Opsin and Retinal Genomics in Salmonid Fishes: Implications for Phylogeny and Retinal Development

By

Stephen G. Dann

B.Sc., Saint Francis Xavier University, 1997,

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

In the Department of Biology

© Stephen G. Dann 2003,
University of Victoria
All Rights reserved. This dissertation may not be reproduced in whole or in part, by photocopying or other means, without the permission of the author.

Supervisor: Dr. Craig W. Hawryshyn

Abstract

Positive selection can be demonstrated by statistical analysis when non-synonymous nucleotide substitutions occur more frequently than synonymous substitutions (dN>dS). This pattern of sequence evolution has been observed in the rhodopsin gene of cichlids. Mutations in opsin genes resulting in amino acid (aa) replacement appear to be associated with the evolution of specific color patterns and the evolution of courtship behaviors. In fish, aa replacements in opsin proteins have improved vision at great depths in deep-sea species. Salmonids experience diverse light environments during their migratory life-history. Furthermore, sexual selection has resulted in species-specific male and female coloration during spawning. To look for evidence of positive selection in salmonid opsins, the RH1, RH2, LWS, SWS1 and SWS2 genes were sequenced from six Pacific salmon species as well as Atlantic salmon. These salmonids include landlocked and migratory species and species that vary in their coloration during spawning. In each opsin gene comparison from all species sampled, traditional dN:dS analysis did not indicate positive selection. However, the more sensitive Creevey-McInerney statistical analysis indicated that RH1 and RH2 experienced positive selection early during the speciation of salmonids.

Misexpression of opsins has been linked to apoptosis of photoreceptor cells in the vertebrate retina. Misexpression includes knockout of opsin expression, expression of an opsin with mutant aa sequence, mutation of opsin promoter/regulatory sequence, or mutation of a transcription factors aa sequence responsible for opsin regulation. Salmonid fish lose their ultraviolet-sensitive (UVS) cones through post-natal developmental

apoptosis mediated by thyroid hormone. In order to identify genetic mechanisms that may play a role in the loss of UVS cones, the transcriptional regulation of the SWS1 opsin in the rainbow trout (*Oncorhynchus mykiss*) was investigated. The Transfac database was interrogated with promoter sequence acquired by genome walking PCR using MatInspector V2.2 to identify putative transcription factor binding sites. Putative binding sites for AP-1 (c-jun) and NF-κB were found in the SWS1 opsin promoter and were chosen for further investigation due to their high MatInspector scores, their established role in photoreceptor apoptosis, and their relative exclusion from other opsin promoters. NF-κB and c-jun proteins were visualized in rainbow trout retinal tissue using immunohistochemistry and c-jun was identified in rainbow trout retinal protein homogenate by immunoblot. A chromatin immunoprecipitation-polymerase chain reaction technique was employed to examine the interaction of c-jun and NF-κB proteins with their proposed binding sites in the opsin promoters. Both NF-κB and c-jun were found to bind exclusively to the SWS1 opsin promoter. Given the role of NF-κB and c-jun during photoreceptor apoptosis, the influence of their activity through thyroid hormone and their selective binding to the SWS1 opsin promoter in rainbow trout, these transcription factors represent good candidates of mechanisms underlying UVS cone degeneration in salmonids.

Developmental and physiological changes in the retina of salmonid fishes occur during smoltification, a metamorphic event associated with thyroid hormone that prepares salmon for oceanic migration. These changes include loss of ultraviolet-sensitive (UVS) cone photoreceptors, switching of visual pigments, alterations in thyroid hormone regulation, and associated changes in behavior. This model provides an

PCR are used to identify a previously uncharacterized gene transcript in *Oncorhynchus mykiss* under developmental regulation in the retina during smoltification, *rtp12.5*. This unique cDNA encodes a putative protein 112 amino acids long similar to a hypothetical human open reading frame located on chromosome 14.Q24.2. Differential expression was confirmed by RNA dot blot and *in situ* hybridization. Also presented are the *O. mykiss sep15* cDNA sequence and a description of its expression in the vertebrate retina. Considering the expression pattern within retinal tissue observed by *in situ* hybridization, *rtp12.5* may be under TH regulation and may be involved in neuronal remodeling of the retina during loss of UVS cones.

Table of Contents

List of Tables

List of Figures

Acknowledgements

The author wishes to acknowledge the contributions of several people during the progression of this research from start to finish. Mr. William (Ted) Allison was instrumental in the direction of the research and interpretation of data. His wealth of literary knowledge provided me guidance and insights. Dr. David B. Levin was an indispensable resource when it came to learning and adapting techniques, new ideas, and troubleshooting. Without Dr. Levin's lab and facilities, I would still be stuck on making a pipettor work. Ms. Sarah Barber, now an employee of the BCCA genomics research center, has always listened attentively when things were rough. Ms. Pauline Tymchuk and Ms. Eleanore Floyd have both been constant sources of support in cutting through the administrative bureaucracy and in dealing with day-to-day graduate student tribulations. Members of the Hawryshyn lab, past and present, provided technical assistance and constant critiques of my research ideas. The members of the Glickman sequencing lab ensured processing of my data. My friends and family (Dad, Kathy, Michael, Jessica, Abigail, Michelle, Hugh, Griffen, Trisha, Mike H., Sonja, Jason, Mike P. Cari, and Sam) who put up with more than their fair share of my frustrations but who also shared in my jubilation when things went well. Dr. Craig W. Hawryshyn gave me the opportunity and freedom to explore my research interests while somehow managing to keep me on course to the finished product. Ms. Victoria L. Kyle has been with me through thick and thin. Thanks for waiting hon; I am grateful for your patience and the opportunity to fully realize our mutual commitment.

Dedication

It is an honour and pleasure to be able to dedicate the research contained herein to my late mother. Without her warmth, her strength, her dedication, and her encouragement I would never have aspired to this feat. I miss you with all my heart mom and if I could trade everything bound together here for just a chance to have you back I would. I hope you are pleased by what I have presented here, I wish I could show you a copy in person although I am sure by now you have been over it an infinite number of times. I love you,

Stephen

List of Abbreviations

A_1 – 11-cis retinal conjugated opsin
A_2 – 3,4-dehydroretinal conjugated opsin,
aa – amino acids
AAV – Adeno-associated virus
AC – Amacrine cell
AP-1 – Activating Protein-1
ATP – Adenosine triphosphate
BC – Bipolar cell
BSA – Bovine serum albumin
bp – base pair(s),
cDNA – DNA complementary to RNA
ChIP – Chromatin immunoprecipitation
CMV – Cytomegalovirus
DDRT-PCR – Differential display RT-PCR
dN – NS substitutions per NS site
dS – S substitutions per S site
DNA – Deoxyribonucleic acid
GC – Ganglion cell
GCL – Ganglion cell layer
GFP – Green fluorescent protein
GMP – Guanidine monophosphate
GDP – Guanidine diphosphate
GTP – Guanidine triphosphate
HC – Horizontal cell
HMM – Hidden Markov model
INL – inner nuclear layer
IPL – Inner plexiform layer
LWS – Long wavelength sensitive opsin
ME – Minimum evolution
MWS – Mid wavelength sensitive opsin
MMLV – Moloney murine leukemia virus,
MSP – Microspectrophotometry
NCBI – National Center for Biotechnology Information (USA)
NF-κB – Neurotrophic factor-kappa B
NRL – Neural retina leucine zipper
NS – Nonsynonymous mutation
NSI – Nonsynonymous invariable mutation
NSV – Nonsynonymous variable mutation
O - Opsin
ONL – Outer nuclear layer
OPL – Outer plexiform layer
ORF – open reading frame
PBS – Phosphate buffered saline
PDE – Phosphodiesterase
PCR – Polymerase chain reaction
PNR – Photoreceptor specific nuclear receptor (i.e. NR2E3)
PR – Photoreceptor
RACE – Rapid amplification of cDNA ends
RK – Rhodopsin kinase
RT-PCR – Reverse transcription-PCR
RH1 – Rhodopsin Type 1
RH2 – Rhodopsin Type 2
RNA – Ribonucleic acid
RP – Retinitis Pigmentosa
RPE – Retinal Pigmented Epithelium
rtp12.5 – Rainbow trout protein 12.5 kDa
S – Synonymous mutation
SECIS – Selenocysteine insertion sequence
Sep15 – 15 kDa selenoprotein
SI – Synonymous invariable mutation
SV – Synonymous variable mutation
SWS1 – Short wavelength sensitive opsin type 1
SWS2 – Short wavelength sensitive opsin type 2
T – Transducins
TF – Transcription factor
TH – Thyroid hormone
THR – Thyroid hormone receptor
UTR – untranslated region(s)
UV – ultraviolet
UVS – Ultraviolet sensitive cones
VA – Vertebrate ancient opsin
XGal – 5-bromo-4-chloro-3-indolyl b-d-galactopyran-oside,
λ_{max} – Maximum wavelength of absorption.

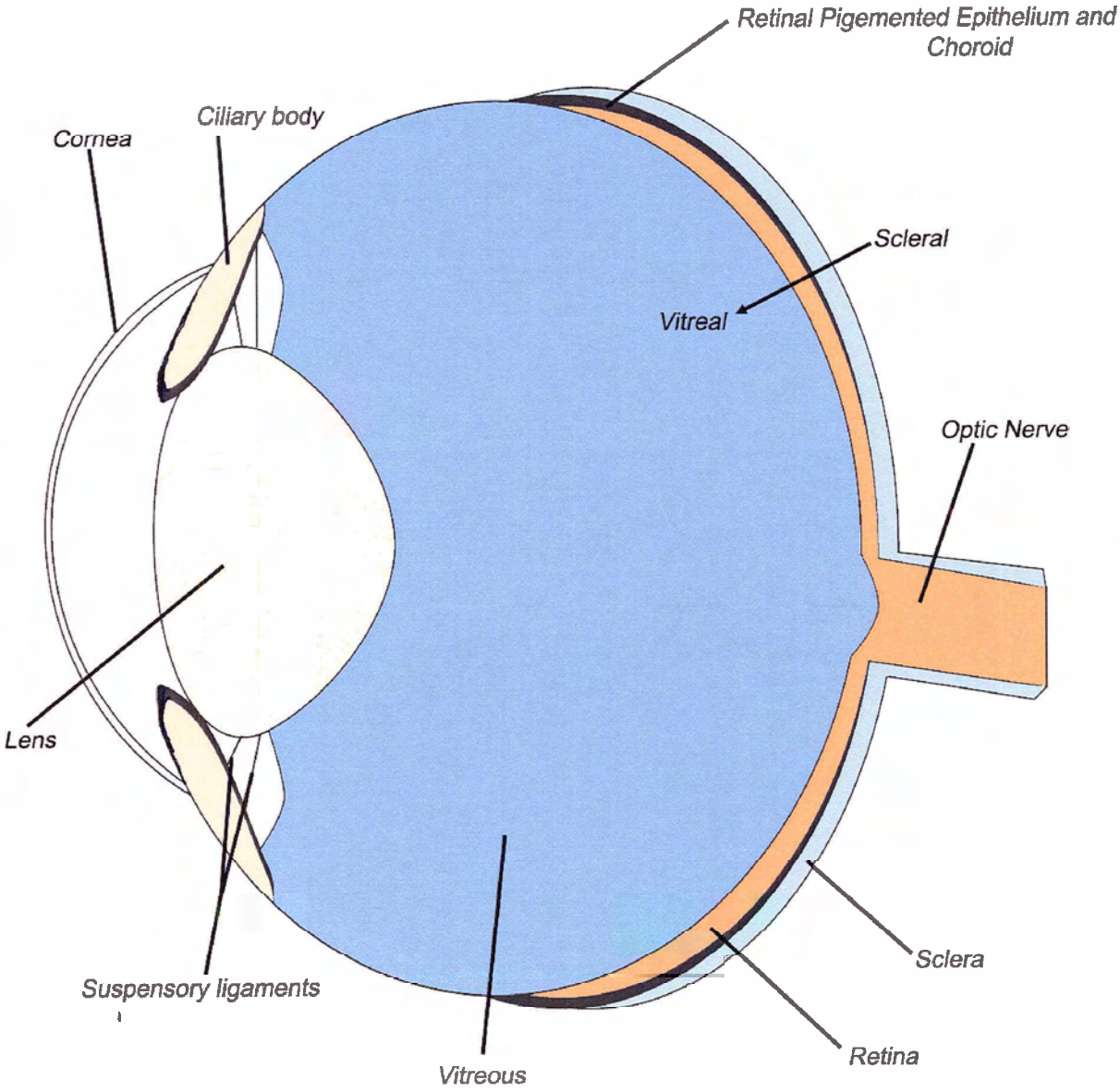
1. Introduction

1.1 Eye Structure

Virtually all animals employ visual sense with specialized organs, the eyes, which transduce light signals into a perceived image through neuronal processing. All vertebrate eyes are based on a common structural plan where light passes through a hole in the iris, the pupil, forming an inverted image on the retina (Wolff and Last, 1968; Figure 1). Light is focused onto the retina by the cornea, which is the exterior, clear membrane formed by neural crest cells, and by the lens, a tough but flexible tissue resulting from expression of crystallin by lens precursor cells (Kepler, 1604). The lens is suspended by suspensory ligaments and can be moved and shaped to focus light in different conditions by smooth ciliary muscle. The vertebrate eye is contained in, and protected by, a tough fibrous tissue called the sclera, also known as the white of the eye in humans. The shape of the eye is maintained by liquid pressure exerted by the gelatinous vitreous, secreted by the ciliary body. When discussing the eye, the terms vitreal and scleral are used to explain the position of the structure of interest. A structure that is found more interior is referred to as vitreal while a structure found more exterior is scleral. The choroid, the next layer vitreal in relation to the sclera, contains the trabecular meshwork of blood vessels that provide oxygen to eye tissues. Continuing vitreal is the retinal pigmented epithelium (RPE), which protects retinal neurons from light damage with melanin deposits resulting in the dark pigmentation, and nourishes retinal cells with protein and vitamin derived factors. Finally, the neural retina is found vitreal to the RPE, and this layer is ultimately responsible for perception of light and some neural signal before the neural message is sent to the visual cortex in the brain via the optic nerve.

Figure 1.
The basic anatomy of a cross section through the vertebrate eye.

