear in the trout retina during natural ontogeny has been identified (Beaudet *et al.*, 1997; Hawryshyn *et*

al., 2003a, See also Chapter 3), and this appears to represent regeneration from retinal stem cells (Chapter 3). Furthermore, a high level of cell proliferation has been identified in the INL of rainbow trout retinae (Julian *et al.*, 1998). These proliferating inner nuclear layer cells (PINCs) have been shown to be modulated by surgical injury (Faillace *et al.*, 2002). The PINCs have subsequently been identified in other teleosts (Vihtelic & Hyde, 2000; Otteson *et al.*, 2001; Cid *et al.*, 2002), including other salmonid fish (Candal *et al.*, 2001). Thus an abundance of proliferating cells that can produce photoreceptors exist in rainbow trout retina and could account for regeneration of structure when the albino trout are allowed to recover from damaging light (Allen & Hallows, 1997). Indeed proliferation was up-regulated in albino zebrafish, following light-induced apoptosis (Vihtelic & Hyde, 2000). However, data from zebrafish do not speak directly to the above hypotheses regarding rainbow trout. In zebrafish light induced a substantial loss of ONL nuclei and cone cell death (Vihtelic & Hyde, 2000), and the mechanisms whereby the number of ONL nuclei is maintained in trout thus remained unknown.

Observations during both sets of experiments (Series 1 and 2) demonstrated a qualitative increase in TUNEL labelling of rod nuclei in each of the 20 fish receiving light damage. Both TUNEL labelling and pyknotic nuclei were in the vitreal portion of the ONL, and were observed rarely in peripheral regions of the retina, where light damage is less pronounced (current study, Allen *et al.*, 2001). In Series 1, this TUNEL labelling was increased at the first time-point examined, 2 days, and continued to be elevated at subsequent time-points. The high levels

of TUNEL labelling were significantly different between 0 and 10 days of light damage when each location was summed for a given individual. In Series 2, TUNEL labelling was more consistently observed, and significantly increased in each location in the fish that were not-pretreated, and significantly increased in fish that were pretreated when each location was summed for a given individual. The number of ONL nuclei did not decrease within the first 10 days of light damage, despite substantial loss of ROS. Thus three separate experiments over two years demonstrated a significant increase in TUNEL labelling in rod nuclei after 10 days of damaging light treatment. No such increase in TUNEL labelling nor ROS loss were observed in normally pigmented trout. Taken together, these data confirm the hypothesis that rods are dying and being replaced by the proliferating cells present in the retina.

Indeed, my labelling for PCNA demonstrated that light can modulate proliferation in albino fish, despite no effect on pigmented fish at the time-points tested. When the entire span of the retina was considered (excluding the CGZ) significant decreases in PCNA labelling were measured after 10 days of light damage (Fig 3B). Subsequently the PCNA labelling significantly increased, possibly in response to the peak of death that occurred at 10 days (Compare Fig. 3A & 3B). When I quantified PCNA labelling in the central retina only, the area where ROS loss and TUNEL labelling were most substantial, I noted a similar pattern but also a significant increase in PCNA labelling in the INL by 5 days of light treatment (Fig 3C). This is consistent with data (Julian, 1997 Ph.D. Thesis; described in Faillace *et al.*, 2002) that showed increases in PCNA labelling in the

same brood stock of albino trout as in the current work, and using previously described light treatment regimes (Allen & Hallows, 1997; Allen *et al.*, 1999; Allen *et al.*, 2001).

One unexplained aspect of the data is the decrease in proliferation observed at 10 days of light damage in Series 1. This is coincident with the peak of TUNEL labelling. Thus the decrease in proliferation may represent a state where the retina is in shock due to damage, and subsequently is able to recover its proliferative potential by day 20. The decrease in proliferation at day 10 was repeated in series 2, and bears further investigation. Regardless, the result does not affect the interpretation of the overall result: proliferation was modulated by light damage, including increases in central retinal proliferation subsequent to detection of cell death.

The impressive effect of light on cell death in albino trout was not always mirrored by a dramatic increase in cell proliferation, although important significant increases were observed. It should be considered that the methods used allow only a "snap-shot' of proliferative events that occurred. For example proliferation in trout retina varies in a circadian rhythm (Julian *et al.*, 1998), and an increase in proliferative rate may be more or less apparent during another portion of the day. The number of PCNA-positive nuclei in normally pigmented trout retina was significantly higher in the hours before sunrise as compared to other times (Julian *et al.*, 1998), whereas sampling in the current experiments occurred closer to mid-day. Further experiments should incorporate such considerations and utilize

cell fate mapping to demonstrate that new rods are being generated, and that the rate of this process is modulated by damaging light.

Some rod nuclei were lost in albino trout by twenty days of light exposure (Series 1), although cones remained intact. This result was different than previous examinations of albino trout, which were maintained in brighter light conditions prior to exposure to high intensity light and demonstrated no decrease in the number of ONL nuclei (Allen & Hallows, 1997; Allen *et al.*, 1999; Allen *et al.*, 2001). The result has several interesting implications: 1) It is a further demonstration that rods of albino trout can be eliminated by the current light regime, i.e. the treatments used kills rods. My working hypothesis is that prior to this 20 day timepoint proliferative events were sufficient to replace dying rods; 2) This observation creates a potential avenue of research where cone-dominated retinae are desirable, as a substantial amount of cone-enriched retina can be obtained; 3) Maintaining fish in moderately high intensity light prior to treatment with damaging light has a protective effect and/or enhances regenerative capacity of the retina.

To investigate if the latter implication was valid, as has been demonstrated in albino rodents (Li *et al.*, 2001, 2003), two groups of albino trout were treated to the same damaging light regime (experiment Series 2). One group remained in dim light prior to treatment (not-pretreated) whereas the other group was maintained in moderate intensity light for ten days prior to treatment (pretreated). Fish were sacrificed after 10 days of treatment, which was the timepoint in the Series 1 with the largest modulations in TUNEL and PCNA labelling. Indeed, I

observed significant differences between pretreated and not-pretreated groups for both PCNA and TUNEL labelling. I also observed disorganized and ectopic rod opsin expression in the ONL and INL, reminiscent of observations in zebrafish (Vihtelic & Hyde, 2000). Ectopic rod opsin expression may represent degenerating rods and/or new rods that are being generated to replace rods and maintain the number of ONL nuclei. Regardless, TUNEL, PCNA and rod opsin data all support the hypothesis that photic history of the fish can modulate the retina's susceptibility to damage and/or its regenerative capacity. The decrease in TUNEL labelling supports a protective effect of pre-treatment, as there was significantly less rod apoptosis in the pretreated group. Furthermore, the PCNA data are not consistent with the pretreated fish having increased regenerative capacity. This represents first report where photic history modulates light-induced damage in a non-mammalian vertebrate.

Chapter 5:

Adaptive Value of the UVS Cone Ontogeny

5.1 Abstract

In addition to salmonids, ultraviolet-wavelength sensitive (UVS) cones are also lost during the ontogeny of several other fishes. I am curious if the loss of UVS cones has any selective advantage to the fish. Previous hypotheses suggested that the lack of UVS cones (e.g. in deeper waters) may be directly related to the lack of UV light reaching the retina. My data on UVS cone distributions demonstrate that this explanation cannot be complete: UVS cones remain in the dorsal retina of some salmonids during their demersal stages (Chapter 2), and sample a downward field-of-view with a lower quantity of UV photons. The abundance of UV quanta in the environment and the various functions attributed to UV vision argue strongly that it could have selective advantages in large fish (Losey *et al.*, 1999); the reappearance of UVS cones late in life history is consistent with this argument. Here, I consider two primary hypotheses, that are not exclusive of one another, as to the selective advantages to losing UV vision as the fish undergo transition to new photic environments and new predator-prey relationships. First, loss of UVS sensitivity could be part of a refinement of visual sensitivity: losing UVS cones could change the wavelength discrimination or spatial resolution of the remaining cone types, or the disappearance of cones could allow retinal space for new rod photoreceptors to function in increasingly scotopic environments. The second hypothesis predicts that the loss of UVS cones reduces the detection of scattered, non-target

photons. This is based on an old hypothesis (Walls & Judd, 1933a, b) that speculates the abundance of UV photons scattered into the light path (UV light scatters more than longer wavelength light) degrades the target image, and detection of these photons decreases the signal to noise ratio. The hypothesis remains viable for explaining the benefits of ocular filters, but has not been applied previously to the loss of UVS cones during ontogeny. In this new hypothesis, the driving factor in loss of UVS cones is the need to detect predators and prey at increasing distances as the fish gets larger.

Losing the UVS cones may be an effective alternative (or complement) to increasing yellow intraocular filters during ontogeny, as these filters will negatively impact the scotopic visual system (Walls & Judd, 1933a; Heinermann, 1984), The importance of the latter is demonstrated by fishes that have evolved occlusable yellow filters that block UV light during the day and permit higher photon transmission during scotopic conditions (Heinermann, 1984). Further, this strategy has advantages in that yellow filters may eliminate the potential to regenerate UV sensitivity later in life-history.

5.2 Introduction

Visual ecology has sought an understanding of how organisms have evolved to tune their visual sensitivity (and their body colouration) to their visual tasks and photic environment (Lythgoe, 1979). Several strategies within the visual system have been employed by fishes over evolutionary time, particularly including amino acid replacements in opsins that mediate tuning of a pigment's absorptive properties (reviewed in Yokoyama, 1997; Yokoyama, 2000), which appears to

have occurred amongst salmonids (Dann *et al.*, 2004a). Differential tuning within an individual can also occur during ontogeny, adjusting for varied habitats an individual experiences. Mechanisms can include chromophore shifting (Bridges, 1972; Beatty, 1984), which occurs in salmonids (Bridges & Yoshikami, 1970). Differential opsin expression is also known to occur in some fishes during ontogeny (Archer *et al.*, 1995; Carleton & Kocher, 2001)[9]. Other examples of organisms tuning their visual system to the environment during ontogeny include retinal development that occurs in larval fish as they metamorphose and migrate to deeper waters. This includes fusion of larval single cones into double cones (Sandy & Blaxter, 1980) and addition of rods into pure-cone embryonic retinae during metamorphosis and migration to deeper waters (Sandy & Blaxter, 1980; Johns & Fernald, 1981; Evans & Fernald, 1990; Helvik *et al.*, 2001a; Helvik *et al.*, 2001b).

It may be that the loss of UVS cones is adaptive to the fish's changing environment. Thus, the evolution of this ontogenetic trait may be of considerable interest to visual ecologists (Beaudet & Hawryshyn, 1999). This thesis has been the most thorough examination of the loss of UVS sensitivity and UVS cones in any species to date, combining measurement of UV sensitivity with examinations of opsin gene expression. Furthermore, this thesis has added substantial evidence to the previously hypothesized (Kunz *et al.*, 1994) mechanism of UVS cone disappearance, apoptotic programmed cell death (PCD). This thesis has

[9] In the context of the current thesis topic, it is noteworthy that salmonids possess multiple copies of opsin classes (particularly MWS opsins) in their genomes (Hisatomi *et al.*, 1994; Minamoto & Shimizu, 2003). This means that opsin shifting should be considered a possibility for salmonids (no information is currently available regarding *expression* of multiple copies of salmonid opsin classes).

also investigated the previously proposed (Browman & Hawryshyn, 1994b; Beaudet *et al.*, 1997; Hawryshyn *et al.*, 2003a) reappearance of UVS cones, and introduces a new mechanism to tune visual sensitivity to photic environment: addition of new cone photoreceptors from retinal progenitor cells into an established, mature retina. In this chapter I will consider previous hypotheses regarding why salmonid UVS cones are lost and regenerated, which is a unique ontogeny amongst all animals examined to date.

5.3 Roles of UV vision

UV vision is defined herein as the presence of functional visual pigments with a wavelength of maximal absorbance (λ_{max}) characteristically at 355-390 nm. This definition refers to the principal absorption band (α-band) of the visual pigment. This definition distinguishes UVS visual pigments from all other vertebrate visual pigments , which also absorb in the UV range, where the shoulder of their sensitivity (the β-band) absorbs light, at approximately 30% of the efficiency of their α-band that absorbs at longer wavelengths. The presence of UV vision thus not only has the potential to increase detection of UV light, but characteristically creates the probability that UV light plays a distinct role in colour perception (reviewed by Hawryshyn & Beauchamp, 1985; Neumeyer, 1985; Losey *et al.*, 1999).

UV light scatters to a larger degree than longer wavelength light. This occurs through both molecular (Raleigh) scattering and through particle (Mie) scattering (Loew & McFarland, 1990; Kirk, 1994; Loew, 1999). The amount of scatter is difficult to predict or quantify for a given medium, as it is affected by the

particle size and composition. Overall statements about the amount of scattered light a fish experiences are difficult to estimate because of heterogeneity in its environment. Regardless, shorter wavelength light scatters more than longer wavelength light, and the relative amount of scatter is less wavelength dependant for Mie scattering. It can be approximated by the functions nm^{-4} for Raleigh scattering, and nm^{-1} for Mie scattering (Loew & McFarland, 1990; Kirk, 1994; Loew, 1999). The scattering of light is considered the limiting factor for visual systems underwater, regardless of the particulate and dissolved matter (Lythgoe, 1979, 1985; Loew & McFarland, 1990). Losey *et al.* (1999) conservatively estimate that despite scattering, UV light penetrates bodies of water sufficiently to be biologically active at depths exceeding 100 m. Spectral energy distribution measurements have led to estimates in salmonid fishes that UVS cone photoreceptors are active and sensitive down to depths of up to 15m in the marine environment and 18m in lake habitats (Novales Flamarique *et al.*, 1992, 1993; Novales Flamarique & Hawryshyn, 1997).

UV vision is thought to have a variety of roles in teleosts. It should be stated that there is no need to assume that UV vision is a particularly special ability with a suite of functions differing from the other cone photoreceptors. For example, one can argue that UVS cones simply extend the ability of the visual system into shorter wavelengths of light. Similarly, UVS cones may be interacting with the other three cone types to produce colour vision. The presence of the UVS cone implies tetrachromatic colour vision (compared to trichromatic colour vision in animals with three cone classes). Goldfish have been shown to be

tetrachromatic, using a psychophysical paradigm to investigate wavelength discrimination (Neumeyer, 1985, 1986; Neumeyer & Arnold, 1989; Neumeyer, 1992). The presence of neurons in higher visual centres that are spectrally opponent and receive UV input is highly suggestive that rainbow trout also possess tetrachromatic vision (Coughlin & Hawryshyn, 1994a).

UV vision has been demonstrated to play a role in foraging. Fishes can feed on plankton when illumination is limited to the UV (Loew *et al.*, 1993; Browman *et al.*, 1994). It is likely that UV vision allows increased detection of pelagic prey that would fail to transmit UV light, and thus appear dark against the bright, scattered UV light. This could include the detection of otherwise transparent fishes and planktonic invertebrates (reviewed in Johnsen, 2001).

Conspecific signalling also utilizes UV light. The reflectance patterns of fish include UV light, and unique colours that include UV light are likely apparent to fish that cannot be observed by humans (Losey *et al.*, 1999; Marshall *et al.*, 2003a). Such signalling could be advantageous for communication at short ranges, because the signals would not be conspicuous at longer ranges due to scattering of UV light (Losey *et al.*, 1999; Losey, 2003; Marshall *et al.*, 2003a). Both guppies and swordtails have recently been examined using behavioural paradigms, and were found to use UV light during mating (Karino & Matsunaga, 2002; Smith *et al.*, 2002; Cummings *et al.*, 2003; White *et al.*, 2003).

It is also noteworthy that polarized light detection is often associated with UV vision. In salmonids, cyprinids and damselfish it appears that UV sensitivity plays a central role in the detection of the polarization of light (Hawryshyn &

McFarland, 1987; Hawryshyn *et al.*, 1990; Hawryshyn & Bogler, 1990; Parkyn & Hawryshyn, 1993; Coughlin & Hawryshyn, 1995; Hawryshyn, 2000; Parkyn & Hawryshyn, 2000; Hawryshyn *et al.*, 2002; Hawryshyn *et al.*, 2003b). Each of the above roles of UV vision (e.g. feeding, signalling to conspecifics) may be augmented by polarized light detection. Additionally, the detection of celestial polarized light could serve as navigational cues for migrating fishes (Hawryshyn, 2000, 2003; Parkyn *et al.*, 2003).