Labels indicate anatomical structures as described in the text. Of particular note is the scleral to vitreal directional arrow used to describe orientation of eye tissue layering (Adapted from Rodieck, 1998).


Retinal Pigemented Epithelium and Choroid
Ciliary body
Cornea
Scleral
Vitreal
Optic Nerve
Lens
Suspensory ligaments
Vitreous
Sclera
Retina

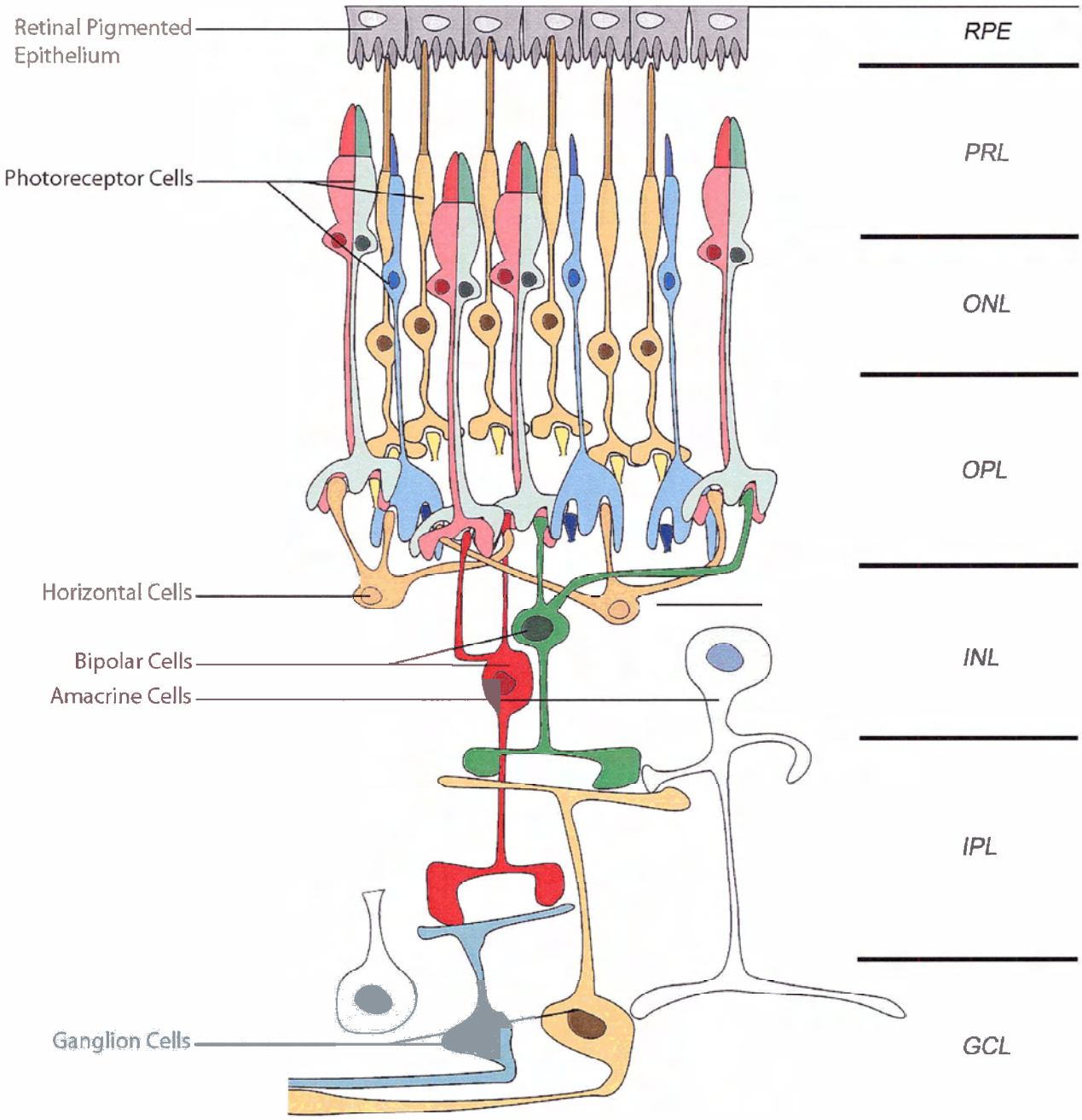
1.2 The Retina

The retina consists of six layers. Listed scleral to vitreal the layers are the photoreceptor (PR) layer, the outer nuclear layer (ONL), the outer plexiform layer (OPL), the inner nuclear layer (INL), the inner plexiform layer (IPL) and the ganglion cell layer (GCL) (Figure 2). The PR layer consists of the inner and outer segments of the cone and rod PRs. The ONL contains the nuclei of these PRs while the OPL contains the synaptic fibers and connections that the PRs make with horizontal cells (HC) and bipolar cells (BC). The INL contain the nuclei of these HC and BC, while the HC and BC efferent fibers make connections with amacrine cell (AC) and ganglion cell (GC) synaptic terminals in the IPL. The GCL consists mainly of GC nuclei; the GCL and the INL also contain nuclei of different classes of AC (Cajal, 1893; Schuman and Meyers, 1969).

Light is first detected by the PRs in the PR layer via the visual cycle biochemical cascade, discussed in detail below. The light is transduced into a neural signal by the PRs and then modified by HCs depending on the strength of neural signal received by surrounding sub-classes of photoreceptors. This modification is the commonly observed neural circuit paradigm, lateral inhibition, which results in different forms of color opponency and represents the first level of neural processing of the visual signal (Dowling, 1987). Upon modification, the neuronal signal is passed to the BCs, which often contact more than one PR cell terminal but contact only one PR-type. There are at least four BC groups based on their morphology and the type of PR they contact and two types within each group, i.e. those BCs responsible for processing an increase in light stimulus (ON-BC) and those responsible for processing a decrease in light stimulus (OFF-BC). The changes in light intensity detected by BCs are passed on to the ACs, which further process the visual signal using lateral inhibition although the mechanisms that determine inhibition or excitation are not as clear as for HCs. Finally, GCs receive the neural signal from

Figure 2.
Cellular anatomy of the vertebrate retina.

The retina consists of several layers as described in the text. The photoreceptor layer (PRL) contains the inner and outer segments of rod and cone photoreceptors. Photoreceptor outer segments are enveloped and nourished by the retinal pigment epithelium (RPE). The outer nuclear layer (ONL) consists of the photoreceptor nuclei while the outer plexiform layer (OPL) contains the synapses of photoreceptors, bipolar cells and horizontal cells. The inner nuclear layer (INL) contains the nuclei of bipolar, horizontal, and some amacrine cells and the inner plexiform layer (IPL) contains the synaptic connections of these cells with the ganglion cells. The ganglion cell layer (GCL) is predominantly ganglion cell nuclei although some amacrine cells reside here as well. The axons of the ganglion cells travel to the optic nerve eventually projecting to several targets in the vertebrate central nervous system (Adapted from Rodieck, 1998).


Retinal Pigmented Epithelium
Photoreceptor Cells
Horizontal Cells
Bipolar Cells
Amacrine Cells
Ganglion Cells
RPE
PRL
ONL
OPL
INL
IPL
GCL

the BCs and their efferent fibers transfer this message via the optic nerve to the lateral geniculate nucleus in the brain, a relay station for the visual sense that sends the message to various regions of the striate visual cortex (Polyak, 1941; Rodieck, 1973). The visual cortex uses the visual sense to produce a suite of responses including, but not limited to, hunger, arousal, proprioception, emotion, fight/flight, etc.

All of the retinal cell types consist of different sub-classes depending on the cell-specific connections they make, their morphology, or their synaptic physiology and each have distinguished functions in visual processing. For the purpose of this discussion, I will focus on PRs, specifically the PR specific opsin protein that characterizes PR sub-class identity. Light photons are initially detected in the outer segments of PRs, which have specialized morphology depending on the wavelength or intensity of light encountered by the retina. Retinae are considered duplex if they contain both rod PRs and cone PRs (Ebrey and Koutalos, 2001). That is, the retina must be able to distinguish between the scotopic (dim, grey conditions) and the photopic (color, bright conditions) using rods and cones respectively. Although some animal's visual sense is limited by this rudimentary distinction of light, others are able to distinguish several wavelengths of light employing different photopic cone PR types. Multiple cone PRs and the separate rod PRs for scotopic conditions result in color vision such as that perceived by humans (Graham and Hartline, 1935).

1.3 The Visual Cascade

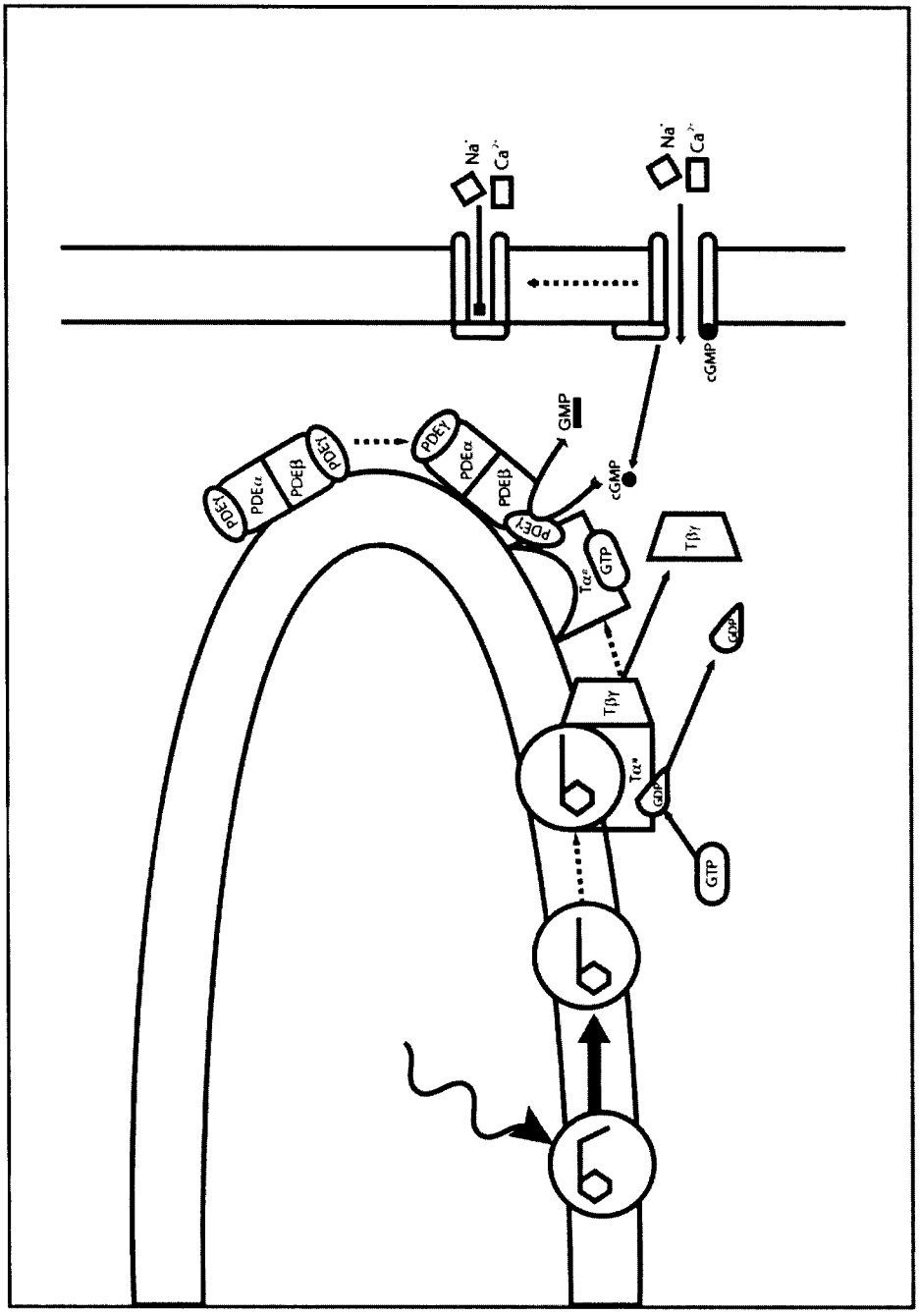
The outer segments of rods consist of cell membrane derived discs while cone outer segments are composed of multiple invaginations of the cell membrane. It is in these discs and invaginations that the visual cascade takes place, ultimately resulting in PR hyperpolarization (Hubel, 1988) Figure 3). The visual cycle within rod PRs has been clarified in recent years due to the advances made in biochemical and physiological laboratory techniques including PCR, *in*

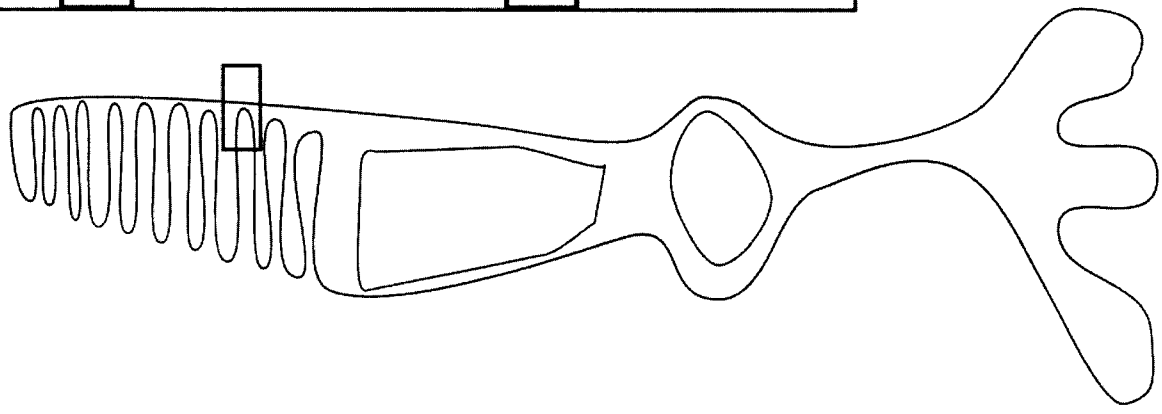
vitro expression systems, protein purification techniques, patch clamp recordings, etc. Membrane bound apoproteins called opsins are found throughout the discs/invaginations of PRs. These apoproteins are conjugated to an organic chromophore which, upon absorbing a light photon, changes conformation to an excited energy state. Vitamin A derived chromophores, either an 11-*cis* retinal (A_1) or a 3,4-dehydroretinal (A_2) conjugated to a lysine at position 296 (in human RH1), are common to all vertebrate opsins (Lipkin and Obukhov, 2000). The chromophore captures light quanta and produces a conformational change in the opsin protein. In the case of 11-*cis* retinal, the chromophore is photoisomerized to all-*trans* retinal upon absorption of light energy. The activated opsin protein created by photoisomerization is responsible for the initiation of the visual cascade within the photoreceptor, ultimately leading to visual perception (Graham and Hartline, 1935). Chromophore excitation results in a slightly larger activated opsin protein that can temporarily bind the membrane bound G-protein transducin made of three subunits T_α, T_β and T_γ. This binding event results in the loss of a GDP molecule from the T_α and replacement with GTP. The replacement of GDP with GTP results in separation of activated T_α from $T_{\beta\gamma}$. Activated T_α binds a cGMP phosphodiesterase (PDE) that is made of four subunits, 1 PDE_α, 1 PDE_β and 2 PDE_γ.