Thus many studies have addressed "Why have UV vision", and clearly identified that UV vision has utility in fishes. However, an important question remains largely unanswered: "Why not have UV vision?". This question has been identified for more than a decade (Goldsmith, 1990), but remains largely unaddressed in the literature. Given the ontogenetic loss of UVS cones that this thesis has examined, the question is pertinent to understanding the evolution of this ontogenetic event in salmonids. From an alternate viewpoint, one might expect that study of the question in an ontogenetic context might provide insight into why UV vision is not more prevalent in vertebrates. Thus, rather than focus on mechanisms that *allow* UV vision, it may be fruitful to consider the mechanisms by which UV vision has been reduced. With these concepts in mind, I turn to a summary of mechanisms whereby UV vision is lost, followed by discussion of some novel hypotheses regarding the adaptive significance of the UVS cone loss.

5.4 Loss of UV Vision

I will consider the loss of UV vision over two timescales: i) evolutionary time; ii) ontogenetic development.

The examination of the disappearance of UV vision over evolutionary time may provide insight into several aspects of visual ecology, and thus an understanding of how visual system features have evolved to sample the photic environment. Further, it may be that such concepts are valuable to shaping larger evolutionary theory, similar to the contributions made by studying regressive evolution of eyes and other features in cave animals (Culver, 1982; Culver *et al.*, 1995; Jeffery & Martasian, 1998).

Early vertebrates are thought to have possessed four cone photoreceptor mechanisms, including UVS cones and thus UV vision (Bowmaker, 1998; Shi & Yokoyama, 2003). The lack of UV vision in contemporary vertebrates can be the result of various traits, which often occur together.

Most obvious is the lack of UVS visual pigment, which may include loss of the gene from the genome. The lack of UVS visual pigment in contemporary vertebrates can occur through a mutation of the opsin to a non-functional pigment, as has occurred in dolphins (Fasick *et al.*, 1998) and coelacanths (Yokoyama *et al.*, 1999). Presumably, control of opsin expression, i.e. the lack of expression of a functional UVS opsin, could also account for lack of UV vision, as occurs during ontogeny (see below), however relaxation of selection pressure and genetic drift would then be expected to lead to a non-functional protein (as exemplified by cavefish, see Yokoyama *et al.*, 1995; Parry *et al.*, 2003).

The other major way in which UV vision has been lost over evolutionary time has been the evolution of ocular filters. Yellow filters, blocking short wavelength light are expressed in the ocular tissues of many vertebrates and invertebrates. The filters can occur in the cornea, lens, vitreous, or photoreceptors themselves. The retinal vascular supply may also act as a filter. A further example of the evolution of filter is the macular pigment of vertebrates, which is a yellow pigment centred over the fovea of diurnal primates (see reviews by Walls & Judd, 1933a; Heinermann, 1984; Douglas & McGuigan, 1989; Douglas & Marshall, 1999; Losey *et al.*, 2003)

During ontogeny of several fishes there appears to be a loss of UV vision. This may occur through increased yellow pigmentation in ocular structures. This is not solely an aging process where tissue degradation leads to a reduction from optimal performance. In fishes, the pigments are not photo-oxidation products, but represent several spectrally distinct pigments. Indeed, transmission of the fish lens can remain fairly constant throughout the fish's life, or even increase its transmissivity presumably through a dilution of pigment as the lens grows (reviewed in Heinermann, 1984; Hawryshyn *et al.*, 1989; Thorpe & Douglas, 1993; Douglas & Marshall, 1999).

Another mechanism whereby fish might lose UV sensitivity during ontogeny is through opsin shifting. In this hypothetical idea, the complement of photoreceptors remains the same, but the opsin(s) it expresses change during development, with an overall reduction in the amount of UVS opsin expressed. Quantifications of mRNA levels in retinal homogenates of cichlids lend

preliminary support to this idea. Differential opsin expression occurs both between species (Carleton *et al.*, 2000; Carleton & Kocher, 2001) and likely during ontogeny of individual species (Carleton *et al.*, 2002). Although the existence of unidentified opsins and any changes in photoreceptor mosaic in these cichlids might confound this interpretation. Opsin shifting during ontogeny is known to occur in the rods of various eels (Wood & Partridge, 1993; Hope *et al.*, 1998; Zhang *et al.*, 2000; Zhang *et al.*, 2002), and MSP evidence supports opsin shifting in tuna (Loew *et al.*, 2002) and black bream (Shand *et al.*, 2002). Thus the existence of opsin shifting during ontogeny of fishes is well-established, although the role this plays in the loss of UV vision has only tentatively been established (in cichlids).

Finally, a clearly identified mechanism to lose UV sensitivity during ontogeny is the loss of the UVS cone. This is has been examined in greatest detail in rainbow trout, including examinations of opsin protein and mRNA expression in coordination with electrophysiological tests of UV sensitivity (See Chapters 2 and 3). This appears to be representative of other species in *Oncorhynchus* and *Salmo*, in that they lose their UVS cones, based upon morphologically identified cone types (in plastic or paraffin sections), electrophysiology, and MSP (Furst, 1904; Lyall, 1957b, a; Ahlbert, 1976; Bowmaker & Kunz, 1987; Hawryshyn *et al.*, 1989; Browman & Hawryshyn, 1992; Beaudet *et al.*, 1993; Browman & Hawryshyn, 1994b; Novales Flamarique & Hawryshyn, 1996; Novales Flamarique, 2000; Browman *et al.*, 2001). It also appears that smelt lose their UVS cones (Reckel *et al.*, 2003). However the identity of UVS cones in smelt is

putative, based upon their location in the cone mosaic, and the presumption of their loss is inferred from their limited distribution across the retina in the single life history stage examined (Reckel *et al.*, 2003). The smelt is in a genus basal to *Salmo* and *Oncorhynchus,* and complements information on the loss of putative UVS cones from grayling (*Thymallus thymallus*) and brook trout (*Salvelinus fontinalis*) (Bathelt, 1970). Furthermore, putative UVS cones appear to be lost during ontogeny of the Cisco (*Coregonus albula*), because fry of an unidentified species possess them (Eigenmann & Shafer, 1900) whereas older specimens do not (Lyall, 1957b; Ahlbert, 1969), as reviewed by Ahlbert (1976). Therefore it appears that loss of UVS cones is ubiquitous in salmoniformes, although the timing and extent of the loss seems to vary.

UVS cones have also been shown to be lost during ontogeny in a few other teleost fishes. UVS cones also appear to be lost from the retina of yellow perch (*Perca flavescens*) (Loew & Wahl, 1991) and some, but not all, cyprinid fishes. UVS cones are lost from the retina of the rudd (*Scardinius erythrophthalmus*) (Scholes, 1975; Whitmore & Bowmaker, 1989). It has been suggested that UVS cones are lost from goldfish (*Carassius auratus auratus*), although this is probably not a complete loss. Sensitivity to UV light, as measured by ERG, has been reported to decrease with age in goldfish (Chen & Stark, 1994). However, it is noteworthy that UV sensitivity can be measured in large goldfish using psychophysical means (Hawryshyn & Beauchamp, 1985; Hawryshyn, 1991b, a; Neumeyer, 1992; Fratzer *et al.*, 1994). The loss of UVS cones has been suggested to explain why fewer UVS cones are found in larger goldfish during

MSP or electrophysiology (Bowmaker *et al.*, 1991; Palacios *et al.*, 1998). Fewer UVS cones are found relative to SWS cones as measured by *in situ* hybridization in larger goldfish (Stenkamp *et al.*, 2001). Furthermore, the distribution of goldfish cones labelled by an antibody against the human blue opsin is limited in larger goldfish (Sharma, 1991). It has subsequently been demonstrated that the human blue opsin and the goldfish UVS (*i.e.* SWS1) opsin share the greatest sequence identity (Raymond *et al.*, 1993; Hisatomi *et al.*, 1996), thus the antibody used by Sharma (1991) likely labelled UVS cones. The UV sensitivity in large goldfish (Hawryshyn & Beauchamp, 1985; Hawryshyn, 1991b, a; Neumeyer, 1992; Fratzer *et al.*, 1994) may be mediated by UVS cones that are not lost (as in salmonids, see Deutschlander *et al.*, 2001), or it may represent differences based on the sources of the fish, which are highly domesticated (as suggested by Bowmaker *et al.*, 1991). In contrast, it is established that UVS cones are not lost from another cyprinid, the zebrafish (*Danio rerio*), as determined by ERG, *in situ* hybridization, and immunohistochemistry (Raymond *et al.*, 1996; Hughes *et al.*, 1998; Saszik *et al.*, 1999; Vihtelic *et al.*, 1999). Furthermore, with collaborators from the Hawryshyn lab, I have observed that UVS cones do not disappear from zebrafish retinae when the fish were treated with thyroid hormone (Temple *et al.*, 2003; Allison *et al. In prep*) in an identical manner to the treatment that induces UVS cone loss in rainbow trout (as per Chapter 3). This is despite our observation of dramatic changes in skin colouration and a shift in the chromophore used by photoreceptors, which demonstrated that TH was affecting

zebrafish retinal gene expression (Temple *et al.*, 2003; Allison *et al.*, In preparation).

The mechanism of UVS cone loss appears to be apoptotic programmed cell death (PCD) in both Atlantic salmon (*Salmo salar*) (Kunz *et al.*, 1994) and rainbow trout (*O. mykiss*) (See chapter 3). It remains unclear if this can be generalized to other teleosts where UV cones are lost. I view the loss as a purposeful, developmental event (*i.e.* not a necrosis) in these other fishes, and thus suggest that PCD of UVS cones is most likely occurring in other teleosts as well.

A future direction of research should be to examine if some of the above mechanisms of loss of UV vision occur in parallel. For example, the co-occurrence of UVS cone loss with decreased transmissivity of ocular media to UV light should be further investigated. Some information on species comparisons is available (Losey *et al.*, 2003; Marshall *et al.*, 2003b), but the timing of these events during ontogeny is largely unknown.

5.5 Adaptive significance of losing UVS cones

The loss of UV vision during ontogenetic and evolutionary time can occur through various mechanisms. Little theoretical progress has been made on the functional significance of blocking UV light through intraocular filters since Walls and Judd published their speculations on intraocular filters in 1933.

Three primary reasons were listed (Walls & Judd, 1933a, b) to explain how yellow intraocular filter may be useful: 1) reduce retinal damage by UV light; 2) increase visual acuity by reducing chromatic aberration of the lens; 3)

enhancement of detail by absorption of scattered light[10]. These hypotheses remain the central considerations for the benefits of intraocular filters in recent investigations (Heinermann, 1984; Douglas & Marshall, 1999; Losey *et al.*, 2003).

5.5.1 Past speculation on why UVS cones are lost during ontogeny

In contrast to changes in ocular media transmissivity, there has been surprisingly little speculation regarding the benefits of losing UV vision through the loss of UVS cones. Thus, there has been little consideration of selective factors that have led to the evolution of UVS cone loss. Discussions of UVS cone loss during teleost ontogeny have mentioned changes in the fish's prey, and (apparently) presumed that on new prey the utility of UV vision will be reduced to a level where the UVS cone regresses (Bowmaker & Kunz, 1987; Bowmaker, 1990; Browman & Hawryshyn, 1994b; Kunz *et al.*, 1994). Several authors have also speculated that a reduction of UV quanta reaching the retina, through decreased UV light reaching the eye and/or through decreased transmissivity of the ocular media for UV light, makes the utility of the UVS cone limited (Douglas *et al.*, 1989; Whitmore & Bowmaker, 1989; Bowmaker, 1990; Browman & Hawryshyn, 1994b; Kunz *et al.*, 1994; Beaudet & Hawryshyn, 1999).

The latter two concepts contribute little to understanding an evolutionary mechanism, but point out factors that correlate to the loss of UVS cones. The implication (or unstated assumption) is a view that the lack of utility leads to

[10] Walls & Judd (1933) also considered the possibility that coloured filters could enhance (or at least modify) colour vision when the filters take the form of coloured oil droplets contained within photoreceptors. The discussion of this topic is relevant to reptiles and birds; because fish do not possess colour oil droplets, this type of benefit will not be considered further here.

regression of the feature. Indeed this could be the case, where the metabolic costs of maintaining UVS cones outweigh their utility.

The metabolic cost of maintaining UVS cones could be higher than the other cone classes, if UVS cones sustain more damage than other cone classes. Thus UVS cones could be disappearing because they absorb too much light and this leads to light-induced damage. This concept strikingly contrasts the aforementioned hypothesis that UVS cones disappear because they do not receive enough light. This hypothesis expands on the implication that UVS cones are lost because they are expensive to maintain, and has not appeared in the literature previously. The hypothesis is formed based on the higher energy per photon in shorter wavelength radiation. Whereas cellular damage from UV light is typically associated with DNA damage, it seems unlikely that UVS cones are exposed to more UV light than the other (adjacent) photoreceptor classes or other retinal neurons. However, it has recently been hypothesized that light-induced damage of photoreceptors also occurs through pathways requiring opsin absorbing the light (Hao *et al.*, 2002). These pathways are not yet understood, thus one can imagine that absorption of higher energy photons may lead to increased photoreceptor damage. One might speculate that the pathways could involve photochemical (e.g. oxidative) damage and/or photothermal effects (e.g. see review by Glickman, 2002). This hypothesis could be tested by maintaining fish in photic regimes with differing UV content, and testing if there is differential loss of UVS cones or differential damage of UVS cones compared to other photoreceptors at the EM level. However, this experimental design presumes

that damage itself is the molecular signal leading to UVS cone disappearance. The signal may be a developmental program (e.g. endocrine control of development by increases in TH) to signal loss of UVS cones that has evolved because metabolic costs of maintaining UVS cones are high. The dorsal distribution of UVS cones in large rainbow trout (Chapter 2) may be considered consistent with this hypothesis, however the ventral distribution of putative UVS cones in smelt (Reckel *et al.*, 2003) is not. The increasing depth salmonids utilize when UVS cones are absent (see Section 5.5.2) is also not consistent with this hypothesis. The lack of UVS cone loss in several fish species (e.g. zebrafish) suggests that the utility of UVS cones can outweigh the metabolic costs in at least some situations. Explorations of differential light-induced damage to teleost photoreceptor classes have suggested that UVS cones are most resilient to damage. These experiments have used light with low UV content and examined damage using opsin immunohistochemistry in albino zebrafish (Vihtelic & Hyde, 2000), and used EM to investigate photoreceptor damage when UV light is applied to goldfish (Chen *et al.*, 1999b). These preliminary observations could be expanded on by use of differential light regimes on a species where UVS cones are clearly lost during ontogeny.

High metabolic costs leading to loss of UVS cones is a similar argument to those made for the regression of eyes in cave fishes (and other cave-dwelling organisms, e.g. invertebrates. Reviewed in Culver, 1982; Culver *et al.*, 1995; Fong *et al.*, 1995). Typically, regressive evolution is a framework that invokes genetic drift to explain loss of function in genes. It should be noted, however, that

the loss of the UVS cone during ontogeny cannot be readily explained by genetic drift. Genetic drift leading to mutation of the opsin or some other structural gene would not be maintained in the population because it would be selected against: the UVS cone is functional early in life history. Therefore if the lack of utility of the UVS cone, combined with its high metabolic expense, is held as a tenable explanation for some species, then it must include the evolution of a signalling mechanism that instructs the UVS cone to die. Indeed, an apoptotic *programmed* cell death mechanism appears to have evolved (See Chapter 3 Kunz *et al.*, 1994). Two types of signalling mechanism can be envisaged that lead to PCD: 1) the expression of (developmental patterning) genes which instruct the UVS cone, and not the other cone classes, to die at a particular point during the fishes life history; 2) one can imagine that if a second UVS opsin had been expressed late in ontogeny of ancestral fish, then mutation of the second copy to a non-functional opsin, that is lethal to the UVS cone, might act to drive cell death when its expression is increased later in life history. In summary, I believe that current evidence cannot eliminate the hypothesis that the high metabolic cost of maintaining a population of photoreceptors is a factor that favoured the evolution of UVS cone loss; for this hypothesis to be tenable, however, one must assume that UVS cones have limited utility in large fish.

At least for some species, however, a lack of utility for the UVS cones in large fish can be refuted. A substantial population of UVS cones is maintained in large rainbow trout (*O. mykiss,* see Chapter 2) and smelt *Osmerus eperlanus.* The results from smelt are tentative, as they are based solely on histology, and the

identity of the cone class is putative, and the idea that the UVS cones are disappearing is based upon their limited distribution in the ventrotemporal retina, and thus assumes they were ubiquitously distributed in the retina of younger individuals. In rainbow trout, however, the situation is clearer: large trout have UVS cones (as defined by expression of UVS opsin) in a substantial portion of their retina (Chapter 2). The distribution of these cones is in the dorsotemporal retina (Chapter 2), which is consistent with histological examinations of large rainbow trout (Martens, 2000; Hawryshyn *et al.*, 2003a). Furthermore, the data are consistent with optic nerve recordings where directional stimuli showed that sensitivity to UV light remains intact in the dorsal retina of large trout (Deutschlander *et al.*, 2001). The latter data also demonstrated that the retained UVS cones are functional.

The topological distribution of UVS cones in rainbow trout is particularly relevant to the question of whether UV photons reach the photoreceptors of large fish in sufficient quantity for the UVS cone to be useful. The population of UVS cones that is retained in large trout is in the dorsal retina which samples a ventral field of view (*e.g.* light from below the fish). Because the highest intensity light (*i.e.* the highest number of UV photons) is coming from above the fish, this strongly supports the argument that sufficient photons must be reaching the ventral retina to support UVS cone function. Thus the distribution of UVS cones is exactly opposite what one would predict if the number of photons reaching the retina were a factor driving UVS cone loss. Therefore, a lack of UV photons reaching the photoreceptors cannot be a complete explanation for why UVS

cones are lost during ontogeny. Furthermore, UV sensitivity should be expected to have various roles in these large fish, including detection of prey and con-specific communication (reviewed above). The data generated from this thesis allow the conclusion that UVS cones would have utility in large trout; therefore, there must be some selective advantage to losing UVS cones in trout, and possibly in other fish that lose UVS cones as well. This will be discussed in the next section. My speculations regarding the selective advantages of losing the UVS cone can be divided into two categories that are not mutually exclusive of each other: 1) re-tuning the visual system; 2) increasing visual acuity by decreasing detection of scattered UV photons.

5.5.2 Why lose UVS cones? Re-tuning the visual system

One selective advantage of losing UVS cones may be a re-tuning of the visual system, mediated through an effect upon the remaining photoreceptors. This may be expected during the time fish lose their UVS cones, as it appears that this is associated with the time when fish migrate to deeper water, including marine environments in anadromous populations. Several authors have presumed that the loss of UVS cones in salmonids is associated with fish moving to deeper waters (Lyall, 1957b, a; Ahlbert, 1976; Loew & McFarland, 1990; Beaudet & Hawryshyn, 1999). This seems reasonable as salmonids have deeper waters available to them in ocean and lake environments compared to the rivers and streams they occupied when UVS cones were ubiquitously distributed in the retina. Sampling from research vessels and commercial catches indicate that salmonids use deeper water in marine systems than is available to them in

streams and rivers (Groot & Margolis, 1991). After the first year of life in the marine environment, most Pacific salmon take up a pelagic existence, while two species (chinook and coho) can have shelf-resident and offshore variants (Groot & Margolis, 1991). Little information is available about daily habits of salmonids regarding the depths they utilize. However, data storage tags have recently been used to collect environmental temperature the fish experiences at regular time-points (e.g. each minute) (Walker *et al.*, 2000; Reddin *et al.*, 2004). The observed diurnal fluctuations in temperature, compared with oceanographic temperature sensors, indicate that salmonids must be making diurnal deep dives in marine waters (Walker *et al.*, 2000; Reddin *et al.*, 2004). The shallowest dives amongst the salmonids investigated to date, and most relevant to this thesis, come from ocean-going *O. mykiss* (steelhead salmon) that make diurnal dives to approximately 50 meters (Walker *et al.*, 2000). The dives occur during daylight hours, and salmonids are at the surface during night (Walker *et al.*, 2000; Reddin *et al.*, 2004). Thus the salmonids are performing visual tasks in an increased amount of scotopic environment when UVS cones are absent. The dives are believed to be associated with feeding, a task which relies on an optimized visual system. Further data on the marine ecology of salmon will greatly benefit interpretations regarding visual system development and its adaptive value to new visual tasks and photic environments. Regardless, at the time many fish (and at least salmonids) lose their UVS cones, they experience a different photic environment, and different predator/prey relationships.

One can argue that the remaining photoreceptors might perceive colours differently without the UVS cone present. For example, some fish skin reflects light that human observers perceive as yellow, but the reflected light can (but doesn't always, depending on the colour of the fish) contain a substantial amount of UV light that humans do not perceive. This reflected UV light is believed to be perceived by fish with UV vision, along with the longer wavelength light, to form a unique perceived colour that is not familiar to humans (Losey *et al.*, 1999; Losey, 2003). Losing UVS cones would change the perception of this colour (similar statements can be made for other colours, yellow is an arbitrary example). Considering the new predator-prey relationships and new photic environments that the fish are experiencing as the UVS cones are lost (e.g. switching from planktivory to piscivory, and moving to deeper waters) it may be that the absence of the UVS cone is advantageous. Beyond the perception of colour, such advantages could include improvements in the spatial resolution or adaptation of the retina. Goldfish UVS cones appear to be intermediate in these regards when compared to rods and the other cone classes (Hawryshyn, 1991b, a).

Furthermore, UVS cones appear to be a critical participant in the polarization sensitivity (PS) of some fish, including several that lose UVS cones (e.g. salmonids and goldfish, Hawryshyn & McFarland, 1987; Hawryshyn *et al.*, 1990; Parkyn & Hawryshyn, 1993; Coughlin & Hawryshyn, 1995; Novales Flamarique *et al.*, 1998; Hawryshyn, 2000; Parkyn & Hawryshyn, 2000; Hawryshyn *et al.*, 2002; Parkyn *et al.*, 2003). Thus, as rainbow trout lose their UVS cones they also lose their ability to orient to polarized light (Hawryshyn *et al.*, 1990; Hawryshyn,

2000). PS has several putative advantages, including increasing contrast of prey targets, con-specific communication, and allowing detection of celestial polarized light for use as a navigational cue. However, the perception of polarized light is believed to be mediated by the same sets of photoreceptors that are mediating colour vision. The efficiency of photoreceptors in their absorption of a photon can thus be affected by both the wavelength and polarization of the light. Thus, confounds to the perception of colours are possible when the stimuli vary in their polarization character (Hawryshyn, 2000; Hawryshyn *et al.*, 2003b). If one imagines situations where the accurate discrimination of a target by wavelength or intensity is more important than the perception of the polarization content of the target, then loss of the UVS cone could theoretically be advantageous through removal of confounds in the task.

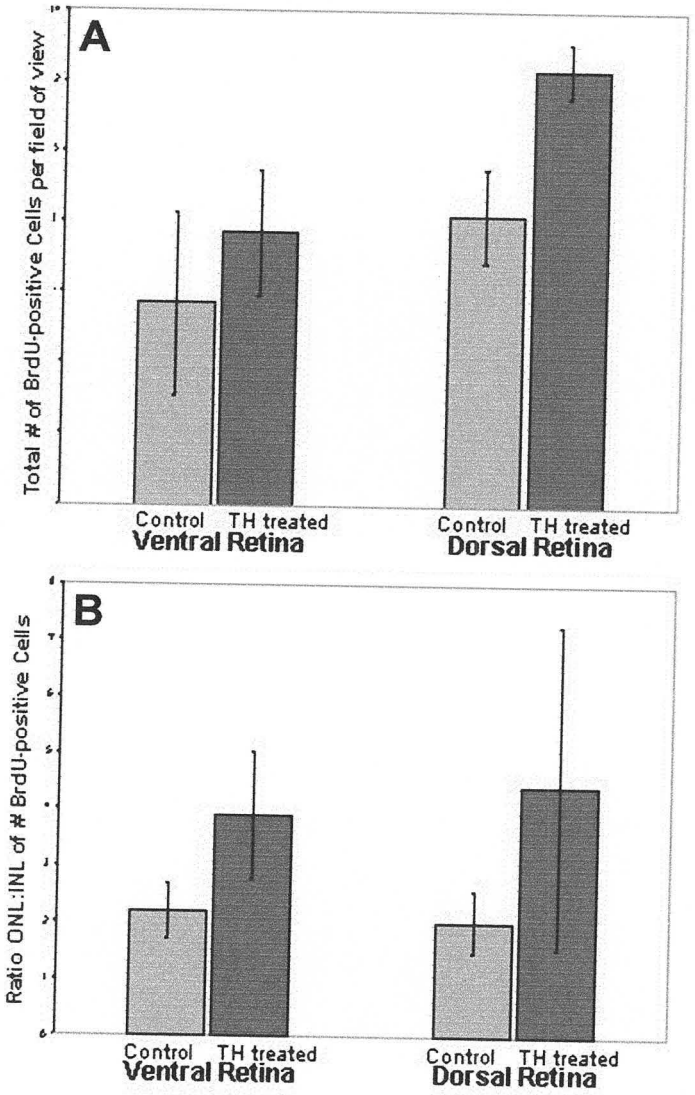
Preliminary support for the contention that the remaining cone mechanisms change when UVS cones disappear comes from work I have completed with Kathy Veldhoen and Craig Hawryshyn. We have recently observed that the levels of SWS, MWS, and LWS cone opsin proteins from retinal homogenates increase substantially (increases ranged from 1.4-fold to 2.5-fold increases of each protein) during TH treatment (Allison, Veldhoen & Hawryshyn, *in preparation*). It remains to be seen if similar changes in opsin levels occur during natural development or in locations where UVS cones are lost. Furthermore, the response of trout to the offset of light stimuli (this acts as a shadow detector, detecting dark prey on a light background), mediated primarily by the MWS cone

mechanism, has been shown to change during smoltification in rainbow trout (Beaudet *et al.*, 1993).

The loss of UVS cones could also create space in the retina for more rods to be added. This would have obvious advantages to fish moving to deeper waters, inhabiting increasingly scotopic environments. Indeed, in salmonids rods are known to fill in the spaces left by disappearing UVS cones (Lyall, 1957b; Ahlbert, 1976; Kunz *et al.*, 1994). It is debatable, however, whether this is substantially different than other fishes that also increase the number of rods in their retina as the retina grows, but have not been observed to lose their UVS cones. Arguing in favour of UVS cone disappearance being beneficial to rod density, it has been observed that the increase in rod number during growth is greater in the ventral salmonid retina (Ahlbert, 1976), and this is coincident with the location of UVS cone disappearance. Furthermore, I have observed that when rainbow trout are treated with thyroid hormone, there is an increase in BrdU labelled rods (Figure 20) in some areas of the retina, which accompanies the loss of the UVS cone. This is consistent with rods being created at an increased rate at a time similar to the loss of UVS cones. It remains to be tested, however, if the same effect can be observed during natural development. The latter point cannot be dismissed, because retinoic acid treatment of embryonic zebrafish can lead to increased rod production (Hyatt *et al.*, 1996; Perkins *et al.*, 2002). It can be argued that a loss of UVS cones is not a strict requirement for increasing the number of rods in fish retina. However I would argue that without loss of cones any increase in rods would necessarily decrease the ability to maximize cone density and/or size. This

would be detrimental to cone visual acuity and/or photon capture. This latter point returns the argument to my above speculation that UVS cone loss could be advantageous to the remaining cones, and could be re-stated: The loss of UVS cones may be advantageous because it allows rods to be added to the retina with less impact upon the potential to increase cone size or packing.

Figure 20. BrdU delivered during the beginning of TH treatment reveals more rods labelled in fish receiving TH treatment, consistent with more rods coming into the retina when UVS cones are disappearing. **A.**Thyroid Hormone (TH) treatment increases the number of cells generated in the dorsal retina, consistent with results of TH treatment on *Xenopus* (Beach & Jacobson, 1979; Marsh-Armstrong *et al.*, 1999). **B.**Thyroid Hormone (TH) treatment changes the types of cells generated, increasing the number of ONL cells (*i.e.* rods) relative to the number of INL cells. This is occurring near the same time that TH treatment produces UVS cone death. Error bars are 1 standard error, n=4.


A
Total # of BrdU-positive Cells per field of view
Control
TH treated
Ventral Retina
Control
TH treated
Dorsal Retina
B
Ratio ONL:INL of # BrdU-positive Cells
Control
TH treated
Ventral Retina
Control
TH treated
Dorsal Retina

The hypothesis that rod number increases are dependent on UVS cone loss could be tested by: 1) examining the number of rods per cone mosaic unit in salmonids during ontogeny; 2) comparing the areas of retina where UVS cones disappear to those where they do not; 3) further experiments could utilize caspase inhibitor to block UVS cone disappearance during TH treatment to investigate if the increase in rods I have observed is dependent on UVS cone disappearance.

5.5.3 Why lose UVS cones? Decreasing detection of scattered UV photons

An alternative, although not mutually exclusive, line of inquiry regarding hypothetical selective advantages to UVS cone loss is to reconsider the benefits of losing UV vision as outlined by Walls & Judd (1933). I believe that one of their speculations has remained unrecognized as being highly relevant to the current discussion: UV-blocking filters decrease detection of scattered UV light. Walls and Judd speculated that the abundance of UV photons scattered into the light path (UV light scatters more than longer wavelength light, and this is particularly apparent in aqueous media) degrades the target image, and detection of these photons decreases the signal:noise ratio. The hypothesis remains viable for explaining the benefits of ocular filters (Heinermann, 1984; Douglas & Marshall, 1999; Losey, 2003; Losey *et al.*, 2003; Siebeck *et al.*, 2003) but has not been applied previously to the loss of UVS cones during ontogeny. In this new hypothesis, the driving factor in loss of UVS cones is the need to detect predators and prey at increasing distances as the fish gets larger. Indeed it may be that UV vision, when present, acts only over short distances and allows tasks

such as conspecific communication using signals that are not visible to predators viewing from longer distances (Losey, 2003).

Losing the UVS cones may be an effective alternative (or complement) to increasing yellow intraocular filters during ontogeny, as these filters will negatively impact the scotopic visual system (Walls & Judd, 1933a, b; Heinermann, 1984; Losey *et al.*, 2003). Walls and Judd (1933) noted that "Any colour-filter is worse than useless in scotopic vision" (p.674). This is because any ocular filter will decrease the amount of light reaching the retina, and for scotopic vision it is assumed that maximizing photon capture is the highest priority of the visual system. This concept is supported by contemporary workers in the field (Heinermann, 1984; Douglas & Marshall, 1999; Losey *et al.*, 2003). The contention that ocular filters are detrimental to scotopic vision is highlighted by the observation that several fish have evolved occlusable filters. These filters are typically corneal chromatophores. Occlusable filters block short wavelength light transmission during the day and alter their form to increase light transmission during dim-light conditions (reviewed in Appleby & Muntz, 1979; Heinermann, 1984; Douglas & Marshall, 1999; Siebeck *et al.*, 2003).

Not detecting scattered UV light by developing the retina in a manner to lose UVS cones, as compared to the increase in yellow pigment of the ocular filters, has another important advantage: the UVS cone loss is reversible. Rather than having a permanent UV blocking filter, the fish is able to regenerate its UV sensitivity late in life history. This argument relies on the UVS cones having a

particular function in sexually mature salmonids, which will be reviewed in the next section.