Upon binding the activated T_α a PDE_γ is displaced revealing the PDE catalytic site. This catalytic site results in hydrolysis of cGMP to GMP by the breakage of an ester bond between phosphorus and oxygen in cGMP. The effect of this cascade is the reduction in intercellular cGMP concentration (Hecht, 1937). In its resting state the Na^+ and Ca^{2+} flow into a PR produces a dark current of -34 pA. The inflow of Na^+ and Ca^{2+} is balanced by a constant outflow of K^+ in the inner segment; however the Na^+/ Ca^{2+} channels are gated by cGMP. A drop in the concentration of cGMP due to photoactivation of an opsin protein results in closing of Na^+/ Ca^{2+} channels. This, coupled with the loss of K^+ down its concentration gradient, results in a net loss

Figure 3.
Opsin activation and the visual cascade within the invaginations of a vertebrate cone photoreceptor.

Light quanta (*hv*) absorbed by 11-*cis* retinal bound to inactivated opsin (O_i) results in a change of state to all-*trans* retinal. The higher energy chromophore causes the opsin to bulge (O_a) and bind transducin-α (Tα*). When transducin binds O_a the transducin βγ subunits (Tβγ) are released and GTP replaces GDP on the α subunit. The binding of GTP results in Tα's ability to displace the γ subunit of Phosphodiesterase (PDEγ) from PDE α and β. The displacement of PDEγ reveals PDE's active site where cGMP is converted to GMP. The drop in cytoplasmic cGMP concentration results in closing of Na^+/Ca^{2+} cGMP gated channels and subsequent hyperpolarization of the photoreceptor. This hyperpolarization is propagated past the inner segment, nucleus towards the synapse pedicle by the gated-K^+ outflow down its concentration gradient. This cascade can be extrapolated to the discs of rod photoreceptors where the same cascade takes place albeit activated by a more sensitive opsin (rhodopsin) and different isoforms of transducin, PDE, etc. (Adapted from Ebrey and Koutalos, 2001).


Na+
Ca2+
Na+
Ca2+
cGMP
GMP
PDEγ
PDEα
PDEβ
PDEγ
cGMP
GTP
Tαβ
Tβγ
GDP
Tβγ
Tα*
GDP
GTP



of positive charge and hyperpolarization of the PR. This hyperpolarization results in a decrease in glutamate release from the PR synapse followed by classical neuronal depolarization and signal propagation in the BC and GC (Abney, 1913).

Upon activation, an opsin molecule is able to bind to rhodopsin kinase (RK), which uses ATP to phosphorylate the apoprotein. Although phosphorylation alone does not inactivate the photoisomerized opsin it allows another protein, arrestin, to tightly bind, replacing the RK. Arrestin removes the all trans-retinal from the opsin protein and another cytoplasmic protein, retinal dehydrogenase, converts it to all-*trans* retinol (Vitamin A). At this point, the converted chromophore is shuttled to the RPE by inter-photoreceptor retinoid binding protein while the phosphorylated opsin protein is dephosphorylated by rhodopsin phosphatase. The all-*trans* retinol is regenerated to 11-*cis* retinal through the action of two enzymes: retinyl ester isomerase converts all-trans retinol to 11-*cis* retinol which 11-*cis* retinol dehydrogenase converts to 11-*cis* retinal. T_{α} is inactivated by hydrolysis of GTP to GDP, a process that is facilitated by RGS9. The inactivation of transducin ends the stimulation of PDE, returning cGMP levels to those found in the dark state (Valberg and Lee, 1991).

Light adaptation and recovery in PRs is generally dependent on Ca^{2+} concentration (Ebrey and Koutalos, 2001). The reduction in levels by the closing of cGMP gated channels results in an increase in guanylate cyclase activity through a negative feedback loop. The increased cyclase activity increases cGMP levels by converting free GTP to cGMP. This counteracts the light-stimulated PDE activity resulting in recovery and/or light adaptation. Furthermore, the RK is inhibited by high Ca^{2+} levels through recoverin, a co-enzyme of RK. Finally, the cGMP gated channels themselves are inhibited by high levels of Ca^{2+} through calmodulin activity.

Although each of the proteins and protein subunits involved in phototransduction can have isoforms specialized for specific PR types only opsins have been shown to adapt their

function based directly on their PR. In fact, PRs are generally characterized based on the type of opsin gene they express but there are a number of exceptions to this rule (eg. MWS/SWS1 coexpression in mouse PRs). That is, the opsin gene expressed determines the wavelength of light that the corresponding PR will absorb. This discussion focuses on opsin proteins, their regulation and role in salmonid retinal development.

Unless otherwise stated the vast majority of information gathered to produce sections *1.1-1.3* is found in two excellent sources for the vision scientist, Rodieck (1998) and WebVision (http://webvision.med.utah.edu/ (Kolb *et al.*, 2003).

1.4 Opsin Structure

Opsin visual pigments mediate absorption of light by photoreceptors in both vertebrate and invertebrate animals. These 39 kDa, G-protein coupled receptors, consist of single polypeptide chains containing seven trans-membrane alpha helices. Mutations and gene duplication events in vertebrate evolution have produced groups of opsins that absorb specific light wavelengths: violet/ultraviolet- (SWS1 opsin), short wavelength- (SWS2), mid and long wavelength-sensitive (MWS/LWS), type 1 rhodopsin (RH1) and type 2 rhodopsin (RH2). Humans have one SWS1 opsin (λ_{max} 426), two MWS/LWS opsins (λ_{max} 530 & λ_{max} 555) for photopic vision, as well as a RH1 used specifically for scotopic vision (λ_{max} 506) (Merbs and Nathans, 1992). Visual pigment genes have been identified and characterized in a variety of fishes (Helvik *et al.*, 2001; Johnson *et al.*, 1993). This includes the opsin genes of zebrafish and goldfish, important models of retinal development and function in vertebrates (Stenkamp *et al.*, 1996; Vihtelic *et al.*, 1999).

Characterization of amino acid substitution effects on opsin function have been studied in each of the visual pigment types, resulting in explanations for differences in function between

MWS and LWS pigments, SWS1 and SWS2 pigments and RH1 and RH2 pigments (Chang *et al.*, 1995; Cowing *et al.*, 2002b; Yokoyama and Radlwimmer, 1999; Yokoyama and Radlwimmer, 2001; Yokoyama and Tada, 2003). Functional studies on opsins between closely related species have also identified instances of molecular tuning of opsins (Cowing *et al.*, 2002a; Fasick and Robsinson, 1998; Ma *et al.*, 2001; Takahashi and Ebrey, 2003; Yokoyama, 2000a; Yokoyama *et al.*, 2000; Yokoyama and Shi, 2000; Yokoyama and Tada, 2000). The acquisition of opsin sequence data has allowed for reliable phylogenetic derivation of visual pigments from several genera of vertebrates (Bowmaker, 1998; Yokoyama, 1996; Yokoyama, 2000b; Yokoyama, 2000c).

Although an amino acid (aa) residue analogous to lysine-296 is present in all eukaryote visual opsins, substitutions of several aa residues surrounding the chromophore have occurred during evolution. Amino acid replacements at phosphorylation or glycosylated residues, paired with those that affect chromophore placement, can alter a visual pigment's spectral tuning (Yokoyama, 2000b). Amino acid replacements in opsin molecules provide the foundation for a visual system capable of discriminating spectral stimuli. Spectral tuning of opsins can also occur through differential use of A_1/A_2 chromophores. A_1 chromophore conjugated to an opsin confers a shorter wavelength of maximum absorption. Mixing of A_1 and A_2 within the retina, even within the same photoreceptor, can broaden an animal's range of visual sensitivity (Palacios *et al.*, 1998).

1.5 Salmonid Visual System

Pacific salmon, *Oncorhynchus* sp., diverged during the middle Pliocene, 5 to 6 million years ago, and subsequently evolved diverse life-history strategies (Shedlock *et al.*, 1992; Wilson, 1997). Some species migrate from freshwater to the marine environment at smoltification[1], while others remain land-locked. The timing of smoltification is varied in

Oncorhynchus sp. For example, *O. gorbuscha* (pink salmon) becomes salt tolerant immediately after hatching while *O. nerka* (sockeye salmon) will spend upwards of three years in freshwater lakes before oceanic migration. Representatives from the genus *Oncorhynchus* sp. are found over a wide geographic range, from both the Asian and North American sides of the Pacific Ocean, and from the Alaskan Aleutian Islands to the Southern tip of Baja, Mexico (Groot and Margolis, 1991; Wilson, 1997). Some salmonids exhibit an extensive geographic range experiencing vastly differing spectral environments (ocean-going) while others remain relatively confined to one specific habitat (landlocked). Examination of spectral energy distribution of freshwater and marine environments and their effectiveness in stimulating the cone photoreceptor complement in salmonid fishes has been previously reported (Novales Flamarique and Hawryshyn, 1993; Novales Flamarique *et al.*, 1992). These studies indicate that all cone photoreceptors are active and sensitive down to depths of up to 15m in the marine environment and 18m in lake habitats. Freshwater habitats favor passage of longer (red) wavelengths of light while saltwater habitats allow passage of shorter (blue) wavelengths (Alexander *et al.*, 1994).

Previous research shows that salmonid retina undergo structural remodeling and changes in spectral sensitivity during post-natal maturation to compensate for a change in habitat (Beaudet *et al.*, 1993; Deutschlander *et al.*, 2001; Hawryshyn *et al.*, 1989). The loss of UV photosensitivity corresponds to the loss of UV-sensitive (UVS) cones in the rainbow trout (*Oncorhynchus mykiss*) retina through apoptosis (Allison *et al.*, 2003; Kunz *et al.*, 1994). UVS cones found in rainbow trout are homologous to the S-cones found in mammalian retina in that both express the SWS1 opsin. The UVS cones in *O. mykiss* degenerate during smoltification. Premature loss of UV photosensitivity was observed in rainbow trout treated with exogenous thyroid hormone (TH), a hormone noted for its crucial role in development and metamorphosis of vertebrates (Browman and Hawryshyn, 1992; Browman and Hawryshyn, 1994). TH is the ligand for thyroid hormone

receptors (THR) localized in cell nuclei that function as inducible transcription factors (TF) (Baniahmad, 2002). There are two types of THR, α and β, with at least two isoforms of each that can enhance or inhibit transcription by binding to thyroid response elements in gene promoter regions (Harvey and Williams, 2002; Yen, 2001). Plate *et al.* (2002) illustrated the activation of TH in the retina from its inactive circulating conformation by deiodinases in the retina.

UV vision in salmonids mediates sensitivity to plane polarized light, used in navigation, migration and foraging (Hawryshyn, 2000; Hawryshyn, 2003; Novales Flamarique and Hawryshyn, 1997). Not only do these species exploit spectrally diverse habitats, they also employ a dynamic A_1/A_2 chromophore ratio in photoreceptor function permitting a broader spectral range of visual sensitivity. Salmon migration into salt water, resulting in exposure to shorter light wavelengths, is paired with a shift from A_2- to A_1- dominated opsins resulting in the tuning of photoreceptors to the bluer spectral environment (Alexander *et al.*, 1994).

Oncorhynchus sp. also undergo morphological changes at sexual maturation, including a change in coloration from silver to red (Groot and Margolis, 1991; Wilson, 1997). Spawning salmon deposit carotenoid pigment in the skin to produce bright red displays for mate selection (Foote *et al.*, In Press-Animal Behavior; Olsen and Mortensen, 1997; Steven, 1947). Female salmon engaging in mate selection exploit the males' red skin color as an assessment of fitness, while also serving as an aggressive display trait to other males (de Gaudemar, 1998). Since Pacific salmon are typically semelparous one would expect that mate selection exhibits a high degree of specificity based on recognition of traits such as coloration. Behavioral field studies by Foote *et al.* (In Press-Animal Behavior) have shown that a specific color of a female decoy can elicit spawning behavior in male sockeye. Thus, the carotenoid deposits in the female skin may create a species-specific spectral reflection that triggers spawning in the males.

Studies of the opsin characteristics in the cichlid fish have recently revealed various mechanisms of spectral tuning in the retina. These mechanisms include differential expression of opsin gene ortho- and paralogues as measured by both microspectrophotometry (MSP) and quantitative RT-PCR (Carleton *et al.*, 2000; Carleton and Kocher, 2001). Sequenced RH1 genes from representative cichlid pairs were found to have significantly more NS mutations than S mutations, suggesting that positive selection had taken place (Sugawara *et al.*, 2002). Sampling of the LWS opsin gene from cichlids illustrated that high variation in LWS sequence between species relative to variation in SWS2-B between species was likely due to selection-driven fixation of acquired mutation (Terai *et al.*, 2002). Previous behavioral experiments have described the sexually selected coloration of male cichlids for females making mate choices (Seehausen *et al.*, 1999a; Seehausen *et al.*, 1999b; Seehausen and van Alphen, 1998; Seehausen *et al.*, 1999c). The wide range of coloration in cichlids and the resulting behavioral biases may explain the resulting tuning of cichlid visual pigments through differential expression and positive selection although it is not known how these are related.