Testing these ideas regarding functional benefits of losing the UVS cones is a difficult undertaking. It could be accomplished through comparing the ability of trout to detect targets at large distances, when the targets are detected by retinal areas with and without UVS cones (e.g. in large rainbow trout where UVS cones remain only in a portion of the retina). This approach suffers several confounds introduced by other asymmetries in the retina (reviewed in Chapter 1) including differences in photoreceptor density and visual pigment identity. Furthermore, depending on the paradigm used to assess the perception of stimuli (e.g. attack distance), there may be confounds introduced by differences in the motivation of the fish to attack prey in certain directions. Alternatively, transgenic modification could be used to block the signals for UVS cone cell death, or express the UVS opsin in photoreceptors that survive in large fish. This would allow testing of the ability to detect prey (or other targets) at large distances in fish with and without UV sensitivity. These tests could be refined by testing fish in media with differential UV scattering properties, or by increasing the quantity of UV photons in the down-welling light.

5.6 Functions of regenerated UV sensitivity

Regeneration of UVS cones seems like a substantial metabolic investment at a time when salmonids are expending energy for a return migration to spawning grounds. Other aspects of the salmonid photoreception, *i.e.* chromophore

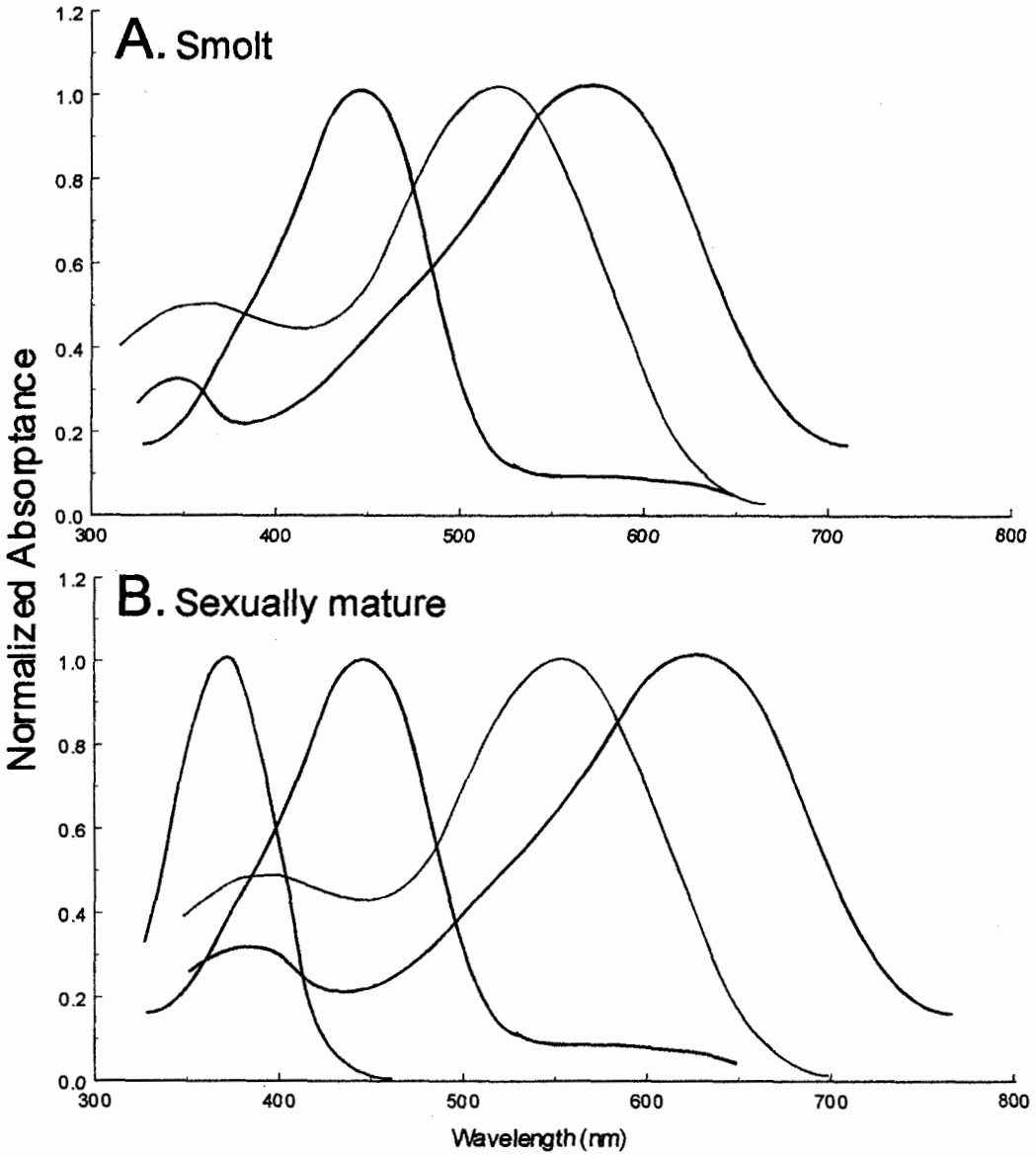
content, also change during salmonid spawning migration (Bridges, 1964; Beatty, 1966, 1984) in a manner that may tune the visual system for its new environment and/or tasks. Therefore I speculate that the ability to detect UV light has importance in the late stages of the salmonid life cycle. The hypothesis presented above suggests that UVS cones operate best when the target is at short distances. This implies that tasks mediated by regenerated UVS cones might occur over short distances or in small bodies of water. This would be consistent with smaller bodies of water and courtship behaviour occurring at this stage of the salmonid life cycle, where UV and polarized light signals might be viable. The regenerated UV sensitivity may play a role in polarized light detection, which salmonids can orient to as potential cues for migration (Hawryshyn *et al.*, 1990; Hawryshyn & Bogler, 1990; Hawryshyn, 2000; Parkyn & Hawryshyn, 2000; Parkyn *et al.*, 2003). Additionally, reflected light from conspecifics could contain polarized light or UV signals used in mating. UV light has been suggested to play a role in short-distance conspecifics communication (Losey, 2003) and plays a role in the mating signals of some fish (Karino & Matsunaga, 2002; Smith *et al.*, 2002; Cummings *et al.*, 2003; White *et al.*, 2003). The colouration of salmonids can dramatically change to beautiful hues during spawning, and these hues alone can elicit a mating response (Foote *et al.*, *In press*). Indeed, comparative examinations of salmonid opsins consider body colouration during mating as having a role in opsin evolution (Dann *et al.*, 2004a). It is noteworthy that the UVS cone may be mediating visual tasks that are not substantively different from those mediated by the other cone classes;

regardless, the increase in UVS cone density over the retina at sexual maturity could increase the spatial resolution of the photopic system, similar to the hypothesized role of new rods (Johns & Fernald, 1981; Johns, 1982; Fernald, 1990) added into the stretching teleost retina during adult growth. Further, the shift to vitamin A_2-based chromophore during spawning migration will expand the visual sensitivity into longer wavelengths (Beatty, 1984). The concomitant increase in UV sensitivity will broaden the spectral sensitivity in an impressive manner. The range of photoreceptor λ_{max} in ocean-going salmonids with A_1-based pigments and lacking UVS cones would be from about 430 to 570 nm (Hawryshyn *et al.*, 2001). During spawning migration, an increase in UVS cone density and a switch to A_2-based pigments could increase the spread of λ_{max} to a range from 370 to 617 nm (Hawryshyn *et al.*, 2001). This represents an almost doubled breadth amongst cone λ_{max}, *i.e.* an increased breadth of 117 nm (Figure 21).

5.7 Summary

In summary, it appears that there is ample reason to believe that UV vision would be an advantageous character in large fishes, and this is exemplified by the point that UVS cones regenerate late in life history of salmonids. Previously, workers in this field have speculated that fish lose their UVS cones because not enough photons reach the photoreceptors for the UVS cones to have utility. However this explanation cannot be complete: I have demonstrated that UVS cones are retained in the dorsal retina of large trout, where fewer photons would be received compared to the areas where UVS cones are lost.

Figure 21. The change in chromophore from A_1- to A_2-based (Beatty, 1966; Bridges, 1967) combined with the regeneration of UVS cones (see Chapter 3) that both occur at sexual maturity of salmonids would be expected to substantially broaden the fish's spectral sensitivity. **A.** The cone spectra that exist in salmonid smolts, e.g. ocean-going fish. **B.** The expected cone spectra present in sexually mature fish. Notably, the sensitivity in B is also expected in small fish, the transition to A_1-based chromophore may occur as the fish prepare for smoltification (Alexander *et al.*, 1994), a time when they lose UVS cones (Chapter 2), although the change in visual pigment may be coincidental and actually driven by an annual cycle.


A. Smolt
B. Sexually mature
Normalized Absorptance
Wavelength (nm)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
300
400
500
600
700
800

This led me to consider the question of whether or not the loss of UVS cones might have a selective advantage. Possible selective advantages, which have remained unrecognized in the literature, may include a tuning of the visual system to its new tasks as the fish move to new predator/prey relationships and deeper (*e.g.* marine) waters. The tuning of the visual system may include alterations in the properties of remaining cone photoreceptors. This concept might be extended to rods, as the balance of current evidence suggests that UVS cone loss enables an increase in rod photoreceptors. Finally, I have recognized that loss of UVS cones may confer important advantages to the visual system by reducing detection of UV photons that are scattered into the light path and reduce target detection. This is similar to long-standing hypotheses regarding benefits of UV blocking pigments in ocular filters. The principle attribute that makes this strategy an effective complement to UV blocking filters is that it does not hinder dim-light vision. This is critical as the fish are moving to deeper waters. Further, for at least some species, this strategy allows UVS cone regeneration to restore UV vision for tasks such as navigation or for conspecific communication during mating.

Literature cited

Adams, B. A., Cyr, D. G. & Eales, J. G. (2000). Thyroid hormone deiodination in tissues of American plaice, *Hippoglossoides platessoides*: characterization and short-term responses to polychlorinated biphenyls (PCBs) 77 and 126. *Comparative Biochemistry and Pysiology. Part C, Pharmacology, Toxicology and Endocrinology* **127,** 367-378.

Adamus, G., Zam, Z. S., Arendt, A., Palczewski, K., McDowell, J. H. & Hargrave, P. A. (1991). Anti-rhodopsin monoclonal antibodies of defined specificity: characterization and application. *Vision Research* **31,** 17-31.

Ahlbert, I. (1969). The organization of the cone cells in the retinae of four teleosts with different feeding habits (*Perca fluviatilis* L., *Lucioperca lucioperca* L., *Acerina cernua* L. and *Coregonus albula* L.). *Arkiv For Zoologi* **22,** 445-481.

Ahlbert, I.-B. (1976). Organization of the cone cells in the retinae of salmon (*Salmo salar*) and trout (*Salmo trutta trutta*) in relation to their feeding habits. *Acta Zoologica (Stockh)* **57,** 13-35.

Alexander, G. (1998). The role of thyroid hormone in visual pigment changes in juvenile coho salmon (*Oncorhynchus kisutch*). In *Department of Biological Sciences*, pp. 170. Simon Fraser University, Burnaby, BC.

Alexander, G., Sweeting, R. & McKeown, B. (1994). The Shift in Visual Pigment Dominance in the Retinae of Juvenile Coho Salmon (*Oncorhynchus kisutch*): an Indicator of Smolt Status. *Journal of Experimental Biology* **195,** 185-197.

Alexander, G., Sweeting, R. & McKeown, B. A. (2001). The effects of 3,4,3'-triiodo-l-thyronine on didehydroretinol synthesis by isolated coho salmon retinal pigment epithelial cells. *General and Comparative Endocrinology* **123,** 192-202.

Alexander, G. P., Sweeting, R. M. & McKeown, B. A. (1998). The effect of thyroid hormone and thyroid hormone blocker on visual pigment shifting in juvenile coho salmon (*Oncorhynchus kisutch*). *Aquaculture* **168,** 157-168.

Ali, M. A. (1959). The ocular structure, retinomotor and photo-behavioral responses of juvenile pacific salmon. *Canadian Journal of Zoology* **37,** 965-996.

Ali, M. A. (1964). Stretching of the retina during growth of salmon (*Salmo salar*). *Growth* **28,** 83-89.

Ali, M. A. & Kobayashi, H. (1968). Electroretinogram-flicker fusion frequency in albino trout. *Experientia* **24,** 454-455.

Allen, D. M. (1977). Measurements of serum thyroxine and the proportions of rhodopsin and porphyropsin in rainbow trout. *Canadian Journal of Zoology* **55,** 836-842.

Allen, D. M. (1995). Solar pruning of ros in albino trout. *Investigative Ophthalmology & Visual Science* **36,** S516-S516.

Allen, D. M. & Hallows, T. E. (1997). Solar pruning of retinal rods in albino rainbow trout. *Visual Neuroscience* **14,** 589-600.

Allen, D. M., Hendricks, G. C. & Hallows, T. E. (2001). Peripheral rods evade light damage in albino trout. In *Retinal Degenerative Diseases and Experimental Therapy.* ed. Hollyfield, J. G. & *al, e.* Kluwer Academic/Plenum Publishers, New York.

Allen, D. M., McFarland, W. N., Munz, F. W. & Poston, H. A. (1973). Changes in the visual pigments of trout. *Canadian Journal of Zoology* **51,** 901-914.

Allen, D. M. & Munz, F. W. (1983). Visual pigment mixtures and scotopic spectral sensitivity in rainbow trout. *Environmental Biology of Fishes* **8,** 185-190.

Allen, D. M., Pipes, C., Deramus, K. & Hallows, T. E. (1999). A comparison of light-induced rod degeneration in two teleost models. In *Retinal Degenerative Diseases and Experimental Therapy.* ed. Hollyfield, J. G. & al, e., pp. 337-350. Kluwer Academic/Plenum Publishers, New York.

Allison, W. T., Dann, S. G., Helvik, J. V., Bradley, C., Moyer, H. D. & Hawryshyn, C. W. (2003). Ontogeny of ultraviolet-sensitive cones in the retina of rainbow trout (*Oncorhynchus mykiss*). *Journal of Comparative Neurology* **461,** 294-306.

Allison, W. T., Haimberger, T. J., Hawryshyn, C. W. & Temple, S. E. (*In Preparation*). Visual Pigment Shifting in Zebrafish: Evidence for a rhodopsin - porphyropsin interchange system. **in prep**.

Appleby, S. J. & Muntz, W. R. A. (1979). Occlusable yellow corneas in Tetraodontidae. *Journal of Experimental Biology* **83,** 249-259.

Archer, S., Hope, A. & Partridge, J. C. (1995). The molecular basis for the green-blue sensitivity shift in the rod visual pigments of the European eel.

Proceedings of the Royal Society of London. Series B: Biological Sciences **262,** 289-295.

Awatramani, G., Wang, J. & Slaughter, M. M. (2001). Amacrine and ganglion cell contributions to the electroretinogram in amphibian retina. *Visual Neuroscience* **18,** 147-156.

Barthel, L. K. & Raymond, P. A. (2000). In situ hybridization studies of retinal neurons. *Methods in Enzymology* **316,** 579-590.

Bathelt, v. D. (1970). Experimentelle und vergleichend morphologische untersuchungen am visuellen system von teleostei. *Zoologische Jahrbücher. Abteilung für Anatomie und Ontogenie der Tiere.*

Bayliss, L. E., Lythgoe, R. J. & Tansley, K. (1936). Some new foms of visual purple found in sea fishes with a note on the visual cells of origin. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **120,** 95-113.

Beach, D. H. & Jacobson, M. (1979). Influences of thyroxine on cell proliferation in the retina of the clawed frog at different ages. *Journal of Comparative Neurology* **183,** 615-623.

Beatty, D. D. (1966). A study of the succession of visual pigments in Pacific salmon (*Oncorhynchus*). *Canadian Journal of Zoology* **44,** 429-455.

Beatty, D. D. (1984). Visual pigments and the labile scotopic visual system of fish. *Vision Research* **24,** 1563-1573.

Beaudet, L., Browman, H. I. & Hawryshyn, C. W. (1993). Optic nerve response and retinal structure in rainbow trout of different sizes. *Vision Research* **33,** 1739-1746.

Beaudet, L. & Hawryshyn, C. W. (1999). Ecological aspects of vertebrate visual ontogeny. In *Adaptive mechanisms in the ecology of vision.* ed. Archer, S. N., Djamgoz, M. B. A., Loew, E. R., Partridge, J. C. & Vallerga, S. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Beaudet, L., Novales Flamarique, I. & Hawryshyn, C. W. (1997). Cone photoreceptor topography in the retina of sexually mature Pacific salmonid fishes. *Journal of Comparative Neurology* **383,** 49-59.

Bilotta, J. & Saszik, S. (2001). The zebrafish as a model visual system. *International Journal of Developmental Neuroscience* **19,** 621-629.

Blackshaw, S., Fraioli, R. E., Furukawa, T. & Cepko, C. L. (2001). Comprehensive analysis of photoreceptor gene expression and the identification of candidate retinal disease genes. *Cell* **107,** 579-589.

Bowmaker, J. K. (1990). Visual pigments of fishes. In *The visual system of fish.* ed. Douglas, R. H. & Djamgoz, M. B. A. Chapman And Hall, Ltd., London.

Bowmaker, J. K. (1998). Evolution of colour vision in vertebrates. *Eye* **12,** 541-547.

Bowmaker, J. K. & Kunz, Y. W. (1987). Ultraviolet receptors, tetrachromatic colour vision and retinal mosaics in the brown trout (*Salmo trutta*): age-dependent changes. *Vision Research* **27,** 2101-2108.

Bowmaker, J. K., Thorpe, A. & Douglas, R. H. (1991). Ultraviolet-sensitive cones in the goldfish. *Vision Research* **31,** 349-352.

Braisted, J. E., Essman, T. F. & Raymond, P. A. (1994). Selective regeneration of photoreceptors in goldfish retina. *Development* **120,** 2409-2419.

Braisted, J. E. & Raymond, P. A. (1992). Regeneration of dopaminergic neurons in goldfish retina. *Development* **114,** 913-919.

Braisted, J. E. & Raymond, P. A. (1993). Continued search for the cellular signals that regulate regeneration of dopaminergic neurons in goldfish retina. *Brain Research. Developmental Brain Research* **76,** 221-232.

Branchek, T. (1984). The development of photoreceptors in the zebrafish, *Brachydanio rerio.* II. Function. *Journal of Comparative Neurology* **224,** 116-122.

Branchek, T. & Bremiller, R. (1984). The development of photoreceptors in the zebrafish, *Brachydanio rerio.* I. Structure. *Journal of Comparative Neurology* **224,** 107-115.

Brett, J. R. & Ali, M. A. (1958). Some observation on the structure and photomechanical responses of the Pacific salmon retina. *Journal of the Fisheries Research Board of Canada* **15,** 815-829.

Bridges, C. D. (1956). The visual pigments of the rainbow trout (*Salmo irideus*). *Journal of Physiology* **134,** 620-629.

Bridges, C. D. (1964). Periodicity of Absorption Properties in Pigments Based on Vitamin A_2 from Fish Retinae. *Nature* **203,** 303-304.

Bridges, C. D. (1975). Storage, distribution and utilization of vitamins A in the eyes of adult amphibians and their tadpoles. *Vision Research* **15,** 1311-1323.

Bridges, C. D. & Yoshikami, S. (1970). Distribution and evolution of visual pigments in salmonid fishes. *Vision Research* **10,** 609-626.

Bridges, C. D. B. (1967). Photopigments in the char of Lake Windermere (*Salvelinus willughbii* (Günther), forma *autumnalis* and forma *vernalis*). *Nature* **214,** 205-206.

Bridges, C. D. B. (1972). The rhodopsin-porphyropsin visual system. In *Handbook of Sensory Physiology*, vol. VII. ed. Dartnall, H. J. Springer-Verlag, Berlin.

Bridges, W. R. & von Limbach, B. (1972). Inheritance of albanism in rainbow trout. *Journal of Heredity* **63,** 152-153.

Browman, H. & Hawryshyn, C. (1994a). Retinoic Acid Modulates Retinal Development in the Juveniles of a Teleost Fish. *Journal of Experimental Biology* **193,** 191-207.

Browman, H., Novales-Flamarique, I. & Hawryshyn, C. (1994). Ultraviolet photoreception contributes to prey search behaviour in two species of zooplanktivorous fishes. *Journal of Experimental Biology* **186,** 187-198.

Browman, H. I. & Hawryshyn, C. W. (1992). Thyroxine induces a precocial loss of ultraviolet photosensitivity in rainbow trout (*Oncorhynchus mykiss*, Teleostei). *Vision Research* **32,** 2303-2312.

Browman, H. I. & Hawryshyn, C. W. (1994b). The developmental trajectory of ultraviolet photosensitivity in rainbow trout is altered by thyroxine. *Vision Research* **34,** 1397-1406.

Browman, H. I., Holmqvist, B., Forsell, J., Ebbesson, L. & Alm, P. (2001). Life-stage dependent changes and thyroid hormone-mediated plasticity in the retinal ultraviolet photoreceptor cell complement of Atlantic salmon (*Salmo salar*). *American Zoologist* **41,** 1398-1399.

Cameron, D. A. & Easter, S. S., Jr. (1995). Cone photoreceptor regeneration in adult fish retina: phenotypic determination and mosaic pattern formation. *Journal of Neuroscience* **15,** 2255-2271.

Candal, E., Anadon, R., Caruncho, H. C. & Rodriguez-Moldes, I. (2001). Proliferation, cell death and reelin expression during development of the

retina in a teleost, the common trout (*Salmo trutta fario*). *International Journal of Developmental Biology* **45,** S69-S70.

Carleton, K. L., Harosi, F. I. & Kocher, T. D. (2000). Visual pigments of African cichlid fishes: evidence for ultraviolet vision from microspectrophotometry and DNA sequences. *Vision Research* **40,** 879-890.

Carleton, K. L. & Kocher, T. D. (2001). Cone opsin genes of African cichlid fishes: tuning spectral sensitivity by differential gene expression. *Molecular Biology and Evolution* **18,** 1540-1550.

Carleton, K. L., Spady, T. C. & Kocher, T. D. (2002). Visual sensitivities of cichlid fishes: using DNA to see through the eyes of a fish. *Integrative and Comparative Biology* **42,** 1206-1206.

Chen, C. K., Burns, M. E., Spencer, M., Niemi, G. A., Chen, J., Hurley, J. B., Baylor, D. A. & Simon, M. I. (1999a). Abnormal photoresponses and light-induced apoptosis in rods lacking rhodopsin kinase. *Proceedings of the National Academy of Sciences of the United States of America* **96,** 3718-3722.

Chen, D. M., Dong, G. & Stark, W. S. (1999b). Ultraviolet light damage and reversal by retinoic acid in juvenile goldfish retina. In *Retinal Degenerative Diseases and Experimental Therapy.* ed. Hollyfield, J. G., Anderson, R. E. & LaVail, M. M., pp. 325-336. Plenum, New York.

Chen, D. M. & Stark, W. S. (1994). Electroretinographic analysis of ultraviolet sensitivity in juvenile and adult goldfish retinas. *Vision Research* **34,** 2941-2944.

Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162,** 156-159.

Chuang, J. C. & Raymond, P. A. (2002). Embryonic origin of the eyes in teleost fish. *Bioessays* **24,** 519-529.

Cid, E., Velasco, A., Ciudad, J., Orfao, A., Aijon, J. & Lara, J. M. (2002). Quantitative evaluation of the distribution of proliferating cells in the adult retina in three cyprinid species. *Cell and Tissue Research* **308,** 47-59.

Clarke, P. G., Posada, A., Primi, M. P. & Castagne, V. (1998). Neuronal death in the central nervous system during development. *Biomedicine and Pharmacotherapy* **52,** 356-362.

Coughlin, D. J., Forry, J. A., McGlinchey, S. M., Mitchell, J., Saporetti, K. A. & Stauffer, K. A. (2001). Thyroxine induces transitions in red muscle kinetics and steady swimming kinematics in rainbow trout (*Oncorhynchus mykiss*). *Journal of Experimental Zoology* **290,** 115-124.

Coughlin, D. J. & Hawryshyn, C. W. (1994a). The contribution of ultraviolet and short-wavelength sensitive cone mechanisms to color vision in rainbow trout. *Brain, Behavior and Evolution* **43,** 219-232.

Coughlin, D. J. & Hawryshyn, C. W. (1994b). Ultraviolet sensitivity in the torus semicircularis of juvenile rainbow trout (*Oncorhynchus mykiss*). *Vision Research* **34,** 1407-1413.

Coughlin, D. J. & Hawryshyn, C. W. (1995). A cellular basis for polarized-light vision in rainbow trout. *Journal of Comparative Physiology A-Sensory Neural and Behavioral Physiology* **176**.

Crescitelli, F. (1991). The scotopic photoreceptors and their visual pigments of fishes: functions and adaptations. *Vision Research* **31,** 339-348.

Culver, D. C. (1982). Regressive evolution. In *Cavelife*. ed. Culver, D. C., pp. 56-76. Harvard University Press, Cambridge, MA.

Culver, D. C., Kane, T. C. & Fong, D. W. (1995). *Adaptation and natural selection in caves: the evolution of Gammus minus*. Harvard University Press, Cambridge.

Cummings, M. E., Rosenthal, G. G. & Ryan, M. J. (2003). A private ultraviolet channel in visual communication. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **270,** 897-904.

Cyr, D. G. & Eales, J. G. (1996). Interrelationships between thyroidal and reproductive endocrine systems in fish. *Reviews in Fish Biology and Fisheries* **6,** 165-200.

Cyr, D. G., Idler, D. R., Audet, C., McLeese, J. M. & Eales, J. G. (1998). Effects of long-term temperature acclimation on thyroid hormone deiodinase function, plasma thyroid hormone levels, growth, and reproductive status of male Atlantic cod, *Gadus morhua. General and Comparative Endocrinology* **109,** 24-36.

Dann, S. G., Allison, W. E. & Hawryshyn, C. W. (2002). Upstream sequence of retinal opsin genes capitulates transcriptional control of those genes in *Oncorhynchus mykiss*. *Investigative Ophthalmology & Visual Science* **43,** S1011-S1011.

Dann, S. G., Allison, W. T. & Hawryshyn, C. W. (2003a). Examination of ontogenetic changes in vision in Pacific salmon using retinal opsin genomics. *Investigative Ophthalmology and Visual Science* **44,** S4539-S4539.

Dann, S. G., Allison, W. T., Levin, D. B. & Hawryshyn, C. W. (2003b). Identification of a unique transcript down-regulated in the retina of rainbow trout (*Oncorhynchus mykiss*) at smoltification. *Comparative Biochemistry and Physiology. Part B, Biochemistry and Molecular Biology,* 849-860.

Dann, S. G., Allison, W. T., Levin, D. B., Taylor, J. S. & Hawryshyn, C. W. (2004a). Salmonid opsin sequences undergo positive selection and indicate an alternate evolutionary relationship in *Oncorhynchus. Journal of Molecular Evolution* **58,** *In Press.*

Dann, S. G., Allison, W. T., Veldhoen, K., Johnson, T. & Hawryshyn, C. W. (2004b). Chromatin immunoprecipitation assay on the rainbow trout opsin proximal promoters illustrates binding of NF-κB and c-jun to the SWS1 promoter in the retina. *Experimental Eye Research, In Press.*

de la Rosa, E. J. & de Pablo, F. (2000). Cell death in early neural development: beyond the neurotrophic theory. *Trends in Neurosciences* **23,** 454-458.

Deutschlander, M. E., Greaves, D. K., Haimberger, T. J. & Hawryshyn, C. W. (2001). Functional mapping of ultraviolet photosensitivity during metamorphic transitions in a salmonid fish, *Oncorhynchus mykiss. Journal of Experimental Biology* **204,** 2401-2413.

Devlin, R. H., Biagi, C. A., Yesaki, T. Y., Smailus, D. E. & Byatt, J. C. (2001). Growth of domesticated transgenic fish. *Nature* **409,** 781-782.

Dong, W., Teraoka, H., Yamazaki, K., Tsukiyama, S., Imani, S., Imagawa, T., Stegeman, J. J., Peterson, R. E. & Hiraga, T. (2002). 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity in the zebrafish embryo: local circulation failure in the dorsal midbrain is associated with increased apoptosis. *Toxicol Sci* **69,** 191-201.

Douglas, R. H., Bowmaker, J. K. & Kunz, Y. W. (1989). Ultraviolet vision in fish. In *Seeing Contour and Colour.* ed. Kulikowski, J., pp. 601-616. Pergamon Press.

Douglas, R. H. & Marshall, N. J. (1999). A review of vertebrate and invertebrate optical filters. In *Adaptive mechanisms in the ecology of vision.* ed. Archer, S. N., Djamgoz, M. B. A., Loew, E. R., Partridge, J. C. & Vallerga, S. Kluwer Academic Publishers, Dordrect, The Netherlands.

Douglas, R. H. & McGuigan, C. M. (1989). The spectral transmission of freshwater teleost ocular media--an interspecific comparison and a guide to potential ultraviolet sensitivity. *Vision Research* **29,** 871-879.

Eales, J. G. (1995). Regulation of thyroidal status in fish. *Netherlands Journal of Zoology* **45,** 175-180.

Easter, S. S. (1992). Retinal growth in foveated teleosts: nasotemporal asymmetry keeps the fovea in temporal retina. *Journal of Neuroscience* **12,** 2381-2392.

Easter, S. S. & Hitchcock, P. F. (2000). Stem cells and regeneration in the retina: What fish have taught us about neurogenesis. *Neuroscientist* **6,** 454-464.

Eigenmann, C. H. & Shafer, G. D. (1900). The mosaic of single and twin cones in the retina of fishes. *American Naturalist* **34,** 109-118.

Engstom, K. (1960). Cone types and cone arrangements in the retina of some cyprinids. *Acta Zoologica (Stockh)* **41,** 277-295.

Engstom, K. (1963). Cone types and cone arrangements in teleost retinae. *Acta Zoologica (Stockh)* **44,** 179-243.

Evans, B. I. & Fernald, R. D. (1990). Metamorphosis and fish vision. *Journal of Neurobiology* **21,** 1037-1052.

Faillace, M. P., Julian, D. & Korenbrot, J. I. (2002). Mitotic activation of proliferative cells in the inner nuclear layer of the mature fish retina: regulatory signals and molecular markers. *Journal of Comparative Neurology* **451,** 127-141.

Fasick, J. I., Cronin, T. W., Hunt, D. M. & Robinson, P. R. (1998). The visual pigments of the bottlenose dolphin (*Tursiops truncatus*). *Visual Neuroscience* **15,** 643-651.

Fernald, R. D. (1990). Teleost vision: seeing while growing. *Journal of Experimental Zoology. Supplement* **5,** 167-180.

Firsov, M. L., Govardovskii, V. I. & Donner, K. (1994). Response univariance in bull-frog rods with two visual pigments. *Vision Research* **34,** 839-847.

Fischer, A. J. & Reh, T. A. (2001). Muller glia are a potential source of neural regeneration in the postnatal chicken retina. *Nature Neuroscience* **4,** 247-252.

Fischer, A. J. & Reh, T. A. (2003). Potential of Muller glia to become neurogenic retinal progenitor cells. *Glia* **43,** 70-76.

Fladmark, K. E., Serres, M. H., Larsen, N. L., Yasumoto, T., Aune, T. & Doskeland, S. O. (1998). Sensitive detection of apoptogenic toxins in suspension cultures of rat and salmon hepatocytes. *Toxicon* **36,** 1101-1114.