Considering the wide geographic distribution of salmon, sexually selected coloration characteristics, and radiation of salmonid species, it was hypothesized that evolution of opsin genes from salmonids parallel the cichlids and undergo positive selection. Our interpretation of adaptive mechanisms of evolution in the opsin gene family will be greatly facilitated through an examination of opsins from the closely related salmon species that range through spectrally diverse habitats, neural remodeling of retinal photosensitivity during post-natal development, and sexually selected traits that adjust color presentation at sexual maturity.

1.6 Regulation of Photoreceptor Differentiation and Opsin Expression

TF control of photoreceptor differentiation and opsin expression has been demonstrated in a variety of systems. For instance, the TFs Chx10 and Pax6 facilitate neuronal differentiation

into non-photoreceptor neurons but inhibit differentiation into photoreceptors (Belecky-Adams *et al.*, 1997; Toy *et al.*, 2002). Chx10 also appears to regulate retinal progenitor proliferation after upregulation by retinoid orphan B receptor (Chow *et al.*, 1998). Wu *et al.* (2001) has shown that regenerating cones in the goldfish retina express the TF Notch 1 and N-cadherin prior to differentiation.

Mutations in the nuclear receptor/transcription factor PNR result in S-cone differentiation suggesting a key role for PNR in S-cone determination (Milam *et al.*, 2002). Similarly, neural retina leucine zipper (NRL) is essential for rod photoreceptor development as the mouse NRL knockout phenotype is an S-cone dominated retina (Mears *et al.*, 2001; Rehemtulla *et al.*, 1996). Other experiments show that NRL and cone-rod homeobox (CRX) act synergistically and that CRX is required for rod opsin expression (Chen *et al.*, 1997; Mitton *et al.*, 2000). Activation of retinoic acid respecters results in an increase in rod photoreceptor differentiation in rat and zebrafish retinae while decreasing cone differentiation (Hyatt *et al.*, 1996; Kelley *et al.*, 1999; Perkins *et al.*, 2002). An earlier study illustrates induction of cone photoreceptor differentiation by TH in rat retinal cell culture (Kelley *et al.*, 1995b). An *in vivo* follow-up study showed that THR-β2 is essential for execution of the M-cone differentiation pathway (Ng *et al.*, 2001). More recent data suggests that THR-β2 and CRX regulate cone opsin expression while PNR suppresses progenitor cell proliferation (Yanagi *et al.*, 2002).

Based on the wealth of research on TF function in developing PRs and the role of TH, a ligand for TFs implicated in PR differentiation, in salmonid UVS cone apoptosis we inferred that SWS1 opsin regulation may be involved in subsequent UVS cone death. PCR based techniques described herein can show that the SWS1 opsin proximal promoter in salmonids is bound by transcription factors with known functions in PR apoptosis.

1.7 Differential Expression in the Parr and Smolt Salmonid Retina

Previous studies have shown that differential expression occurs in retina undergoing photoreceptor apoptosis through the study of Retinitis Pigmentosa (RP) and cDNA screening techniques. Differentially expressed genes in RP include clusterin, secreted frizzled-related protein and TIMP-3 (Jones *et al.*, 2000; Jones *et al.*, 1994; Jones *et al.*, 1992). Clusterin likely has a cytoprotective effect in degenerating photoreceptor cells and is not causally involved in apoptosis (Jomary *et al.*, 1999a; Jomary *et al.*, 1999b). A differential cDNA screening approach illustrated that TIMP-3 expression levels increase in patients with simplex RP (Jones *et al.*, 1994). This study was followed by one that localized TIMP-3 expression to the photoreceptor inner segments and to the ganglion cell layer of retinitis pigmentosa affected retina (Jomary *et al.*, 1995). TIMP-3 is an inhibitor of metalloproteinases responsible for remodeling of the extra cellular matrix. TIMP-3 was also implicated in the homeostasis of the retina as well as neovascularization in the diseased state (Jomary *et al.*, 1997).

Few molecular markers are known that reflect the parr-smolt transition of salmonids. Previous research has implicated genes enriched in the gill and liver including Na^+/K^+ ATPase, cortisol receptor, insulin-like growth factor-1 and transferrin (Hardiman and Gannon, 1996; Mizuno *et al.*, 2001; Sakamoto *et al.*, 1995; Singer *et al.*, 2002). Intracerebral levels of gonadotropin releasing hormone and growth hormone releasing hormone levels have been shown to peak at smoltification in salmonids (Parhar and Iwata, 1996; Parhar *et al.*, 1996). Identification of genes regulated in the retina at smoltification, however, is limited to the SWS1 opsin in apoptotic UVS cones. Thus, our objective was to resolve differences in retinal gene expression before and after smoltification in salmonid retina. To accomplish this Differential Display Reverse Transcription-Polymerase Chain Reaction (DDRT-PCR), created by Liang and Pardee (1992), was employed.

1.7 Thesis Objectives

The salmonid visual system is a relatively novel model of retinal development, degeneration and regeneration. In order to study even the most rudimentary questions regarding retinal mosaic composition, photoreceptor differentiation, visual pigment biochemistry, or expression pattern analysis during smoltification some basic molecular biology techniques were employed. Cloning of fragments of rainbow trout opsin cDNA permitted the mapping of UVS cone reduction during TH treament by *in situ* hybridization paired with a loss of UV-sensitivity as measured by electroretinograms (Allison *et al.*, 2003). The first objective of this thesis was to build on the genetic information gathered in this publication by acquiring the full length cDNA sequence of each of the opsins from rainbow trout retina. These sequences were required to supplement previously obtained electrophysiological and MSP data describing absorption spectra in the rainbow trout retina. By identifying each of the retinal opsin types (i.e. RH1, RH2, SWS1, SWS2, LWS) from the rainbow trout retinal transcriptome, paired with *in situ* hybridization we confirmed that previous retina/photoreceptor absorption characteristics were due to a single opsin type and not mixed expression of more than one opsin in a photoreceptor.

Cloning of opsins in the cichlids and coelocanths has resulted in both statistical and functional evidence that opsin genes of closely related species undergo positive selection upon exploitation of new habitats and species radiation (Carleton and Kocher, 2001; Sugawara *et al.*, 2002; Yokoyama and Tada, 2000). The second objective of this paper was to use the full-length opsin cDNA sequence acquired from rainbow trout to create primers based on untranslated regions (UTRs) that would produce full-length opsin cDNA sequence from other salmonids. Acquisition of these sequences would permit statistical comparison of non-synonymous (NS) to synonymous (S) mutation within salmonids, evolutionary analysis of visual pigments within

teleosts, and evolutionary analyses of salmonids based on opsin sequence changes between species.

The third objective of this thesis revisits the use of the salmonid retina as a model of retinal development and degeneration. Several studies in the past have explored photoreceptor specific differentiation and apoptosis in the context of TF expression. With these studies in mind, and using the full-length opsin cDNA sequence from rainbow trout, the third objective of this thesis was to characterize the opsin promoter regions using genome walking. To determine if there were any biologically relevant TF binding sites in the promoter regions possibly involved in UVS cone apoptosis, an immunoprecipitation-PCR coupled assay was used.

The fourth objective of this dissertation was based on the success of the third objective, the previous research regarding gene expression in smolting salmonids and the cellular and physiological work on the retina done on smolting salmonids. Simply stated the final objective of this thesis was to show that the gene expression profile of the rainbow trout changed during the period that UVS cones and UV-photosensitivity are lost from the retina. DDRT-PCR was employed to identify differentially expressed genes in salmonid retinae and follow up hybridization experiments were used to validate the results.

2. Rainbow Trout (*Oncorhynchus mykiss*) Retinal Opsin cDNA Sequence

2.1 Chapter Introduction

In humans, mutations and gene duplication events that have occurred throughout vertebrate evolution have produced functional visual pigments that are violet/blue- (SWS1- λ_{max} 426), green- (LWS- λ_{max} 530) and red- (LWS- λ_{max} 555) sensitive as well as an opsin that is used specifically for scotopic vision (Rhodopsin RH1- λ_{max} 506) (Merbs and Nathans, 1992). A number of vertebrate species, however, commonly take advantage of an additional visual pigment that is ultraviolet-sensitive (SWS1 opsin). This is in addition to blue- (SWS2 opsin), green- (RH2 opsin), red- (MWS/LWS opsin) sensitive cones and rod (RH1 opsin) photoreceptor specific opsins.

Visual pigment genes have been identified and characterized in a variety of teleost species. This includes the opsin genes of zebrafish and goldfish, important models of both retinal development and function in vertebrates (Chinen *et al.*, 2003; Stenkamp *et al.*, 1996; Vihtelic *et al.*, 1999). Previous studies have also been useful in characterizing the evolution and phylogenetic derivation of visual pigments from several genera of vertebrates (Reviewed in (Ma *et al.*, 2001; Yokoyama, 1995; Yokoyama, 1996; Yokoyama and Shi, 2000). Nucleotide sequence analysis of opsin genes, therefore, can predict both amino acid composition and the relative absorption maxima of the opsin. Inter-specific differences in a specific opsin's predicted amino acid sequence and the observed absorption curves can be interpreted in reference to adaptive evolution of the opsin's ORF.

Using cDNA sequence from members of the opsin gene family is an attractive model to explore adaptive evolutionary processes. Unlike other representative proteins, the substrate that activates an opsin (i.e. light) is dynamic and changes on a recordable scale resulting in varying

selective pressure in different spectral habitats. Spectral tuning of opsin sequences has been investigated by comparison over a relatively broad range of species thereby allowing assumption of amino acid identity responsible for a given absorption spectrum. There are few examples in which amino acid sequence is correlated with changes in habitat and light environment including the coelocanth (Yokoyama, 2000a; Yokoyama and Tada, 2000) and the acquisition of trichromatic vision in primates (Osorio and Vorobyev, 1996).

An examination of closely related species that exist in distinctly different light environments would facilitate interpretation of adaptive mechanisms of evolution in the opsin gene family. The purpose of the current study was to characterize the full-length opsin cDNA sequence from a representative member of the *Oncorhynchus* genus, namely *O. mykiss* (rainbow trout). cDNA library screening as well as 3' and 5' RACE protocols were performed to isolate a member of each of the opsin classes (i.e. SWS1, SWS2, RH1, RH2, and LWS). This groundwork allowed easy characterization of several opsins from related *Oncorhynchus* species from diverse environments using RT-PCR and subsequent evolutionary interpretation.

The data presented in chapter 2 and 3 appears in the manuscript accepted in the *Journal of Molecular Evolution* under the title "Salmonid opsin sequences undergo positive selection and indicate an alternate evolutionary relationship in *Oncorhynchus*." This manuscript was submitted in June of 2003 and is currently accepted. I am first author while Craig Hawryshyn is the senior author. Although I performed the experimental procedures and collected the data for this chapter, the remaining authors (W. T. Allison, D. B. Levin, and J.S. Taylor) made significant intellectual or technical contributions that warranted authorship.

2.2 RNA Isolation

Total RNA isolations were performed on retinal tissue from *O. mykiss* using TRIzol® LS Reagent (Life Technologies, Inc.) with a protocol modified from Chomczynski and Sacchi

(1987). Poly-adenylated mRNA was subsequently isolated using the Promega PolyAttract® mRNA isolation system.

2.2 cDNA library construction and screening

O. mykiss retinal mRNA was used, with the SuperScript™ Plasmid System for cDNA Synthesis and Plasmid Cloning (Invitrogen), to create a cDNA library. Briefly, first and second strand synthesis was performed with SuperScript™ II reverse transcriptase (Invitrogen) with 500ng of mRNA and a poly-T primer (42°C for 1 hour). Ligation of adapters to the cDNA ends allowed subsequent directional ligation into the plasmid vector pSPORT® (Invitrogen). After transformation of *E. coli* with vector/insert, single colonies were used to create macroarray nylon membranes for subsequent plasmid screening (96 colonies per membrane).

O. mykiss rhodopsin probe was created by performing nested RT-PCR on *O. mykiss* retinal total RNA. First round PCR and RT reactions were carried out in the same tube by a two-step process. The PCR reverse primer primed 1.0 μg of total RNA for first strand cDNA synthesis by Superscript™ II reverse transcriptase (Invitrogen). The first strand synthesis reaction was incubated for 1 hour at 42°C, after which PCR continued under the following conditions: one initial denaturation (94°C for 4 minutes), followed by 30 cycles of 94°C (denaturation) for 45 seconds, 60°C for 45 seconds (primer annealing) and 72°C for 60 seconds (extension). One μl of the first round PCR product was used as template for second round PCR, which consisted of an initial denaturation of 94°C for 4 minutes followed by 30 cycles of 94°C for 45 seconds, 65°C for 45 seconds and 72°C for 60 seconds. Table 1 shows the first round PCR and RT primers. The cDNA library macroarrays were screened using standard procedures overnight at 60°C (Sambrook *et al.*, 1989).

2.4 5' and 3' Rapid Amplification of cDNA ends (RACE)

The RACE method was used to isolate the 5'-end of the *O. mykiss* RH1 gene as well as the full-length sequences of the SWS1, SWS2, RH2, and LWS opsin genes. Ambion's FirstChoice™ RLM Race Kit was used with *O. mykiss* mRNA according to the manufacturer's protocol. Template for 5'-RACE PCR was created by first using calf intestinal phosphatase to remove free phosphates from the 5'-end of degraded or incomplete mRNAs, rRNAs or tRNAs. Full length capped mRNAs were digested by tobacco acid pyrophosphatase, leaving the 5'-monophosphate necessary for adapter ligation by RNA ligase. A 45 base RNA adapter was ligated to 5'-end full length mRNA by RNA ligase. First strand cDNA synthesis preformed on the treated *O. mykiss* retinal mRNA by MMLV reverse transcriptase was primed by random hexamers. Primers, complementary to the 5'-adapter and used as the forward primers in the subsequent nested PCR, were supplied with the kit.

Subsequent PCR reactions for the isolation of the 5'-end of the opsin genes were as follows. The first round of PCR was an initial denaturation of 94°C for 4 minutes followed by 30 cycles of 94°C for 45 seconds, 60°C for 45 seconds and 72°C for 60 seconds. The second round of PCR used 1.0 µl of the first round product as template and consisted of an initial denaturation of 94°C for 4 minutes followed by 30 cycles of 94°C for 45 seconds, 60°C for 45 seconds and 72°C for 60 seconds, and one step of 72°C for 5 minutes. Primers, created by multiple sequence alignment of several species' opsin sequences, were used to acquire the opsin gene 5'-end.