Fong, D. W., Kane, T. C. & Culver, D. C. (1995). Vestigialization and Loss of Nonfunctional Characters. *Annual Review of Ecology and Systematics* **26,** 249-268.

Foote, C. J., Brown, G. S. & Hawryshyn, C. W. (*In press*). Female colour and male choice in sockeye salmon: can a pre-existing bias explain convergent evolution between anadromous and non-anadromous morphs. *Animal Behavior.*

Forrest, D., Hallbook, F., Persson, H. & Vennstrom, B. (1991). Distinct functions for thyroid hormone receptors alpha and beta in brain development indicated by differential expression of receptor genes. *EMBO Journal* **10,** 269-275.

Forrest, D., Reh, T. A. & Rusch, A. (2002). Neurodevelopmental control by thyroid hormone receptors. *Current Opinion in Neurobiology* **12,** 49-56.

Franz, V. (1913). *Sehorgan*, vol. 7. Gustav Fischer, Jena.

Fratzer, C., Dorr, S. & Neumeyer, C. (1994). Wavelength discrimination of the goldfish in the ultraviolet spectral range. *Vision Research* **34,** 1515-1520.

Furst, C. M. (1904). Zur kenntnis der histogenese und des washstums der retina. *Lunds Universitets Årsskrift* **40,** 1-45.

Galton, V. A. (1992). The role of thyroid hormone in amphibian metamorphosis. *Trends Endocrinol Metab* **3,** 96-100.

Glickman, R. D. (2002). Phototoxicity to the retina: mechanisms of damage. *Int J Toxicol* **21,** 473-490.

Goldsmith, T. H. (1990). Optimization, constraint, and history in the evolution of eyes. *Quarterly Review of Biology* **65,** 281-322.

Groot, C. & Margolis, L. (1991). *Pacific salmon life histories.* University of British Columbia Press, Vancouver, Canada.

Hafezi, F., Marti, A., Grimm, C., Wenzel, A. & Reme, C. E. (1999). Differential DNA binding activities of the transcription factors AP-1 and Oct-1 during light-induced apoptosis of photoreceptors. *Vision Research* **39,** 2511-2518.

Hafezi, F., Steinbach, J. P., Marti, A., Munz, K., Wang, Z. Q., Wagner, E. F., Aguzzi, A. & Reme, C. E. (1997). The absence of c-fos prevents light-induced apoptotic cell death of photoreceptors in retinal degeneration in vivo. *Nature Medicine* **3,** 346-349.

Hagedorn, M. & Fernald, R. D. (1992). Retinal growth and cell addition during embryogenesis in the teleost, *Haplochromis burtoni*. *Journal of Comparative Neurology* **321,** 193-208.

Hao, W., Wenzel, A., Obin, M. S., Chen, C. K., Brill, E., Krasnoperova, N. V., Eversole-Cire, P., Kleyner, Y., Taylor, A., Simon, M. I., Grimm, C., Reme, C. E. & Lem, J. (2002). Evidence for two apoptotic pathways in light-induced retinal degeneration. *Nature Genetics* **32,** 254-260.

Harris, W. A. & Perron, M. (1998). Molecular recapitulation: the growth of the vertebrate retina. *International Journal of Developmental Biology* **42,** 299-304.

Hasegawa, E. I., Saito, T. & Seki, J. (2002). Composition changes in retinal pigments according to habitat of chum and pink salmon. *Journal of Fish Biology* **61,** 1305-1308.

Hawryshyn, C. W. (1991a). Light-adaptation properties of the ultraviolet-sensitive cone mechanism in comparison to the other receptor mechanisms of goldfish. *Visual Neuroscience* **6,** 293-301.

Hawryshyn, C. W. (1991b). Spatial summation of cone mechanisms in goldfish. *Vision Research* **31,** 563-566.

Hawryshyn, C. W. (2000). Ultraviolet polarization vision in fishes: possible mechanisms for coding e-vector. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* **355,** 1187-1190.

Hawryshyn, C. W. (2003). Polarisation Vision in Fishes. In *Sensory Processing in the Aquatic Environment*. ed. Collin, S. P. & Marshall, N. J. Springer-Verlag, New York.

Hawryshyn, C. W., Arnold, M. G., Bowering, E. & Cole, R. L. (1990). Spatial orientation of rainbow trout to plane-polarized light: The ontogeny of E-vector discrimination and spectral sensitivity characteristics. *Journal of*

Comparative Physiology A-Sensory Neural and Behavioral Physiology **166,** 565-574.

Hawryshyn, C. W., Arnold, M. G., Chaisson, D. J. & Martin, P. C. (1989). The ontogeny of ultraviolet photosensitivity in rainbow trout (*Salmo gairdneri*). *Visual Neuroscience* **2,** 247-254.

Hawryshyn, C. W. & Beauchamp, R. (1985). Ultraviolet photosensitivity in goldfish: an independent u.v. retinal mechanism. *Vision Research* **25,** 11-20.

Hawryshyn, C. W. & Bogler, A. E. (1990). Spatial orientation of trout to partially polarized light. *Journal of Comparative Physiology A-Sensory Neural and Behavioral Physiology* **167,** 691-697.

Hawryshyn, C. W., Haimberger, T. J. & Deutschlander, M. E. (2001). Microspectrophotometric measurements of vertebrate photoreceptors using CCD-based detection technology. *Journal of Experimental Biology* **204,** 2431-2438.

Hawryshyn, C. W. & Harosi, F. I. (1994). Spectral characteristics of visual pigments in rainbow trout (*Oncorhynchus mykiss*). *Vision Research* **34,** 1385-1392.

Hawryshyn, C. W., Martens, G., Allison, W. T. & Anholt, B. R. (2003a). Regeneration of ultraviolet-sensitive cones in the retinal cone mosaic of thyroxin-challenged post-juvenile rainbow trout (*Oncorhynchus mykiss*). *Journal of Experimental Biology* **206,** 2665-2673.

Hawryshyn, C. W. & McFarland, W. N. (1987). Cone photoreceptor mechanisms and the detection of polarized-light in fish. *Journal of Comparative Physiology A-Sensory Neural and Behavioral Physiology* **160,** 459-465.

Hawryshyn, C. W., Moyer, H., Allison, W. E., Haimberger, T. J. & McFarland, W. N. (2002). Polarization vision in coral reef fishes: Multichannel detection capabilities in damselfishes. *Investigative Ophthalmology and Visual Science* **43,** U1298-U1298.

Hawryshyn, C. W., Moyer, H. D., Allison, W. T., Haimberger, T. J. & McFarland, W. N. (2003b). Multidimensional polarization sensitivity in damselfishes. *Journal of Comparative Physiology. A, Sensory, Neural, and Behavioral Physiology* **189,** 213-220.

Heinermann, P. H. (1984). Yellow intraocular filters in fishes. *Experimental Biology* **43,** 127-147.

Helvik, J. V., Drivenes, Ø., Harboe, T. & Seo, H. C. (2001a). Topography of different photoreceptor cell types in the larval retina of Atlantic halibut (*Hippoglossus hippoglossus*). *Journal of Experimental Biology* **204,** 2553-2559.

Helvik, J. V., Drivenes, Ø., Næss, T. H., Fjose, A. & Seo, H. C. (2001b). Molecular cloning and characterization of five opsin genes from the marine flatfish Atlantic halibut (*Hippoglossus hippoglossus*). *Visual Neuroscience* **18,** 767-780.

Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J. & Ish-Horowicz, D. (1995). Expression of a Delta homologue in prospective neurons in the chick. *Nature* **375,** 787-790.

Hisatomi, O., Kayada, S., Aoki, Y., Iwasa, T. & Tokunaga, F. (1994). Phylogenetic relationships among vertebrate visual pigments. *Vision Research* **34,** 3097-3102.

Hisatomi, O., Satoh, T., Barthel, L. K., Stenkamp, D. L., Raymond, P. A. & Tokunaga, F. (1996). Molecular cloning and characterization of the putative ultraviolet- sensitive visual pigment of goldfish. *Vision Research* **36,** 933-939.

Hisatomi, O., Satoh, T. & Tokunaga, F. (1997). The primary structure and distribution of killifish visual pigments. *Vision Research* **37,** 3089-3096.

Hitchcock, P. F. & Cirenza, P. (1994). Synaptic organization of regenerated retina in the goldfish. *Journal of Comparative Neurology* **343,** 609-616.

Hitchcock, P. F., Lindsey Myhr, K. J., Easter, S. S., Jr., Mangione-Smith, R. & Jones, D. D. (1992). Local regeneration in the retina of the goldfish. *Journal of Neurobiology* **23,** 187-203.

Hitchcock, P. F. & Raymond, P. A. (1992). Retinal regeneration. *Trends in Neurosciences* **15,** 103-108.

Hoar, W. (1988). The Physiology of Smolting Salmonids. In *Fish Physiology*, vol. Vol. XI Part B. ed. Hoar, W. & Randall, D., pp. 275-343. Academic Press, San Diego.

Hope, A. J., Partridge, J. C. & Hayes, P. K. (1998). Switch in rod opsin gene expression in the European eel, *Anguilla anguilla* (L.). *Proceedings of the Royal Society of London. Series B: Biological Sciences* **265,** 869-874.

Hughes, A., Saszik, S., Bilotta, J., DeMarco, P. J., Jr. & Patterson, W. F., 2nd. (1998). Cone contributions to the photopic spectral sensitivity of the zebrafish ERG. *Visual Neuroscience* **15,** 1029-1037.

Hunt, D. M., Wilkie, S. E., Bowmaker, J. K. & Poopalasundaram, S. (2001). Vision in the ultraviolet. *Cellular and Molecular Life Sciences* **58,** 1583-1598.

Hyatt, G. A., Schmitt, E. A., Fadool, J. M. & Dowling, J. E. (1996). Retinoic acid alters photoreceptor development in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **93,** 13298-13303.

Ientile, R., Macaione, S., Russo, P., Pugliese, G. & Di Giorgio, R. M. (1984). Phenolic and tyrosyl ring deiodination in thyroxine from rat retina during postnatal development. *European Journal of Biochemistry* **142,** 15-19.

Jeffery, W. R. & Martasian, D. P. (1998). Evolution of eye regression in the cavefish *Astyanax:* Apoptosis and the *Pax-6* Gene. *American Zoologist* **38,** 685-696.

Johns, P. R. (1977). Growth of the adult goldfish eye. III. Source of the new retinal cells. *Journal of Comparative Neurology* **176,** 343-357.

Johns, P. R. (1982). Formation of photoreceptors in larval and adult goldfish. *Journal of Neuroscience* **2,** 178-198.

Johns, P. R. & Fernald, R. D. (1981). Genesis of rods in teleost fish retina. *Nature* **293,** 141-142.

Johnsen, S. (2001). Hidden in plain sight: the ecology and physiology of organismal transparency. *Biological Bulletin* **201,** 301-318.

Julian, D. (1997). Cell birth and fate in the teleost retina, pp. 146. University of California, San Francisco, San Francisco.

Julian, D., Ennis, K. & Korenbrot, J. I. (1998). Birth and fate of proliferative cells in the inner nuclear layer of the mature fish retina. *Journal of Comparative Neurology* **394,** 271-282.

Kamermans, M. & Spekreijse, H. (1995). Spectral behavior of cone-driven horizontal cells in teleost retina. *Progress in Retinal and Eye Research* **14,** 313-360.

Kamermans, M. & Spekreijse, H. (1999). The feedback pathway from horizontal cells to cones. A mini review with a look ahead. *Vision Research* **39,** 2449-2468.

Kampa, E. M. (1953). New forms of visual purple from the retinas of certain marine fishes; a re-examination. *Journal of Physiology* **119,** 400-409.

Karino, K. & Matsunaga, J. (2002). Female mate preference is for male total length, not tail length in feral guppies. *Behaviour* **139,** 1491-1508.

Kastner, R. & Wolburg, H. (1982). Functional regeneration of the visual system in teleosts. Comparative investigations after optic nerve crush and damage of the retina. *Zeitschrift für Naturforschung. Section C, A European journal of biosciences.* **37,** 1274-1280.

Kirk, J. T. O. (1994). *Light and photosynthesis in aquatic ecosystems.* Cambridge University Press, Cambridge.

Knight, J. K. & Raymond, P. A. (1995). Retinal pigmented epithelium does not transdifferentiate in adult goldfish. *Journal of Neurobiology* **27,** 447-456.

Kojima, D., Okano, T., Fukada, Y., Shichida, Y., Yoshizawa, T. & Ebrey, T. G. (1992). Cone visual pigments are present in gecko rod cells. *Proceedings of the National Academy of Sciences of the United States of America* **89,** 6841-6845.

Korenbrot, J. I. & Fernald, R. D. (1989). Circadian rhythm and light regulate opsin mRNA in rod photoreceptors. *Nature* **337,** 454-457.

Krishnamoorthy, R. R., Crawford, M. J., Chaturvedi, M. M., Jain, S. K., Aggarwal, B. B., Al-Ubaidi, M. R. & Agarwal, N. (1999). Photo-oxidative stress down-modulates the activity of nuclear factor-kappaB via involvement of caspase-1, leading to apoptosis of photoreceptor cells. *Journal of Biological Chemistry* **274,** 3734-3743.

Kuan, C. Y., Roth, K. A., Flavell, R. A. & Rakic, P. (2000). Mechanisms of programmed cell death in the developing brain. *Trends in Neurosciences* **23,** 291-297.

Kunz, Y. W. (1987). Tracts of putative ultraviolet receptors in the retina of the two-year- old brown trout (*Salmo trutta*) and the Atlantic salmon (*Salmo salar*). *Experientia* **43,** 1202-1204.

Kunz, Y. W., Wildenburg, G., Goodrich, L. & Callaghan, E. (1994). The fate of ultraviolet receptors in the retina of the Atlantic salmon (*Salmo salar*). *Vision Research* **34,** 1375-1383.

Kurz-Isler, G. & Wolburg, H. (1982). Morphological study on the regeneration of the retina in the rainbow trout after ouabain-induced damage: evidence for

dedifferentiation of photoreceptors. *Cell and Tissue Research* **225,** 165-178.

Kusmic, C., Barsanti, L., Passarelli, V. & Gualtieri, P. (1993). Photoreceptor morphology and visual pigment content in the pineal organ and in the retina of juvenile and adult trout, *Salmo irideus*. *Micron* **24,** 279-286.

Kusmic, C. & Gualtieri, P. (2000). Morphology and spectral sensitivities of retinal and extraretinal photoreceptors in freshwater teleosts. *Micron* **31,** 183-200.

Larison, K. D. & Bremiller, R. (1990). Early onset of phenotype and cell patterning in the embryonic zebrafish retina. *Development* **109,** 567-576.

Lee, S. K., Kim, J. H., Lee, Y. C., Cheong, J. & Lee, J. W. (2000). Silencing mediator of retinoic acid and thyroid hormone receptors, as a novel transcriptional corepressor molecule of activating protein-1, nuclear factor-kappaB, and serum response factor. *Journal of Biological Chemistry* **275,** 12470-12474.

Levine, J. S. & MacNichol, E. F., Jr. (1979). Visual pigments in teleost fishes: effects of habitat, microhabitat, and behavior on visual system evolution. *Sensory Processes* **3,** 95-131.

Li, F., Cao, W. & Anderson, R. E. (2001). Protection of photoreceptor cells in adult rats from light-induced degeneration by adaptation to bright cyclic light. *Experimental Eye Research* **73,** 569-577.

Li, F., Cao, W. & Anderson, R. E. (2003). Alleviation of constant-light-induced photoreceptor degeneration by adaptation of adult albino rat to bright cyclic light. *Investigative Ophthalmology and Visual Science* **44,** 4968-4975.

Li, L. (2001). Zebrafish mutants: behavioral genetic studies of visual system defects. *Developmental Dynamics* **221,** 365-372.

Li, S., Chang, C. J., Abler, A. S., Fu, J., Tso, M. O. & Lam, T. T. (1996). A comparison of continuous versus intermittent light exposure on apoptosis. *Current Eye Research* **15,** 914-922.

Liden, J., Delaunay, F., Rafter, I., Gustafsson, J. & Okret, S. (1997). A new function for the C-terminal zinc finger of the glucocorticoid receptor. Repression of RelA transactivation. *Journal of Biological Chemistry* **272,** 21467-21472.

Loew, E. R. (1995). Determinants of visual pigment spectral location and photoreceptor cell spectral sensitivity. In *Neurobiology and clinical aspects of the outer retina.* ed. Djamgoz, M. B. A., pp. 57-77. Chapman & Hall, London.

Loew, E. R. (1999). Light and photoreception: physical principles. In *Adaptive mechanisms in the ecology of vision.* ed. Archer, S. N., Djamgoz, M. B. A., Loew, E. R., Partridge, J. C. & Vallerga, S., pp. 3-23. Kluwer Academic Publishers, Dordrecht, Germany.

Loew, E. R. & McFarland, W. N. (1990). The underwater visual environment. In *The visual system of fish.* ed. Douglas, R. H. & M., D., pp. 1-43. Chapman and Hall, London.

Loew, E. R., McFarland, W. N. & Margulies, D. (2002). Developmental changes in the visual pigments of the yellowfin tuna, *Thunnus albacares. Marine and Freshwater Behaviour and Physiology* **35,** 235-246.

Loew, E. R., McFarland, W. N., Mills, E. L. & Hunter, D. (1993). A chromatic spectrum for planktonic predation by juvenile yellow perch, *Perca flavescens. Canadian Journal of Zoology* **71,** 384-386.

Loew, E. R. & Wahl, C. M. (1991). A short-wavelength sensitive cone mechanism in juvenile yellow perch, *Perca flavescens. Vision Research* **31,** 353-360.

Lombardo, F. (1968). La rigenerazione della retina negli adulti di un Teleosteo. *Rendiconti / Accademia nazionale dei Lincei, Classe di scienze fisiche, matematiche e naturali.* **45,** 631-635.

Lombardo, F. (1972). Andamento e localizzazione dell mitosi durante la rigenerazione delle retina di un Teleosteo adulto. *Rendiconti / Accademia nazionale dei Lincei, Classe di scienze fisiche, matematiche e naturali.* **63,** 323-327.

Losey, G. S. (2003). Crypsis and communication functions of UV-visible coloration in two coral reef damselfish, *Dascyllus aruanus* and *D. reticulatus. Animal Behaviour* **66,** 299-307.

Losey, G. S., Cronin, T. W., Goldsmith, T. H., Hyde, D., Marshall, N. J. & McFarland, W. N. (1999). The UV visual world of fishes: a review. *Journal of Fish Biology* **54,** 921-943.

Losey, G. S., McFarland, W. N., Loew, E. R., Zamzow, J. P., Nelson, P. A. & Marshall, N. J. (2003). Visual biology of Hawaiian coral reef fishes. I. Ocular transmission and visual pigments. *Copeia,* 433-454.

Lyall, A. H. (1957a). Cone arrangements in teleost retinae. *Quar J Micro Sci* **98,** 189-201.

Lyall, A. H. (1957b). The growth of the trout retina. *Quar J Micro Sci* **98,** 101-110.

Lythgoe, J. N. (1979). *The ecology of vision.* Oxford University Press, Oxford.

Lythgoe, J. N. (1985). Light and vision in the aquatic environment. In *Sensory Biology of Aquatic Animals.* ed. Atema, J., Fay, R. R., Popper, A. N. & Tavolga, W. N., pp. 57-82. Springer-Verlag, New York.

Macaione, S., Di Giorgio, R. M., Nicotina, P. A. & Ientile, R. (1984). Retina maturation following administration of thyroxine in developing rats: effects on polyamine metabolism and glutamate decarboxylase. *Journal of Neurochemistry* **43,** 303-315.

Maier, W. & Wolburg, H. (1979). Regeneration of the goldfish retina after exposure to different doses of ouabain. *Cell and Tissue Research* **202,** 99-118.

Mann, F. & Holt, C. E. (2001). Control of retinal growth and axon divergence at the chiasm: lessons from *Xenopus. Bioessays* **23,** 319-326.

Mansour-Robaey, S. & Pinganaud, G. (1990). Quantitative and morphological study of cell proliferation during morphogenesis in the trout visual system. *Journal fur Hirnforschung* **31,** 495-504.

Marotte, L. R., Wye-Dvorak, J. & Mark, R. F. (1979). Retinotectal reorganization in goldfish--II. Effects of partial tectal ablation and constant light on the retina. *Neuroscience* **4,** 803-810.

Marshall, N. J., Jennings, K., McFarland, W. N., Loew, E. R. & Losey, G. S. (2003a). Visual biology of Hawaiian coral reef fishes. II. Colors of Hawaiian coral reef fish. *Copeia,* 455-466.

Marshall, N. J., Jennings, K., McFarland, W. N., Loew, E. R. & Losey, G. S. (2003b). Visual biology of Hawaiian coral reef fishes. III. Environmental light and an integrated approach to the ecology of reef fish vision. *Copeia,* 467-480.

Marsh-Armstrong, N., Huang, H., Remo, B. F., Liu, T. T. & Brown, D. D. (1999). Asymmetric growth and development of the *Xenopus laevis* retina during metamorphosis is controlled by type III deiodinase. *Neuron* **24,** 871-878.

Martens, G. D. (2000). Topographical changes to the cone photoreceptor mosaic in thyroxin challenged rainbow trout (*Oncorhynchus mykiss*). In *Biology*, pp. 134. University of Victoria, Victoria, BC.

Mears, A. J., Kondo, M., Swain, P. K., Takada, Y., Bush, R. A., Saunders, T. L., Sieving, P. A. & Swaroop, A. (2001). Nrl is required for rod photoreceptor development. *Nature Genetics* **29,** 447-452.

Milchert, L. E., Liberles, D. A. & Elofsson, A. (2002). The salmon genome (and other issues in bioinformatics). *Genome Biology* **3,** 4022.

Millodot, M. (1967). The electroretinogram of the trout. *British Journal of Physiological Optics* **24,** 23-27.

Morin, P. P., Hara, T. J. & Eales, J. G. (1993). Thyroid hormone deiodination in brain, liver, gill, heart and muscle of Atlantic salmon (*Salmo salar*) during photoperiodically-induced parr-smolt transformation. I. Outer- and inner-ring thyroxine deiodination. *General and Comparative Endocrinology* **90,** 142-156.

Moyes, C. D., Sharma, M. L., Lyons, C., Leary, S. C., Leon, M., Petrie, A., Lund, S. G. & Tufts, B. L. (2002). Origins and consequences of mitochondrial decline in nucleated erythrocytes. *Biochim Biophys Acta* **1591,** 11-20.

Muntz, W. R. & Mouat, G. S. (1984). Annual variations in the visual pigments of brown trout inhibiting lochs providing different light environments. *Vision Research* **24,** 1575-1580.

Muntz, W. R. & Northmore, D. P. (1971). Visual pigments from different parts of the retina in rudd and trout. *Vision Research* **11,** 551-561.

Munz, F. W. (1957). The photosensitive retinal pigments of marine and euryhaline teleost fishes. University of California, Los Angeles.

Munz, F. W. & Beatty, D. D. (1965). A critical analysis of the visual pigments of salmon and trout. *Vision Research* **5,** 1-17.

Naka, K. I. & Rushton, W. A. (1966a). An attempt to analyse colour reception by electrophysiology. *Journal of Physiology* **185,** 556-586.

Naka, K. I. & Rushton, W. A. (1966b). S-potentials from colour units in the retina of fish (Cyprinidae). *Journal of Physiology* **185,** 536-555.

Negishi, K., Stell, W. K., Teranishi, T., Karkhanis, A., Owusu-Yaw, V. & Takasaki, Y. (1991). Induction of proliferating cell nuclear antigen (PCNA)-

immunoreactive cells in goldfish retina following intravitreal injection with 6- hydroxydopamine. *Cellular and Molecular Neurobiology* **11,** 639-659.

Neumeyer, C. (1985). An Ultraviolet Receptor as a 4th Receptor Type in Goldfish Color-Vision. *Naturwissenschaften* **72,** 162-163.

Neumeyer, C. (1986). Wavelength discrimination in the goldfish. *Journal of Comparative Physiology A-Sensory Neural and Behavioral Physiology* **158,** 203-213.

Neumeyer, C. (1992). Tetrachromatic colour vision in goldfish: evidence from colour mixture experiments. *Journal of Comparative Physiology A-Sensory Neural and Behavioral Physiology* **171,** 639-649.

Neumeyer, C. & Arnold, K. (1989). Tetrachromatic color vision in the goldfish becomes trichromatic under white adaptation light of moderate intensity. *Vision Research* **29,** 1719-1727.

Ng, L., Hurley, J. B., Dierks, B., Srinivas, M., Salto, C., Vennstrom, B., Reh, T. A. & Forrest, D. (2001). A thyroid hormone receptor that is required for the development of green cone photoreceptors. *Nature Genetics* **27,** 94-98.

Novales Flamarique, I. (2000). The ontogeny of ultraviolet sensitivity, cone disappearance and regeneration in the sockeye salmon *Oncorhynchus nerka*. *Journal of Experimental Biology* **203,** 1161-1172.

Novales Flamarique, I. & Hawryshyn, C. (1996). Retinal development and visual sensitivity of young Pacific sockeye salmon (*Oncorhynchus nerka*). *Journal of Experimental Biology* **199,** 869-882.

Novales Flamarique, I. & Hawryshyn, C. W. (1997). Is the use of underwater polarized light by fish restricted to crepuscular time periods? *Vision Research* **37,** 975-989.

Novales Flamarique, I., Hawryshyn, C. W. & Harosi, F. I. (1998). Double-cone internal reflection as a basis for polarization detection in fish. *Journal of the Optical Society of America A. Optics and Image Science* **15,** 349-358.

Novales Flamarique, I., Hendry, A. & Hawryshyn, C. W. (1992). The Photic Environment of a Salmonid Nursery Lake. *Journal of Experimental Biology* **169,** 121-141.

Novales Flamarique, I., Hendry, A. & Hawryshyn, C. W. (1993). Spectral Characteristics of Salmonid Migratory Routes from Southern Vancouver-Island (British-Columbia). *Canadian Journal of Fisheries and Aquatic Sciences* **50,** 1706-1716.

Olson, A. J., Picones, A., Julian, D. & Korenbrot, J. I. (1999). A developmental time line in a retinal slice from rainbow trout. *Journal of Neuroscience Methods* **93,** 91-100.

Olson, A. J., Picones, A. & Korenbrot, J. I. (2000). Developmental switch in excitability, Ca^{2+} and K^{+} currents of retinal ganglion cells and their dendritic structure. *Journal of Neurophysiology* **84,** 2063-2077.

Organisciak, D. T. & Winkler, B. S. (1994). Retinal light damage - practical and theoretical considerations. *Progress in Retinal and Eye Research* **13,** 1-29.

Orozco, A., Linser, P. & Valverde, C. (2000). Kinetic characterization of outer-ring deiodinase activity (ORD) in the liver, gill and retina of the killifish *Fundulus heteroclitus*. *Comparative Biochemistry and Physiology. Part B, Biochemistry and Molecular Biology* **126,** 283-290.

Otteson, D. C., D'Costa, A. R. & Hitchcock, P. F. (2001). Putative stem cells and the lineage of rod photoreceptors in the mature retina of the goldfish. *Developmental Biology* **232,** 62-76.

Otteson, D. C. & Hitchcock, P. F. (2003). Stem cells in the teleost retina: persistent neurogenesis and injury-induced regeneration. *Vision Research* **43,** 927-936.

Palacios, A. G., Varela, F. J., Srivastava, R. & Goldsmith, T. H. (1998). Spectral sensitivity of cones in the goldfish, *Carassius auratus*. *Vision Research* **38,** 2135-2146.

Parkyn, D. C., Austin, J. D. & Hawryshyn, C. W. (2003). Acquisition of polarized-light orientation in salmonids under laboratory conditions. *Animal Behaviour* **65,** 893-904.

Parkyn, D. C. & Hawryshyn, C. W. (1993). Polarized-light sensitivity in rainbow trout (*Oncorhynchus mykiss*): characterization from multi-unit responses in the optic nerve. *Journal of Comparative Physiology A-Sensory Neural and Behavioral Physiology* **172,** 493-500.

Parkyn, D. C. & Hawryshyn, C. W. (2000). Spectral and ultraviolet-polarisation sensitivity in juvenile salmonids: a comparative analysis using electrophysiology. *Journal of Experimental Biology* **203 Pt 7,** 1173-1191.

Parry, J. W., Peirson, S. N., Wilkens, H. & Bowmaker, J. K. (2003). Multiple photopigments from the Mexican blind cavefish, *Astyanax fasciatus*: a microspectrophotometric study. *Vision Research* **43,** 31-41.

Penn, J. S. (1985). Effects of continuous light on the retina of a fish, *Notemigonus crysoleucas. Journal of Comparative Neurology* **238,** 121-127.

Perez, P., Schonthal, A. & Aranda, A. (1993). Repression of c-fos gene expression by thyroid hormone and retinoic acid receptors. *Journal of Biological Chemistry* **268,** 23538-23543.

Perkins, B. D., Kainz, P. M., O'Malley, D. M. & Dowling, J. E. (2002). Transgenic expression of a GFP-rhodopsin COOH-terminal fusion protein in zebrafish rod photoreceptors. *Visual Neuroscience* **19,** 257-264.

Perron, M., Kanekar, S., Vetter, M. L. & Harris, W. A. (1998). The genetic sequence of retinal development in the ciliary margin of the *Xenopus* eye. *Developmental Biology* **199,** 185-200.

Phillips, R. & Rab, P. (2001). Chromosone evolution in the Salmonidae (Pisces): an update. *Biological Reviews* **76,** 1-25.

Pierce, M. E. (1999). Circadian organization in quail retina: differential regulation of melatonin synthesis and iodopsin gene expression in vitro. *Visual Neuroscience* **16,** 843-848.

Pierce, M. E., Sheshberadaran, H., Zhang, Z., Fox, L. E., Applebury, M. L. & Takahashi, J. S. (1993). Circadian regulation of iodopsin gene expression in embryonic photoreceptors in retinal cell culture. *Neuron* **10,** 579-584.

Plate, E. M., Adams, B. A., Allison, W. T., Martens, G., Hawryshyn, C. W. & Eales, J. G. (2002). The effects of thyroxine or a GnRH analogue on thyroid hormone deiodination in the olfactory epithelium and retina of rainbow trout, *Oncorhynchus mykiss*, and sockeye salmon, *Oncorhynchus nerka. General and Comparative Endocrinology* **127,** 59-65.

Polyak, S. L. (1957). *The vertebrate visual system; its origin, structure, and function and its manifestations in disease with an analysis of its role in the life of animals and in the origin of man, preceded by a historical review of investigations of the eye, and of the visual pathways and centers of the brain. Edited by Heinrick Klüver.* University of Chicago Press, Chicago.

Puzianowska-Kuznicka, M., Damjanovski, S. & Shi, Y. B. (1997). Both thyroid hormone and 9-cis retinoic acid receptors are required to efficiently mediate the effects of thyroid hormone on embryonic development and specific gene regulation in Xenopus laevis. *Molecular and Cellular Biology* **17,** 4738-4749.

Raff, M. C., Barres, B. A., Burne, J. F., Coles, H. S., Ishizaki, Y. & Jacobson, M. D. (1993). Programmed cell death and the control of cell survival: lessons from the nervous system. *Science* **262,** 695-700.

Raymond, P. A. (1985). Cytodifferentiation of photoreceptors in larval goldfish: delayed maturation of rods. *Journal of Comparative Neurology* **236,** 90-105.

Raymond, P. A. (1991). Retinal regeneration in teleost fish. *CIBA Foundation Symposium* **160,** 171-186; discussion 186-191.

Raymond, P. A. (1995). Development and morphological organization of photoreceptors. In *Neurobiology and clinical aspects of the outer retina.* ed. Djamgoz, M. B. A., pp. 1-23. Chapman & Hall, London.