3'-RACE was accomplished by first synthesizing cDNA from *O. mykiss* retinal mRNA primed by a 46 bp adapter at 42°C for one hour with MMLV reverse transcriptase. This cDNA was used as template in nested PCR to acquire the 3'-ends of SWS1, SWS2, LWS, and RH2 cDNA. For each of the opsin genes the first round of PCR consisted of an initial denaturation of

94°C for 4 minutes followed by 30 cycles of 94°C for 45 seconds, 60°C for 45 seconds and 72°C for 60 seconds. The second round of PCR used 1.0 µl of first round reaction as template under the following conditions: 94°C for 4 minutes initial denature step, 30 cycles of 94°C for 45 seconds, 60°C for 45 seconds and 72°C for 60 seconds, and one step of 72°C for 5 minutes. 3'-RACE primers were designed based on sequence acquired from the 5'-RACE products. Table 1 shows opsin gene specific primers and adapters used for RACE. Primers used to acquire the 5'-end of RH1 opsin were also used as reverse primers in the opsin probe PCR.

2.5 Cloning and Sequencing

5'- and 3'- RACE PCR fragments were cloned into the vector pGem® (Promega Corporation) using the pGem®-T Easy cloning kit (Promega Corporation) according to the manufacturer's protocol. Ligation reactions were used to transform electrocompetent *E. coli* (ElectroMAX DH10B™ Cells - Invitrogen) via electroporation. Putative positive (white) colonies were screened by PCR using primers designed to the vector's M13 binding sites using the picked bacterial colony as both template for PCR and inoculants for overnight cultures. The standard dideoxy sequencing method employed IRDye™700 and IRDye™800 labeled primers and was preformed on a NEN® Global IR2 DNA Sequencer System (Li-cor). To ensure a lack of PCR or RT error in the sequence data three clones from independent PCR reactions for each 5' and 3' fragment were picked and sequenced.

5'- and 3'-RACE PCR fragments were assembled for each opsin sequence by overlapping sequence at the 3'-end of the 5'-RACE products and the 5'-end of the 3'-RACE products. To ensure that each cDNA previously assembled was from a single contiguous transcript, primers based on the 3' and 5' UTRs were created for each opsin type to produce full-length cDNA PCR products by RT-PCR. PCR products were subsequently cloned and sequenced, and the identity

Table 1.
Primer sequences used in RH1 probe production, 3'- and 5'-RACE, confirmation of results by

RT-PCR and evolutionary RT-PCR as explained in sections 2.2, 2.3 and 2.4 and 3.2

Nested Primer Name	External Primer Sequence (5' to 3')	Internal Primer sequence (5' to 3')
RH1 probe forward	CCAGTTTTCTACGTCCCTATGTC	GGAGCCCATACGAATACCCCC
RH1 probe reverse / RH1 5'-RACE reverse	CGCAAAGAAGGCTGGGATGGT	CATAGGGCATCCAGCACACCA
5'-RACE forward	GCTGATGGCGATGAATGAACACTG	CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG
RH2 5'-RACE reverse	GCGAAGAAGGCAGGGATAGC	CACCAGGAAGCCCATAACCAT
LWS 5'-RACE reverse	CCTTCTCGGCCTTCTGTGTTG	CACGAACAGGGGGAAGAAGC
SWS1 5'-RACE reverse	TAGTCTTTGTTCTCGCTTGTTG	GCCGTAACACAGAATGAAGGAGCAC
SWS2 5'-RACE reverse	TCATCATACAGGAGCGGAACT	CCGAACACCATCACCACCAC
3'-RACE reverse	GCGAGCACAGAATTAATACGACT	CGCGGATCCGAATTAATACGACTCACTATAGG
RH2 3'-RACE reverse	CATTGGGCTGTGCTATTGAGGGCT	TGGACCTGACTACTACACCTTGGC
LWS 3'-RACE reverse	CTTGGGAGAGATGGGTGGTG	CTGGTCTGCTTTCTGGTGTGC
SWS1 3'-RACE reverse	CTGTCCTTGCCTTTGAGAGATATGTG	GGGTCTGGGCTGCTCCTGTGG
SWS2 3'-RACE reverse	GTCTTCCCCCTCTGTTTGGCTGG	GTTCCTCTTCTTCTTCTGCTTCGG
Single round and Evolutionary PCR Primer Names	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
M13 Forward and M13 Reverse	GTAAAACGACGGCCAGT	CAGGAAACAGCTATGACC
1st Round RH2 Opsin Full Length	CTTTCTCTTGCATTCATTGGACATC	CGACAGGGAGTGGAATTGGGAGG
1st Round RH1 Opsin Full Length	CAGAACGGCACAGAAGGAAGCAAC	CGTGAGCCTTTGTCGTTTCCGGG
1st Round LWS Opsin Full Length	GATCAGCAAGACAAGACAACAGAAGC	CTTGCCACTTGGACAGAGATTGATAG
1st Round SWS1 Opsin Full Length	GGGACGGACAAGTAGTGGTCTGAG	GAATCCACCCCAAGACTGTTGGC
1st Round SWS2 Opsin Full Length	GCGATCAACCTTCAAGCTCCTG	GTGTGCATCTATTCTCATCTCTCTTT
2nd Round RH2 Opsin Full Length	GCAAAAGGTTCACAACCAGATCAG	GAAATCAGAGGGTGGGAAGTGCAG
2nd Round RH1 Opsin Full Length	GATCTCATCTTGCCTTCCTCTTGC	GTGGAGCTCGGATTTCATCATTGTC
2nd Round LWS Opsin Full Length	GCCAACCACAAGACAGAGAAAAAGCG	CAAGGGGTGGTTCATAGCAATTTAG
2nd Round SWS1 Opsin Full Length	GGTCTGAGTCAGAGAGGAGCCAAG	GATGGGCTCCTGATGGACTGAGAC
2nd Round SWS2 Opsin Full Length	GGGGAAACCTTGGTAGTGGGGATT	GGCGTCTCTATTTTTATGTTACTTGC
	5' RAC E Adapter	3' RACE adapter
	GCUGAUGGCGAUGAAUGAACACUGCGUUUGC UGGCUUUGAUGAAA	GAGCACAGAATTAATACGACTCACTATAGGTTTTTTTT TTTTVN

of the full-length opsin cDNA sequences was confirmed through a standard nucleotide-nucleotide BLASTn server at NCBI (http://www.ncbi.nlm.nih.gov:80/BLAST/; Altschul *et al.*, 1990) . Each of these fragments was cloned, sequenced and confirmed the 5' and 3' RACE results. Procedures described herein were in accordance with the guidelines established by the Canadian Council on Animal Care. The University of Victoria Animal Care Committee approved all experimental protocols.

2.6 Results

Assembly of nucleotide sequence obtained from cDNA library screening and 5'-RACE produced a full-length cDNA sequence for the rainbow trout RH1 transcript. The 1668 bp RH1 cDNA sequence resulted in a 1068 bp open reading frame (ORF) that predicted a polypeptide sequence of 355 aa.

Assembly of nucleotide sequence obtained from 3'- and 5'-RACE products produced a full-length cDNA sequence for the rainbow trout RH2, LWS, SWS1, and SWS2 transcripts. The RH2 cDNA sequence was 1720 bp long with an ORF of 1041bp that predicted a polypeptide sequence of 346 aa. The LWS cDNA sequence was 1838 bp long with an ORF coding sequence of 1071 bp that predicted an amino acid sequence 356 aa long. The SWS1 cDNA sequence was 1293 bp long with a 1005 bp ORF that predicted an amino acid sequence 334 aa long. The SWS2 cDNA is 1452 bp long with an open reading frame of 1065 bp that predicts an amino acid sequence 354 aa long. The cDNA sequences and predicted amino acid sequences were submitted to Genbank (Accession numbers: AF425072-AF425076).

Opsin sequence identity was confirmed using standard protein BLAST (blastp) on the deduced amino acid sequence. The seven transmembrane rhodopsin protein conserved domain was identified in each opsin type. Each opsin sequence matched closely (i.e. >75% positives identity match) with the corresponding (homologous) opsin sequence from *D. rerio, C. auratus*,

and *T. guttata*. Long distance RT-PCR of total RNA from *O. mykiss* with primers designed from the UTR produced fragments of expected size.

2.7 Discussion

Full-length cDNA sequence for five opsin genes was acquired using 5' and 3' RACE from *Oncorhynchus mykiss*. These sequences represent one gene from each of the five opsin groups within the opsin family i.e. SWS1, SWS2, LWS, RH1, and RH2 and corresponded to previously collected data that indicated λ_{max} values of 371±9.2nm (SWS1 opsin), 432±10.4nm (SWS2 opsin), 519±8.5nm (RH2 opsin), 576±13.9nm (LWS opsin) and 510±2.1nm (RH1 opsin) (Hawryshyn *et al.*, 2001; Hawryshyn and Harosi, 1994). Each of the opsins contained a lysine residue analogous to 296K in human opsins, which binds either 11-cis retinal or 3, 4-dehydroretinal in salmonids. Binding of either chromophore depends on a combination of environmental factors in addition to the developmental state of the animal, and creates a rhodopsin (A_1 - 11-cis retinal) or porphyropsin (A_2 - 3, 4 dehydroretinal) based visual system. Values of λ_{max} listed represent a mixed population of A_1/ A_2 based visual pigments in the photoreceptors.

The sequence comparisons and subsequent functional inferences made in the following sections are based on MSP data from our own laboratory as well as *in vitro* spectrophotometric analyses previously published from other organisms. Although these are both powerful techniques in evaluating visual pigment absorption characteristics, the reflection of the natural state they produce is constrained by experimental technique. MSP is an *in situ* technique that can measure the light absorption characteristics of a photoreceptor's outer segment, and thus the opsin expressed in that photoreceptor. Because the technique is *in situ,* data collected are more likely to reflect the natural state of the opsin. In paired pigment organisms such as rainbow trout,

it is difficult to separate the A_1 and A_2 components from an opsin's absorption characteristics using MSP. *In vitro* assays of reconstituted opsins, however, produce purely A_1 based opsin protein and predict an opsin's absorption characteristic based solely on the A_1 component. One drawback to the reconstitution experiments is the limited interpretation of results in terms of the natural cellular function of the opsin, i.e. protein-lipid interactions as well as phosphorylation state of the protein (Epand, 1998; Kennedy *et al.*, 2001). Goldfish, like the salmonids, employ a paired pigment visual system. Both the MSP and reconstitution studies have yielded similar values for goldfish opsin λ_{max} values from a natural A_1/A_2 mixed retina (Cameron and Powers, 2000; Johnson *et al.*, 1993). Therefore, comparisons of rainbow trout A_1/A_2 opsin MSP data (Hawryshyn *et al.*, 2001; Hawryshyn and Harosi, 1994) to previously published A_1 opsin reconstitution data from other organisms are reasonable. The comparisons described are limited, however, and should be regarded as presumptive until rainbow trout A_1 opsin reconstitution experiments can be completed.

2.8 RH1 opsin

Rainbow trout RH1 absorption as measured by MSP is (510nm). This pigment is red-shifted relative to other teleosts and can be explained by the presence of mixed population of A_1 and A_2 based visual pigment in the retina of rainbow trout. In a retina dominated entirely by A_1, the RH1 λ_{max} would occur at 503 nm (Hawryshyn and Harosi, 1994). Therefore, the predicted aa sequence for the *O. mykiss* RH1 opsin is in agreement with absorbance values and the amino acid sequences of RH1 opsins from other species.

2.9 RH2 Opsin

Previous observation has shown that zebrafish, goldfish, and chum salmon genomes contained multiple green-sensitive opsins both of which belong to the RH2 group (Hisatomi *et*

al., 1994; Johnson *et al.*, 1993; Vihtelic *et al.*, 1999). It is likely that the rainbow trout also have two green-sensitive RH2 opsins. Our investigation, however, found expression of only one, most similar to zebrafish and goldfish type-2. Multiple opsin genes from gene duplication events have been found in tandem array in an organism's genome while only one is expressed. Such is the case for the X-linked human opsin genes, where several copies of the red- and green-sensitive opsin are present on the X-chromosome but commonly only one of each is expressed (Neitz and Neitz, 1995). Similarly, Carleton and Kocher (2001) found that even closely related cichlids differentially express certain opsin genes but almost certainly contain genomic copies of each.

The rainbow trout RH2 gene produces an opsin protein that absorbed maximally at 519 nm in the green spectrum of visible light (Hawryshyn and Harosi, 1994). Most vertebrate RH2 λ_{max} occurs between 467 and 511nm in an A_1 chromophore dominated retina. Two observations possibly account for the red shifted value for rainbow trout RH2. First, rainbow trout used for MSP likely had an A_1/A_2 mixed retina; RH2 opsins associated with an A_2 chromophore would absorb longer wavelengths. Second, of the four substitution sites responsible for the blue shifted absorption of RH2 opsins (Yokoyama, 2000a; Yokoyama and Tada, 2000; Yokoyama *et al.*, 1999), rainbow trout had three of the red shifting amino acids present (123E, 208M, and 164A). The fourth site, 84, although commonly D or N in avian, reptilian and mammalian vertebrates was G in rainbow trout as well as several other teleosts including zebrafish, pufferfish, goldfish and the cichlids.

2.10 LWS opsin

Rainbow trout LWS opsin absorbs maximally at 576nm (Hawryshyn *et al.*, 2001; Hawryshyn and Harosi, 1994). According to Yokoyama and Radlwimmer (1999; 2001), five amino acid sites can distinguish LWS opsin pigment from the MWS pigments (S180, H197,

Y277, T285, and A308). The *O. mykiss* LWS opsin contained each of these five residues at corresponding sites in the aa sequence, namely S177, H194, Y273, T281, and A304. Yokoyama and Radlwimmer (2001) suggest that the λ_{max} of LWS pigments falls between 550-560 nm, thus the 16nm red shift of the *O. mykiss* LWS opsin was likely caused by A_2 absorbance.