Raymond, P. A., Barthel, L. K. & Curran, G. A. (1995). Developmental patterning of rod and cone photoreceptors in embryonic zebrafish. *Journal of Comparative Neurology* **359,** 537-550.

Raymond, P. A., Barthel, L. K., Rounsifer, M. E., Sullivan, S. A. & Knight, J. K. (1993). Expression of rod and cone visual pigments in goldfish and zebrafish: a rhodopsin-like gene is expressed in cones. *Neuron* **10,** 1161-1174.

Raymond, P. A., Barthel, L. K. & Stenkamp, D. L. (1996). The zebrafish ultraviolet cone opsin reported previously is expressed in rods. *Investigative Ophthalmology and Visual Science* **37,** 948-950.

Raymond, P. A., Bassi, C. J. & Powers, M. K. (1988a). Lighting conditions and retinal development in goldfish: photoreceptor number and structure. *Investigative Ophthalmology and Visual Science* **29,** 27-36.

Raymond, P. A. & Hitchcock, P. F. (1997). Retinal regeneration: common principles but a diversity of mechanisms. *Advances in Neurology* **72,** 171-184.

Raymond, P. A. & Hitchcock, P. F. (2000). How the neural retina regenerates. *Results and Problems in Cell Differentiation* **31,** 197-218.

Raymond, P. A., Reifler, M. J. & Rivlin, P. K. (1988b). Regeneration of goldfish retina: rod precursors are a likely source of regenerated cells. *Journal of Neurobiology* **19,** 431-463.

Reckel, F., Hoffmann, B., Melzer, R. R., Horppila, J. & Smola, U. (2003). Photoreceptors and cone patterns in the retina of the smelt *Osmerus eperlanus* (L.) (Osmeridae : Teleostei). *Acta Zoologica* **84,** 161-170.

Reddin, D. G., Friedland, K. D., Downton, P., Dempson, J. B. & Mullins, C. C. (2004). Thermal habitat experienced by Atlantic salmon (Salmo salar L.) kelts in coastal Newfoundland waters. *Fisheries Oceanography* **13,** 24-35.

Reme, C. E., Grimm, C., Hafezi, F., Iseli, H. P. & Wenzel, A. (2003). Why study rod cell death in retinal degenerations and how? *Documenta Ophthalmologica* **106,** 25-29.

Reme, C. E., Grimm, C., Hafezi, F., Marti, A. & Wenzel, A. (1998). Apoptotic cell death in retinal degenerations. *Progress in Retinal and Eye Research* **17,** 443-464.

Reuter, T. E., White, R. H. & Wald, G. (1971). Rhodopsin and porphyropsin fields in the adult bullfrog retina. *Journal of General Physiology* **58,** 351-371.

Rochon-Duvigneaud, A. (1943). *Les yeux et la vision des vertébrés.* Masson, Paris.

Ryder, J. A. (1895). An arrangement of the retinal cells in the eyes of fishes partially simulating compound eyes. *Proceedings of the Academy of Natural Sciences of Philidelphia* **(no volume number assigned),** 161-166.

Sachs, L. M., Damjanovski, S., Jones, P. L., Li, Q., Amano, T., Ueda, S., Shi, Y. B. & Ishizuya-Oka, A. (2000). Dual functions of thyroid hormone receptors during *Xenopus* development. *Comparative Biochemistry and Physiology. Part B, Biochemistry and Molecular Biology* **126,** 199-211.

Sanders, L. H. & Whitlock, K. E. (2003). Phenotype of the zebrafish masterblind (mbl) mutant is dependent on genetic background. *Developmental Dynamics* **227,** 291-300.

Sandy, J. M. & Blaxter, J. H. S. (1980). Study of retinal development in larval herring and sole. *Journal of the Marine Biological Association of the United Kingdom* **60,** 59-71.

Saszik, S., Bilotta, J. & Givin, C. M. (1999). ERG assessment of zebrafish retinal development. *Visual Neuroscience* **16,** 881-888.

Schmitt, E. & Kunz, Y. W. (1989). Retinal morphogenesis in the rainbow trout, *Salmo gairdneri. Brain, Behavior and Evolution* **34,** 48-64.

Scholes, J. H. (1975). Colour receptors, and their synaptic connexions, in the retina of a cyprinid fish. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* **270,** 61-118.

Semple-Rowland, S. L. & Goldstein, E. B. (1981). Segregation of vitamin A_1 and vitamin A_2 cone pigments in the bullfrog retina. *Vision Research* **21,** 825-828.

Shahinfar, S., Edward, D. P. & Tso, M. O. (1991). A pathologic study of photoreceptor cell death in retinal photic injury. *Current Eye Research* **10,** 47-59.

Shand, J., Archer, M. A. & Collin, S. P. (1999). Ontogenetic changes in the retinal photoreceptor mosaic in a fish, the black bream, *Acanthopagrus butcheri. Journal of Comparative Neurology* **412,** 203-217.

Shand, J., Hart, N. S., Thomas, N. & Partridge, J. C. (2002). Developmental changes in the cone visual pigments of black bream *Acanthopagrus butcheri. Journal of Experimental Biology* **205,** 3661-3667.

Sharma, S. C. (1991). Identification of red-green and blue sensitive cones in the goldfish retina by photoreceptor-class specific antibodies. *Society for Neuroscience Abstracts* **17,** 298.

Shi, Y. & Yokoyama, S. (2003). Molecular analysis of the evolutionary significance of ultraviolet vision in vertebrates. *Proc Natl Acad Sci U S A* **100,** 8308-8313.

Shi, Y. B. & Ishizuya-Oka, A. (2000). Thyroid hormone regulation of apoptotic tissue remodeling: implications from molecular analysis of amphibian metamorphosis. *Progress in Nucleic Acid Research and Molecular Biology* **65,** 53-100.

Shi, Y. B., Sachs, L. M., Jones, P., Li, Q. & Ishizuya-Oka, A. (1998). Thyroid hormone regulation of *Xenopus laevis* metamorphosis: functions of thyroid hormone receptors and roles of extracellular matrix remodeling. *Wound Repair and Regeneration* **6,** 314-322.

Siebeck, U. E., Collin, S. P., Ghoddusi, M. & Marshall, N. J. (2003). Occlusable corneas in toadfishes: light transmission, movement and ultrastructure of pigment during light- and dark-adaptation. *Journal of Experimental Biology* **206,** 2177-2190.

Smith, E. J., Partridge, J. C., Parsons, K. N., White, E. M., Cuthill, I. C., Bennett, A. T. D. & Church, S. C. (2002). Ultraviolet vision and mate choice in the guppy (*Poecilia reticulata*). *Behavioral Ecology* **13,** 11-19.

Specker, J. L., Eales, J. G., Tagawa, M. & Tyler, W. A. I. (2000). Parr-smolt transformation in Atlantic salmon: thyroid hormone deiodination in liver

and brain and endocrine correlates of change in rheotactic behavior. *Canadian Journal of Zoology* **78,** 696-705.

St Germain, D. L., Schwartzman, R. A., Croteau, W., Kanamori, A., Wang, Z., Brown, D. D. & Galton, V. A. (1994). A thyroid hormone-regulated gene in *Xenopus laevis* encodes a type III iodothyronine 5-deiodinase. *Proceedings of the National Academy of Sciences of the United States of America* **91,** 11282.

Stenkamp, D. L., Barthel, L. K. & Raymond, P. A. (1997). Spatiotemporal coordination of rod and cone photoreceptor differentiation in goldfish retina. *Journal of Comparative Neurology* **382,** 272-284.

Stenkamp, D. L. & Cameron, D. A. (2002). Cellular pattern formation in the retina: retinal regeneration as a model system. *Molecular Vision* **8,** 280-293.

Stenkamp, D. L., Hisatomi, O., Barthel, L. K., Tokunaga, F. & Raymond, P. A. (1996). Temporal expression of rod and cone opsins in embryonic goldfish retina predicts the spatial organization of the cone mosaic. *Investigative Ophthalmology and Visual Science* **37,** 363-376.

Stenkamp, D. L., Powers, M. K., Carney, L. H. & Cameron, D. A. (2001). Evidence for two distinct mechanisms of neurogenesis and cellular pattern formation in regenerated goldfish retinas. *Journal of Comparative Neurology* **431,** 363-381.

Stockton, R. A. & Slaughter, M. M. (1989). B-wave of the electroretinogram. A reflection of ON bipolar cell activity. *Journal of General Physiology* **93,** 101-122.

Taniguchi, Y., Hisatomi, O., Yoshida, M. & Tokunaga, F. (1999). Evolution of visual pigments in geckos. *FEBS Letters* **445,** 36-40.

Temple, S. E., Allison, W. T., Haimberger, T. J. & Hawryshyn, C. W. (2003). Visual pigment shifting in Zebrafish (*Danio rerio*). *Investigative Ophthalmology & Visual Science* **44,** S713-S713.

Thorgaard, G. H., Bailey, G. S., Williams, D., Buhler, D. R., Kaattari, S. L., Ristow, S. S., Hansen, J. D., Winton, J. R., Bartholomew, J. L., Nagler, J. J., Walsh, P. J., Vijayan, M. M., Devlin, R. H., Hardy, R. W., Overturf, K. E., Young, W. P., Robison, B. D., Rexroad, C. & Palti, Y. (2002). Status and opportunities for genomics research with rainbow trout. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **133,** 609-646.

Thorpe, A. & Douglas, R. H. (1993). Spectral transmission and short-wave absorbing pigments in the fish lens--II. Effects of age. *Vision Research* **33,** 301-307.

Tian, N. & Slaughter, M. M. (1995). Correlation of dynamic responses in the ON bipolar neuron and the b-wave of the electroretinogram. *Vision Research* **35,** 1359-1364.

Veldhoen, K., Beaudet, L., Runions, J., Sharma, S. & Hawryshyn, C. W. (1999). Antibody labeling of the blue-sensitive cones in the retinae of teleost fishes. *Canadian Journal of Zoology* **77,** 1733-1739.

Veldhoen, K. M. (1996). Production of antiserum to a goldfish visual pigment opsin. In *Biology.* University of Victoria, Victoria, BC, Canada.

Veldhoen, K. M., Allison, W. T. & Hawryshyn, C. W. (2003). Proteomic analysis of retinal development using ICAT. *Investigative Ophthalmology & Visual Science* **44,** S547-S547.

Verrier, M.-L. (1928). *Les yeux et la vision des poisons*, vol. Supplement XI. Les Presses universitaires de France, Paris.

Vihtelic, T. S., Doro, C. J. & Hyde, D. R. (1999). Cloning and characterization of six zebrafish photoreceptor opsin cDNAs and immunolocalization of their corresponding proteins. *Visual Neuroscience* **16,** 571-585.

Vihtelic, T. S. & Hyde, D. R. (2000). Light-induced rod and cone cell death and regeneration in the adult albino zebrafish (*Danio rerio*) retina. *Journal of Neurobiology* **44,** 289-307.

von Schantz, M., Lucas, R. J. & Foster, R. G. (1999). Circadian oscillation of photopigment transcript levels in the mouse retina. *Brain Research. Molecular Brain Research* **72,** 108-114.

Wagner, H.-J. (1972). Vergleichende untersuchungen über das muster der sehzellen und horizontalen in der teleosteir-retina (Pisces). *Zeitschrift fur Morphologie der Tierre* **72,** 77-130.

Wald, G. (1941). The visual system of euryhaline fishes. *Journal of General Physiology* **25,** 235-245.

Walker, R. V., Myers, K. W., Davis, N. D., Aydin, K. Y., Friedland, K. D., Carlson, H. R., Boehlert, G. W., Urawa, S., Ueno, Y. & Anma, G. (2000). Diurnal variation in thermal environment experienced by salmonids in the North Pacific as indicated by data storage tags. *Fisheries Oceanography* **9,** 171-186.

Walls, G. L. (1934). The reptilian retina I. A new concept of visual-cell evolution. *American Journal of Ophthalmology* **17,** 892-915.

Walls, G. L. (1942). *The vertebrate eye and its adaptive radiation.* Hafner Publishing Company, New York.

Walls, G. L. & Judd, H. D. (1933a). The Intra-ocular colour-filters of vertebrates. *British Journal of Ophthalmology* **17,** 641-675.

Walls, G. L. & Judd, H. D. (1933b). The Intra-ocular colour-filters of vertebrates. *British Journal of Ophthalmology* **17,** 705-725.

Wenzel, A., Iseli, H. P., Fleischmann, A., Hafezi, F., Grimm, C., Wagner, E. F. & Reme, C. E. (2002). Fra-1 substitutes for c-Fos in AP-1-mediated signal transduction in retinal apoptosis. *Journal of Neurochemistry* **80,** 1089-1094.

White, E. M., Partridge, U. C. & Church, S. C. (2003). Ultraviolet dermal reflexion and mate choice in the guppy, *Poecilia reticulata. Animal Behaviour* **65,** 693-700.

Whitmore, A. V. & Bowmaker, J. K. (1989). Seasonal variation in cone sensitivity and short-wave absorbing visual pigments in the rudd *Scardinius erythrophthalmus. Journal of Comparative Physiology. A, Sensory, Neural, and Behavioral Physiology* **166,** 103-115.

Williams, J. A., Barrios, A., Gatchalian, C., Rubin, L., Wilson, S. W. & Holder, N. (2000). Programmed cell death in zebrafish rohon beard neurons is influenced by TrkC1/NT-3 signaling. *Developmental Biology* **226,** 220-230.

Williams, J. A. & Holder, N. (2000). Cell turnover in neuromasts of zebrafish larvae. *Hearing Research* **143,** 171-181.

Wood, P. & Partridge, J. C. (1993). Opsin Substitution Induced in Retinal Rods of the Eel (*Anguilla Anguilla* (L)) - a Model for G-Protein-Linked Receptors. *Proceedings of the Royal Society of London Series B-Biological Sciences* **254,** 227-232.

Wu, D. M., Schneiderman, T., Burgett, J., Gokhale, P., Barthel, L. & Raymond, P. A. (2001). Cones regenerate from retinal stem cells sequestered in the inner nuclear layer of adult goldfish retina. *Investigative Ophthalmology and Visual Science* **42,** 2115-2124.

Yanagi, Y., Takezawa, S. & Kato, S. (2002). Distinct functions of photoreceptor cell-specific nuclear receptor, thyroid hormone receptor beta2 and CRX in

one photoreceptor development. *Investigative Ophthalmology and Visual Science* **43,** 3489-3494.

Yokoyama, S. (1997). Molecular genetic basis of adaptive selection: examples from color vision in vertebrates. *Annual Review of Genetics* **31,** 315-336.

Yokoyama, S. (2000). Molecular evolution of vertebrate visual pigments. *Progress in Retinal and Eye Research* **19,** 385-419.

Yokoyama, S., Meany, A., Wilkens, H. & Yokoyama, R. (1995). Initial mutational steps toward loss of opsin gene function in cavefish. *Molecular Biology and Evolution* **12,** 527-532.

Yokoyama, S., Zhang, H., Radlwimmer, F. B. & Blow, N. S. (1999). Adaptive evolution of color vision of the Comoran coelacanth (*Latimeria chalumnae*). *Proceedings of the National Academy of Sciences of the United States of America* **96,** 6279-6284.

Zhang, H., Futami, K., Horie, N., Okamura, A., Utoh, T., Mikawa, N., Yamada, Y., Tanaka, S. & Okamoto, N. (2000). Molecular cloning of fresh water and deep-sea rod opsin genes from Japanese eel *Anguilla japonica* and expressional analyses during sexual maturation. *FEBS Letters* **469,** 39-43.

Zhang, H., Futami, K., Yamada, Y., Horie, N., Okamura, A., Utoh, T., Mikawa, N., Tanaka, S., Okamoto, N. & Oka, H. P. (2002). Isolation of freshwater and deep-sea type opsin genes from the common Japanese conger. *Journal of Fish Biology* **61,** 313-324.