2.11 SWS2 Opsin

Rainbow trout SWS2 opsin had a λ_{max} of 432nm, which is at the short wavelength end of the spectrum for SWS2 absorption maxima (435-455nm). Although it is not clear which amino acids conferred blue light-sensitivity, aligning known teleost SWS2 protein sequences (including zebrafish, goldfish, cichlid, and rainbow trout) revealed a conserved amino acid sequence. Future studies will compare SWS2 aa sequence and λ_{max} from other salmonid species to other teleosts in an attempt to narrow the range of aa sites possibly responsible for *O. mykiss* SWS2 λ_{max}.

2.12 SWS1 Opsin

Rainbow trout SWS1, similar to virtually all UV-sensitive pigments, had S and V residues in sites 83 and 84 respectively, corresponding to 90S and 91V in bovine pigment. The trout SWS1 opsin absorbed maximally at 371nm, similar to both goldfish (λ_{max} 359nm) and zebrafish (λ_{max} 362nm). Sixteen amino acid sites indicative of SWS1 opsins where amino acid substitutions occur, and that can alter UV sensitivity, have been identified (Shi *et al.*, 2001; Shi and Yokoyama, 2003). Rainbow trout had 15 of these sites occupied by amino acids similar to other vertebrate SWS1 sequences (L7, F39, F42, T45, F79, S83, V84, Q86, A107, S111, N143, Q145, G177, C178, and S179). Unlike other vertebrate SWS1 pigments, the rainbow trout had a V residue located at position 80 as opposed to a C or V residue found in other species at the corresponding aa sites. The N143 in rainbow trout, similar to N149 of the zebra finch, chameleon and mouse, likely corresponded to the G142 of goldfish and zebrafish. It is possible

that these sites were responsible for the rainbow trout SWS1 red shifted λ_{max} relative to the zebrafish and goldfish. Although the red shift may also be caused by an A_2 based pigment, previous work has shown that switching of the chromophore had a negligible effect on SWS1 λ_{max} (Kawamura and Yokoyama, 1996). Yokoyama (2000b) also suggested that T178 and A179 conferred UV specific absorption characteristics to the SWS1 opsin in goldfish and zebrafish. It is interesting to note that *O. mykiss* as well as *Metriaclima zebra* used C178 and S179 similar to terrestrial vertebrate SWS1 pigments. It is likely that the speciation of the goldfish and zebrafish predated this substitution and could explain the shorter λ_{max} for their SWS1 opsins. Thus C178 and S179 would be an ancestral state where T178 and A179 in goldfish and zebrafish would have been substituted before their speciation.

3. Opsin sequence and analysis from *Oncorhynchus* sp. and *Salmo salar*.

3.1 Chapter Introduction

The loss of UV photosensitivity as a result of UVS cone disappearance from the retina of the salmonids during smoltification is well documented (Allison *et al.*, 2003; Browman and Hawryshyn, 1992; Deutschlander *et al.*, 2001). Furthermore, UVS cones can regenerate in the retina of salmonid fishes at sexual maturity (Beaudet *et al.*, 1997; Hawryshyn *et al.*, 2003). UV vision in salmonids mediates polarization vision, used in navigation, migration and foraging (Hawryshyn, 2000; Novales Flamarique and Hawryshyn, 1997). Salmon also employ a dynamic A_1/A_2 chromophore ratio in photoreceptor function permitting a broader spectral range of visual sensitivity. Coho salmon migration into salt water, resulting in exposure to shorter light wavelengths, is paired with a shift from A_2- to A_1- dominated chromophore ratios resulting in the spectral tuning of photoreceptors to shorter wavelengths (Alexander *et al.*, 1994). Varying A_1/A_2 chromophore ratio has been observed in other species of *Oncorhynchus* although the result of oceanic migration on chromophore ratio has not been determined.

Oncorhynchus sp. also undergo morphological changes at sexual maturation including a change in coloration in some species from silver to red (Clarke *et al.*, 1995; Groot and Margolis, 1991). Sexually mature, spawning sockeye, chum and coho salmon employ carotenoid deposition in the skin to produce red optical signals for mate selection (Foote *et al.*, In Press-Animal Behavior; Olsen and Mortensen, 1997; Steven, 1947). Female salmon engaging in mate selection exploit the males' red pigmented skin color as a test of fitness, which also serves as an aggressive display trait to other males (de Gaudemar, 1998). Pacific salmon typically exhibit semelparity (spawning only once), a life history strategy that requires a high degree of specificity based on recognition of traits such as coloration. Behavioral studies by Foote et al. (In Press-Animal

Behavior) have shown that a deep red coloration of carotenoid pigment can elicit spawning behavior in male sockeye.

Considering the wide geographic distribution of salmon, the sexually selected coloration characteristics, and the radiation of salmonid species, we hypothesized that evolution of opsin genes in salmonids have undergone positive selection similar to the observations in cichlid opsins. The full-length opsin cDNA sequences from *O. tshawytscha* (chinook salmon - oceanic), *O. gorbuscha* (pink salmon - oceanic), *O. kisutch* (coho salmon - oceanic), *O. keta* (chum salmon - oceanic), *O. nerka* (sockeye salmon - oceanic), as well as Atlantic salmon, *Salmo salar* (oceanic) were characterized. Examination of ORF nucleotide sequences by the Creevey-McInerney method revealed instances of positive selection occurring in the RH1 and RH2 opsin genes of salmon (Creevey and McInerney, 2002). This groundwork will facilitate interpretation of opsin composition in photoreceptors of related *Oncorhynchus* sp. acquired by MSP analysis.

3.2 Evolutionary RT-PCR

Evolutionary RT-PCR is the use of a single primer set for amplification of a homologous gene across phylogenetic species in a one step RT-PCR reaction. Primers that amplified single contiguous copies of full-length opsin cDNA sequence from *O. mykiss* were used as first round, single step RT-PCR primers on *O. tshawytscha, O. gorbuscha, O. kisutch, O. keta, O. nerka,* and *Salmo salar* mRNA. Cone opsin genes (RH2, LWS, SWS1, and SWS2) were amplified from *S. salar* retinal mRNA. The *S. salar* RH1 gene cDNA has been previously described (Accession # AAF44620, (Philp *et al.*, 2000). Second round, nested primers were based on UTR sequence adjacent to first round primers thus producing the entire ORF for each opsin. RNA isolations and reverse transcription-PCR was carried out as described in section 2.1 and 2.3, respectively, with the exception that the second round PCR annealing temperature was 65°C for 45 seconds. PCR products obtained were cloned and sequenced as described in section 2.4. Primer sequences for

both first and second round PCR are found in Table 1. Primers were chosen with a predicted melting temperature of at least 65°C, high GC content and minimal primer dimer or hairpin loop structural possibilities. Each of these fragments was cloned, sequenced and confirmed the 5' and 3' RACE results. Procedures described herein were in accordance with the guidelines established by the Canadian Council on Animal Care. The University of Victoria Animal Care Committee approved all experimental protocols.

3.3 Phylogenetic reconstruction

Teleost retinal opsin aa sequences derived from cDNA ORFs were used to reconstruct a phylogenetic tree using the minimum evolution (ME) method rooted with the non-photoreceptor, vertebrate ancient (VA) opsins and the honey-bee UV opsin (Rzhetsky and Nei, 1993). Salmonid opsin aa sequences were concatamerized in the order of LWS-RH1-RH2-SWS1-SWS2 to a total length of 1740 aa and produced a ME phylogenetic tree of the *Oncorhynchus* sp. rooted with *Salmo salar*. ME trees were corrected for multiple substitutions using the Poisson correction for distance and bootstrapped with 1000 replicates (Nei and Kumar, 2000). Phylogenetic analyses were conducted using MEGA version 2.1 (Kumar *et al.*, 2001).

3.4 Creevey-McInerney test for positive selection

Positive selection was tested using Creevey-McInerney analysis with the Crann program (Creevey and McInerney, 2002). This analysis compares the number of S and NS substitutions by creating a neighbor joining tree based on dN values (NS substitutions per NS site) and then determining whether mutations are variable (occur more than once in the tree), or invariable (occur in only one branch of the tree). Four values are compared to those predicted by neutral theory; S variable (SV), S invariable (SI), NS variable (NSV), and NS invariable (NSI) mutations. A substitution is considered variable or non-directional if it occurs once, early in a reconstructed phylogeny, and then again, independently, later in the phylogeny. Invariable or

directional substitutions, on the other hand, occur at a node in the reconstructed phylogeny and remain intact in the subsequent branches. If the NS value is significantly greater than its S counterpart then positive selection is detected as directional (NSI>SI) or non-directional (NSV>SV). Significance was tested by a χ^2 G-test for independence and estimated p-value.

3.5 Opsin sequences and evolution

Full-length opsin gene sequences were obtained from the Pacific salmon, *O. tshawytscha* (Accession # AY214135-AY214139), *O. keta (*Accession # AY214140-AY214144), *O. kisutch* (Accession # AY214145-AY214149), *O. gorbuscha* (Accession # AY214150-AY214154), *O. nerka* (Accession # AY214155-AY214159), and the cone opsin genes from *Salmo salar* (Accession #AY214131-AY214134). Each of the deduced opsin protein sequences contains a lysine residue analogous to 296-K in human opsins, which binds either 11-*cis* retinal or 11-*cis* 3, 4-dehydroretinal in salmonids (Beatty, 1984). Binding of either chromophore depends on a combination of environmental factors and the developmental state of the animal, and creates a rhodopsin (A_1 - 11-cis retinal) or a porphyropsin (A_2 - 3, 4 dehydroretinal) based visual pigment system (Alexander *et al.*, 1994; Beatty, 1984; Provencio *et al.*, 1992).

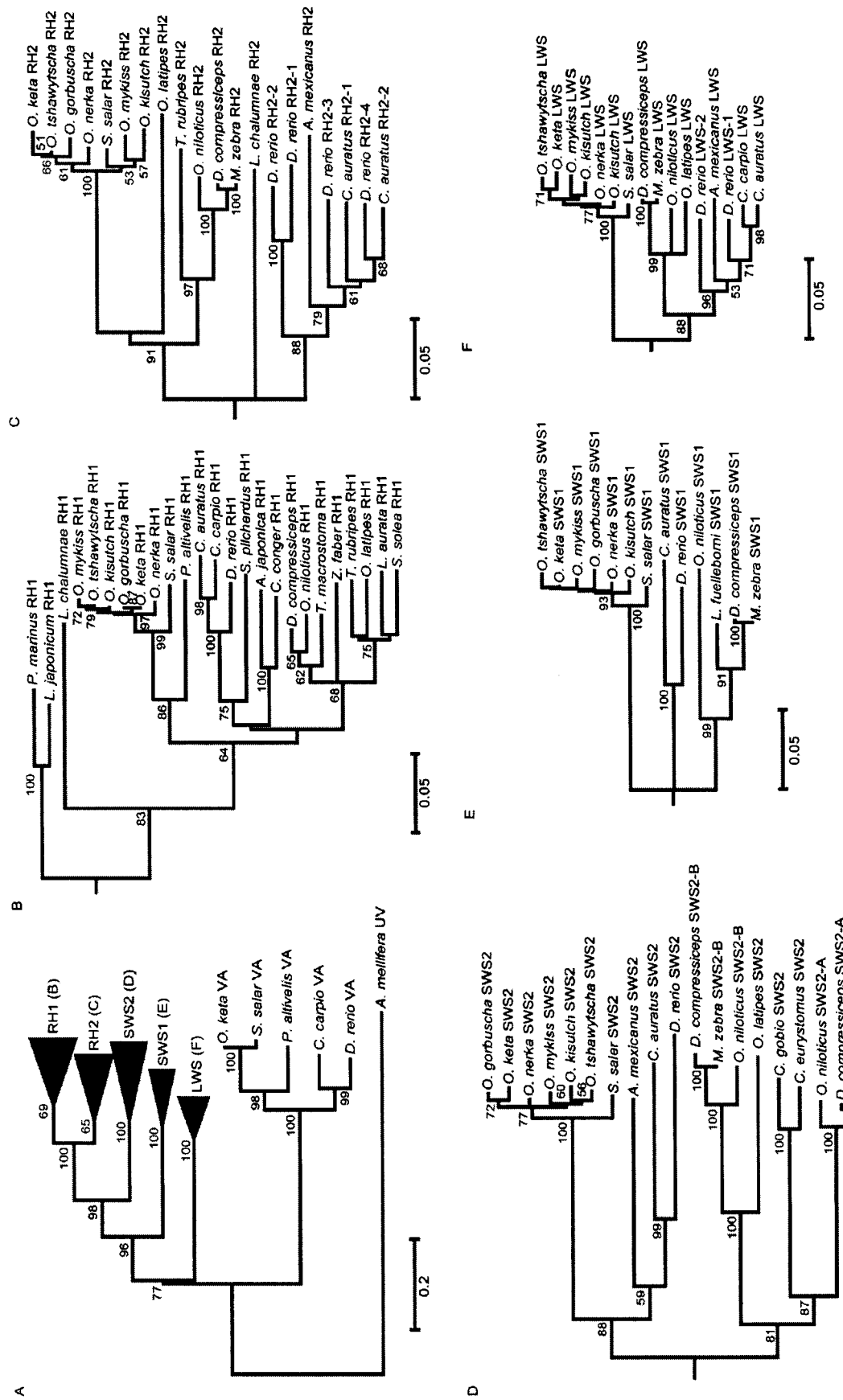
Figure 4 (a-f) illustrates phylogenetic trees reconstructed from amino acid sequences derived from salmonid cDNA sequences. Figure 4a is based on all the opsin sequences and reliably places the photoreceptor opsins into the 5 monophyletic groups previously described (Yokoyama, 2000b). The opsin gene duplication events in zebrafish (Fig. 4c-RH2 and 4f-LWS), goldfish (Fig. 4c-RH2) and the cichlids (Fig4d-SWS2) all occurred independently of the salmonid lineage. Hisatomi *et al.* (1994) described two unique RH2 opsin gene fragments isolated from *O. keta* genomic DNA. Although several clones were sequenced to obtain RH2 opsin sequence from each *Oncorhynchus* sp. only one type of RH2 gene was identified and was found to correspond to the RH2-B genomic fragment reported by Hisatomi *et al.* (1994). While

Figure 4.
Minimum evolution phylogenetic tree of teleost opsin amino acid sequence.

(A) Master tree shows early derivation of contemporary opsin classification out-grouped with the vertebrate ancient opsins and the honeybee UV-sensitive opsin. (B) Teleost RH1 opsin classification. (C) Teleost RH2 opsin classification. (D) Teleost SWS2 opsin classification. (E) Teleost SWS1 opsin classification. (F) Teleost LWS opsin classification.

Accession numbers for non-salmonid vertebrates are: *C. auratus* RH2-1 - L1186, *C. auratus* RH2-2 - L11865, *C. auratus* RH1 - L11863, *C. auratus* LWS - L11867, *C. auratus* SWS1 - D85863, *C. auratus* SWS2 - L11864, *D. rerio* RH2-1 - BAC24129, *D. rerio* RH2-2 - BAC24130, *D. rerio* RH2-3 - BAC24131, *D. rerio* RH2-4 - BAC24132, *D. rerio* RH1 - BAC21668, *D. rerio* LWS-1 - BAC24128, *D. rerio* LWS-2 - BAC24127, *D. rerio* SWS1 - BAC24134, *D. rerio* SWS2 - BAC24133, *P. marinus* RH1 - Q98980, *L. japonicum* RH1 - JN0120, *P. altivelis* RH1 - BAB88652, *C. carpio* - CAA96518, *L. chalumnae* RH1 - AAD30519, *S. pilchardus* RH1 - CAA77259, *A. japonica* RH1 - CAB56647, *C. conger* RH1 - AAB37721, *D. compressiceps* RH1 - BAC02613, *O. niloticus* RH1 - BAC02620, *T. macrostoma* RH1 - BAC02627, *Z. faber* - CAA74832, *T. rubripes* RH1 - AAD54580, *O. latipes* RH1 - BAA19423, *L. aurata* RH1 - CAA77253, *S. solea* RH1 - CAA77254, *O. latipes* RH2 - BAA19420, *T. rubripes* RH2 - AAF44648, *O. niloticus* RH2 - AAF63535, *D. compressiceps* RH2 - AAF63541, *M. zebra* RH2 - AAF63533, *L. chalumnae* RH2 - AAD30520, *A. mexicanus* RH2 - AAB32221, *A. mexicanus* SWS2 - AAB28911, *D. compressiceps* SWS2-A - AAF63540, *D. compressiceps* SWS2-B - AAF63528, *M. zebra* SWS2-A - AAF63525, *M. Zebra* SWS2-B - AAF63529, *O. niloticus* SWS2-A - AAF63527, *O. niloticus* SWS2-B - AAF63531, *O. latipes* SWS2 - BAA19419, *C. gobio* SWS2 - CAD23111, *C. eurystomus* SWS2 - CAD23112, *O. latipes* SWS1 - BAA19422, *D. compressiceps* LWS - AAF63536, *M. zebra* LWS - AAF63537, *O. niloticus* LWS - AAF63539,

O. latipes LWS - BAA19420, *A. mexicanus* LWS - A37440, *C. carpio* LWS - BAB32496, *C. carpio* VA-opsin - AAF74260, *O. keta* VA-opsin - AAK27833, *S. salar* VA-opsin - AAK64514, *D. rerio* VA-opsin - BAA94289, *P. altivelis* VA-opsin - BAB88650, *A. mellifera* UV-opsin - AAC47455. Amino acid sequences were corrected for multiple substitutions by a Poisson correction. Values shown are bootstrap greater than 50% of 1000 replicates. Scale bar values are substitutions per 1000 amino acids.


A
RH1 (B)
RH2 (C)
SWS2 (D)
SWS1 (E)
LWS (F)
O. keta VA
S. salar VA
P. altivelis VA
C. carpio VA
D. rerio VA
A. mellifera UV
0.2
B
P. marinus RH1
L. japonicum RH1
L. chalumnae RH1
O. mykiss RH1
O. tshawytscha RH1
O. kisutch RH1
O. gorbuscha RH1
O. keta RH1
O. nerka RH1
S. salar RH1
P. altivelis RH1
C. auratus RH1
C. carpio RH1
D. rerio RH1
S. pilchardus RH1
A. japonica RH1
C. conger RH1
D. compressiceps RH1
O. niloticus RH1
T. macrostoma RH1
Z. faber RH1
T. rubripes RH1
O. latipes RH1
L. aurata RH1
S. solea RH1
0.05
C
O. keta RH2
O. tshawytscha RH2
O. gorbuscha RH2
O. nerka RH2
S. salar RH2
O. mykiss RH2
O. kisutch RH2
O. latipes RH2
T. rubripes RH2
O. niloticus RH2
D. compressiceps RH2
M. zebra RH2
L. chalumnae RH2
D. rerio RH2-2
D. rerio RH2-1
A. mexicanus RH2
D. rerio RH2-3
C. auratus RH2-1
D. rerio RH2-4
C. auratus RH2-2
0.05
D
O. gorbuscha SWS2
O. keta SWS2
O. nerka SWS2
O. mykiss SWS2
O. kisutch SWS2
O. tshawytscha SWS2
S. salar SWS2
A. mexicanus SWS2
C. auratus SWS2
D. rerio SWS2
D. compressiceps SWS2-B
M. zebra SWS2-B
O. niloticus SWS2-B
O. latipes SWS2
C. gobio SWS2
C. eurystomus SWS2
O. niloticus SWS2-A
D. compressiceps SWS2-A
M. zebra SWS2-A
0.05
E
O. tshawytscha SWS1
O. keta SWS1
O. mykiss SWS1
O. gorbuscha SWS1
O. nerka SWS1
O. kisutch SWS1
S. salar SWS1
C. auratus SWS1
D. rerio SWS1
O. niloticus SWS1
L. fuelleborni SWS1
D. compressiceps SWS1
M. zebra SWS1
0.05
F
O. tshawytscha LWS
O. keta LWS
O. mykiss LWS
O. kisutch LWS
O. nerka LWS
S. salar LWS
D. compressiceps LWS
M. zebra LWS
O. niloticus LWS
O. latipes LWS
D. rerio LWS-2
A. mexicanus LWS
D. rerio LWS-1
C. carpio LWS
C. auratus LWS
0.05

each of the salmonid species may contain multiple copies of each opsin resulting from gene duplication events they may be inactivated and only one is preferentially expressed making isolation of several from cDNA less likely (Carleton and Kocher, 2001; Chinen *et al.*, 2003; Neitz and Neitz, 1995). Several cichlid species contain multiple genomic copies of the SWS2 gene but express a detectable amount of only one SWS2 isoform's gene product (Carleton *et al.*, 2000). Observed substitutions between the aa sequences of salmonids are detailed in Table 2.

Interestingly, the trees reported based on opsin sequence did not reflect the topology predicted from known species-level relationships between salmonids, cyprinids and cichlids. The currently accepted relationship between these three clades is (cyprinid, (cichlid, salmonid)) (McArthur *et al.* 2003). This evolutionary relationship was detected in only the RH2 opsin (Fig. 4c) with a relatively low bootstrap value (65) separating the cyprinids from the cichlid/salmonid clade (Fig. 4a). While the SWS1 opsin relationships appear unresolved, the RH1 (Fig. 4b) and LWS (Fig. 4f) sequences generate monophyletic clades of the cichlids and cyprinids excluding the salmonids with bootstrap values of 64 and 100, respectively. These clades can be traced to specific amino acid identities in the RH1 and LWS polypeptides shared by cichlids and cyprinids but not salmonids. Corresponding to the zebrafish RH1 sequence these sites are 22, 40, 71, 90, 119, 149, 151, 173, 189, 225, 232, 277, 278, 290, 307, and 308, and corresponding to the zebrafish LWS these sites are 27, 53, 58, 82, 97, 142, 158, 214, 219, 226, 231, 238, 303, 338, and 342. The SWS2 tree (Fig. 4d) reveals a cyprinid/salmonid clade excluding the cichlids with a bootstrap value of 100. This relationship can be traced to specific amino acid identities shared by the cyprinids and salmonids but not the cichlids (91, 99, 166, 176, 249, 262, 319, 341, 345, 349, and 352 corresponding to *Oncorhynchus* sp. SWS2). These disparate evolutionary relationships are likely due to independent rates of evolution within the opsins which are a function of the

Table 2.
Amino acid substitutions present in the five opsin types cloned from the Pacific salmon (*Oncorhynchus* sp.) and Atlantic salmon values (*Salmo salar*).

	RH1	RH2	SWS1	SWS2	LWS
Substitutions shared by *Oncorhynchus sp.*	A114G, G124A, K141E, S165A, G197D, V198I, V205I, I209V, S213M, T220S, K248R, Y261F, S264C, A299S, H315N	H39Y, T167A, V256I,	I14V, S26P, A34T, F67Y, I72V, A151G	V46I, C105S, Y107S, G109A, N110Y, L165I, S299T	R4S, L52V, V58I,V132T, R324K
Oncorhynchus mykiss	K141E, I155M	G40C, M45T, F104Y, S210T, S237P, C260R, T271S, L309M	M27I, S288P, F315L	V133M, I164V, I169V	N51I, A177S, A248R, Q250T, M270V
Oncorhynchus tshawytscha	I155M	Y38F, F51L, L109M, S151A, T152S, L174I	E18G, M27I, V54E, N275E	M50I, Y311C	K80E, N86S, F230V, A245G, V246A, A259P
Oncorhynchus gorbuscha	S149T	Y38F, A42V, V43F, L109M, S151A, T152S L174I, M318T	M27K, V53L, N71D, S327C	R111K, A119T, F173Y, V266A	A177S, I237V, D252N
Oncorhynchus nerka	F116C, Y206H, S281T	A151S, T152S, L174V, S210T, T321A, T335A	M27I, V130L, W184R	R111K, Q121L, L175F	A177S
Oncorhynchus kisutch	S38P, I155M	G40C, M45T, F222Y, I264V,T321A, D329G, V343A	D4N, M27I, F49L, R93G	N26I, E144D, G174R	K79E, A248Q, Q257R
Oncorhynchus keta	S149T	S23G, Y38F, F51L, C111S, F149L, S151A, T152S, L174V,		R111K, A119T W185R, M328I	V73G, A177S, F230V, A245G, V246A, A248Q, Q250K, A259P

Table footnote: Substitutions are listed in format of amino acid identity of *Salmo salar* (outgroup), followed by the site # followed by the identity substituted in the species' sequence. For instance, *O. mykiss* RH1 at position 141 contains an E residue whereas *S. salar* contains K at this position. The first row describes substitutions that have been retained in all *Oncorhynchus sp.* compared to *S. salar*.

spectral habitats of the fish. This environmental pressure may have resulted in instances of convergent evolution displayed as unexpected monophyletic clades.

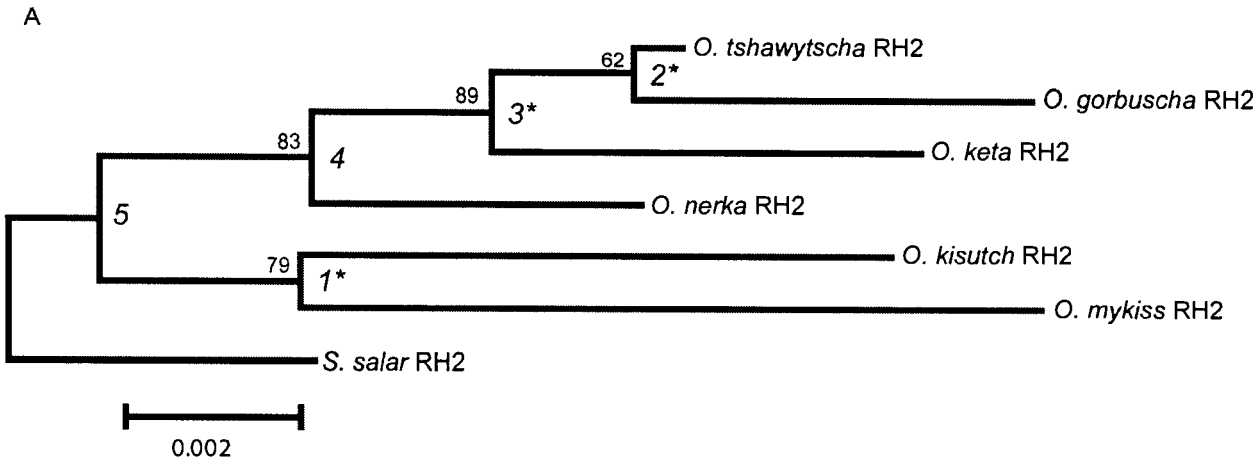
3.6 Positive selection in salmon opsins

The traditional dN:dS comparison between salmonid opsins indicated no positive selection in the sequences sampled (i.e. dS>dN for all opsins; data not shown). The recently developed Creevey-McInerney technique is more sensitive when comparing closely related ORF sequences (Creevey and McInerney, 2002). This test can identify directional positive selection, where advantageous mutations are preserved in all following lineages, and non-directional positive selection, where specific environmental pressures have selected discrete amino acid changes for a single species but not elsewhere in the phylogeny.

Figure 5 (a & b) illustrates the phylogenetic tree and subsequent Creevey-McInerney analysis for RH1 and RH2 opsin ORFs. The values of the four substitution variables, G-test scores, and estimated p-values for each node of the RH1 and RH2 Creevey-McInerney trees are shown in Table 3. Positive selection was not detected in the other opsin sequences and their neighbor joining trees, based on dN values, have short branch lengths and are poorly resolved (bootstrap values <50, data not shown). Although positive selection is not detected within the salmon LWS, SWS1 and SWS2 ORFs, there may be meaningful aa replacements (see section 3.3) that change the opsin's λ_{max}, which will have to be tested by *in vitro* opsin expression/A_1 reconstitution experiments.

Positive non-directional selection was detected in the RH2 opsin ORF (Figure 5a). Specific aa substitutions shared by *O. tshawytscha*, *O. gorbuscha*, and *O. keta* (Y38F, S151A, T152S, L174I), and others shared by *O. mykiss* and *O. kisutch* (G40C, M45T), resulted in the relationship presented in Figure 5a. The branch patterns based on these substitutions were

Figure 5.
Creevey-McInerney analysis of salmonid RH2 and RH1 opsin open reading frames. (A) RH2 opsin analysis reveals non-directional positive selection (*) at three nodes (1, 2, 3). (B) RH1 opsin analysis reveals directional positive selection (**) at one node (5) early in RH1 divergence. Trees were created with neighbor joining analysis of nucleotide ORF based on dN values compared between species. *Salmo salar* was the outgroup in each analysis. Values shown are bootstrap greater than 50% of 1000 replicates. Scale bar values are substitutions per 1000 nucleotides.


A
O. tshawytscha RH2
O. gorbuscha RH2
O. keta RH2
O. nerka RH2
O. kisutch RH2
O. mykiss RH2
S. salar RH2
62
89
83
79
2*
3*
4
5
1*
0.002

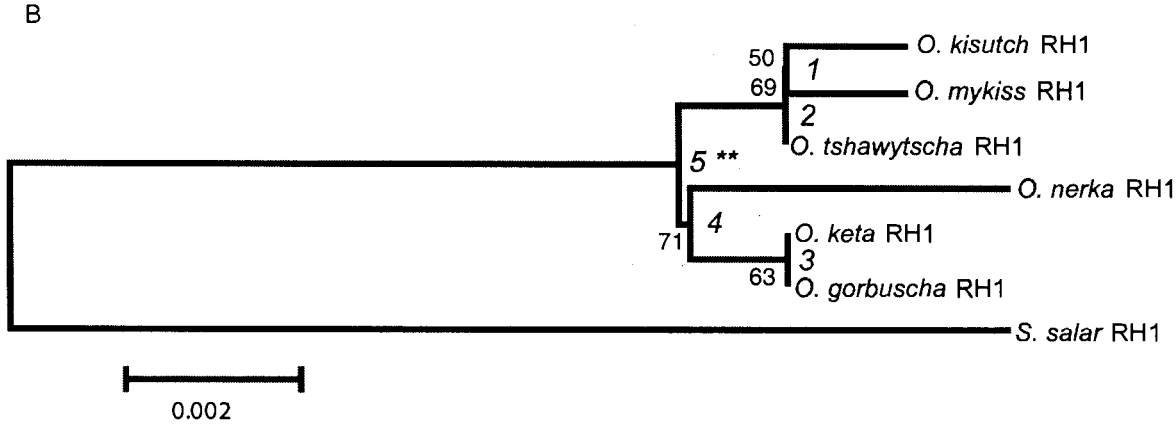

B
O. kisutch RH1
O. mykiss RH1
O. tshawytscha RH1
O. nerka RH1
O. keta RH1
O. gorbuscha RH1
S. salar RH1
50
69
71
63
1
2
5 **
4
3
0.002

Table 3.
Values used for the Creevey-McInerney positive selection analysis on RH1 and RH2 opsin sequences from *Oncorhynchus* sp. outgrouped with *S. salar.*

	NSI	NSV	SI	SV	G-test value	p-value
RH2 Node 1	2	4	9	1	5.10	0.024*
RH2 Node 2	3	8	12	2	8.71	0.0032*
RH2 Node 3	5	12	15	8	4.94	0.026*
RH2 Node 4	2	12	2	16	0.064	0.800
RH2 Node 5	10	24	26	28	3.03	0.082
RH1 Node 1	0	2	0	8	0.00	1.00
RH1 Node 2	1	2	0	8	1.72	0.190
RH1 Node 3	5	12	15	8	1.88	0.170
RH1 Node 4	1	3	1	11	0.493	0.483
RH1 Node 5	17	5	24	22	3.99	0.046**

Table footnote: Node numbers are labeled in Figure 2. * denotes positive non-directional selection where more replacement variable substitutions have occurred than expected from neutral theory. ** denotes positive directional selection where more replacement invariable substitutions have occurred than expected from neutral theory.

accompanied by substitutions in the RH2 opsin that are unique for each species. The occurrence of these unique substitutions at a frequency greater than would be predicted by neutral theory suggests positive non-directional substitutions in the RH2 opsin gene.

Positive selection in the gene responsible for visual detection of green light, RH2, may be related to species recognition and red dermal pigmentation observed in spawning *Oncorhynchus* sp. Skin pigmentation in immature salmonids depends on deposits of the reflective purine guanine, resulting in a primarily silver-green reflected skin color (Staley and Ewing, 1992). Selection of the RH2 opsin may be subject to selective pressures such as intra- and/or inter-specific recognition, and competition for resources. Although not immediately apparent, the relationship between red coloration and adaptive tuning of the RH2 pigment becomes evident when red-green color opponency is considered. In the color opponency model, photoreception in one type of photoreceptor (i.e. RH2 - green) inhibits the response of another (i.e. LWS - red) via horizontal cells in order to maximize the relative signal strength sent to higher visual centers (Kamermans *et al.*, 1991; Stell *et al.*, 1975; Twig *et al.*, 2003). A possible scenario to consider is that if a red light stimulus elicits an energetically expensive spawning response and the RH2 cone is tuned to maximize inhibition on the LWS cone, then only a definitive red light signal will produce enough LWS cone response to overcome opponency and result in spawning behavior.

Parkyn and Hawryshyn (2000) examined the cone λ_{max} spectral sensitivity of several salmonids through optic nerve recordings. They reported that the LWS cone mechanism was indistinguishable from the RH2 cone mechanism in *O. nerka,* while both cone responses were differentiated in *O. mykiss.* The lack of LWS cone response could be due to opponent inhibition from a red-shifted RH2 opsin λ_{max} in *O. nerka.* Although Parkyn and Hawryshyn (2000) report on neuronally processed visual information, it does illustrate a relationship between green and red

photosensitivity within *Oncorhynchus* sp. Further, these differences could be a direct result of the positive selection of the RH2 opsin gene in the salmonids as *O. mykiss* and *O. nerka* appear in separate monophyletic clades (Fig. 5a).

The positive directional selection observed in the salmonid RH1, however, appears to have occurred early in the evolution of *Oncorhynchus* sp. resulting in an aa substitution that occurred in *O. mykiss, O. gorbuscha* and *O. tshawytscha* but not the other Pacific salmon (I155M) (Fig 5b; Where the first residue, I, corresponds to the ancestral state (*Salmo*), the number corresponds to the position in the salmonid RH1 protein and the second residue, M, corresponds to the substitution). If this mutation results in the tuning of salmonid scotopic vision then the species that share the substitution may occupy a similar spectral habitat such as vertical water column position in the ocean, or may have similar feeding habits. It should be noted that in the smelt RH1 sequence, published by Minamoto and Shimizu (2002), site 155 is an M residue. This suggests that an M to I substitution occurred in the salmonid lineage and then a back substitution to M occurred in *O. mykiss, O. gorbuscha* and *O. tshawytscha*. This may be an example of convergent evolution if this mutation results in a significant λ_{max} change of the RH1 opsin. In order to test this hypothesis, *in vitro* opsin expression/A_1 reconstitution experiments paired with site-directed mutagenesis should be performed in future research.

Cloning visual pigment opsin genes from salmon indigenous to the west coast of Canada provides a unique opportunity to examine the effect of diverse evolutionary pressures on the visual system of several closely related species. Previous studies regarding adaptive evolution in vertebrate opsins have been restricted to comparisons based on only a small number of species or only one opsin type. Although evolution and adaptation have allowed Pacific salmon to utilize different niches, migratory behavior, and life history strategies, there was limited change in

amino acid sequence of the opsins between species. The lowest similarity observed was 96% aa identity between *S. salar* and *O. keta* RH1 (15 aa substitutions/354 aa sites) while the highest similarity was 99.4% aa identity between *O. gorbuscha* and *O. nerka* LWS (2 aa substitutions/357 aa sites). The fact that positive selection was documented in the cichlid RH1 opsin by dN:dS analysis may be indicative of their recent radiation in the East African Lakes (Tanganyika, Malawi and Victoria). As opposed to the salmonids, which radiated 6-7 million years ago, the East African Lakes formed only 1 million years ago (Meyer *et al.*, 1990). The S sites in the cichlid opsin ORF have not been saturated in the short period of time since radiation and dN:dS comparisons are sensitive enough to detect positive selection.

3.7 Amino acid substitutions of biological significance

Yokoyama (2000b) lists known specific amino acid changes in vertebrate opsins (i.e. site number and aa identity) that exhibit a λ_{max} shift of more than 5nm. We have found only two changes in the aa sequences in salmonids that are also listed by Yokoyama (2000b): (1) *Oncorhynchus* sp. RH1 opsins contain an F residue at site 261 (corresponding to site 261 in bovine RH1) while *Salmo salar* has a Y residue at the same site. This change appears to be derived in *Oncorhynchus* sp., as other ancestral Salmoniformes species contain a Y residue at site 261 (*S. trutta*, AY158045; *Coregonus autumnalis*, L2494; and *Plecoglossus altivelis*, AB074484). We would expect that this aa replacement would red shift the RH1 λ_{max} in *Salmo* sp. approximately 10nm in comparison to *Oncorhynchus* sp. Munz and Beatty (1965) observed no difference in λ_{max} between A_1 based RH1 of *Salmo* sp. and *Oncorhynchus* sp. (λ_{max} 503 nm) using visual pigment extraction techniques, however slightly longer λ_{max} for both *S. salar* (505 nm) and *S. trutta* (508 nm) (Ali and Wagner, 1974; Allen *et al.*, 1973) have been reported, which could represent population differences in the RH1 sequence. Other osmerids (closely related to *P.*

altivelis) and congeners of *C. autumnalis* examined have A_1-based RH1 λ_{max} between 509-514 nm (Ali and Wagner, 1974), similar to the prediction above. Therefore, we can infer that some of the RH1 aa substitutions shared by *Oncorhynchus* sp. may actually have occurred independently in *S. salar* and caused a blue shift in the RH1 pigment back towards 503nm in an instance of convergent evolution.

Alternatively, the Y261F change described above may not universally affect all opsins, which implies that other aa differences are also required for the Y261F mutation to have an effect. Sequence data from more species of the genus *Salmo* will facilitate the determination of aa replacements that could be specific to *Oncorhynchus* sp. and *Salmo* sp.; (2) aa site 288 of the *O. mykiss* SWS1 gene (corresponding to site 295 of the bovine RH1) was occupied by a P residue, while other members of *Oncorhynchus* sp. and *Salmo salar* contained the more common S residue. This substitution has not been previously examined in any study of SWS1 visual pigment function and evolution in vertebrates. Recently, Yokoyama and Tada (2003) described 7 amino acid substitutions in contemporary SWS2 vertebrate opsins that can alter the λ_{max} *in vitro*. One of these sites, corresponding to position 52 in the bovine RH1, has previously been observed as V, A or G in other vertebrate SWS2 opsin genes. In the salmonid SWS2 opsin a T residue occupies this site. The effect of the SWS1 P295 and SWS2 T52 identities are unknown although it is accepted that variation in λ_{max} of SWS opsins is affected by interactions of sites and is not additive as in the case of RH1, RH2 and LWS. Since these are the only observed variations of aa identity as described by Yokoyama (2000b; 2003), any other differences in opsin λ_{max} observed in future in vitro opsin expression/A_1 reconstitution experiments should be attributed to the remaining substitutions found in Table 2.

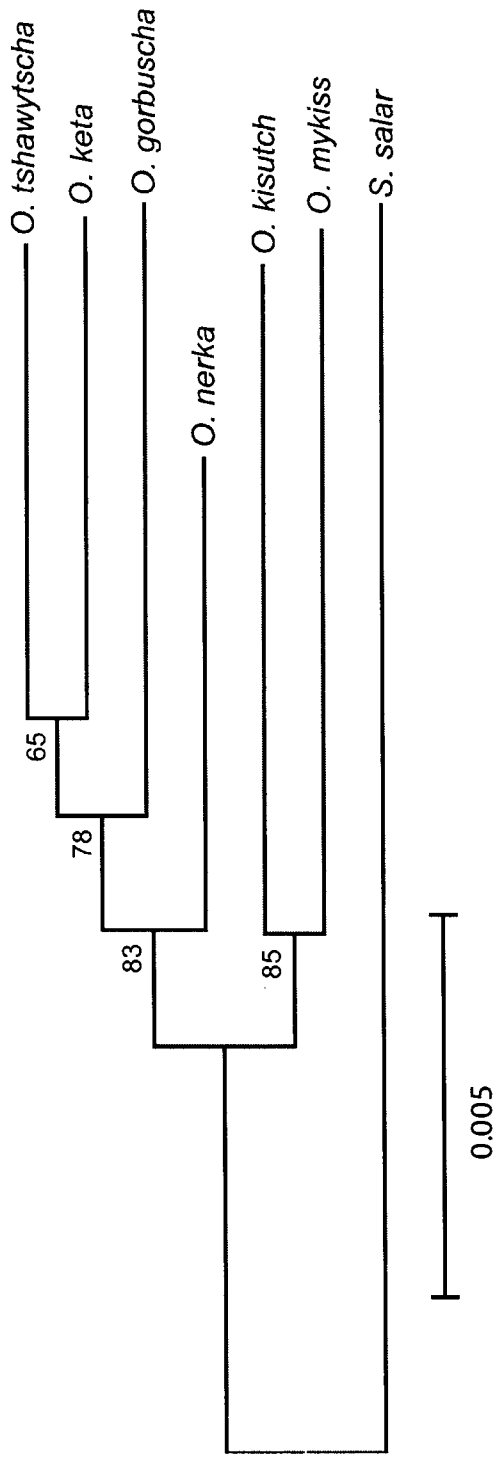
3.8 Salmon evolution specified by opsin sequence

Concatemerized opsin aa sequences described a phylogenetic relationship of the salmonid fishes that did not agree with recent non-coding DNA evolutionary relationships including growth hormone introns (Oakley and Phillips, 1999) and short interspersed repetitive elements (Murata *et al.*, 1993; Murata *et al.*, 1996), as well as allozyme electrophoretic mobility (Osinov and Lebedev, 2000). These established phylogenetic relationships were not supported by similar patterns of opsin sequence phylogeny (Figure 6). Rather, the high degree of sequence similarity in salmonid opsin genes may mask the meta-level phylogeny characteristic of salmonid fishes. An interesting observation from this phylogeny was the relatively high bootstrap values that place *O. mykiss* and *O. kisutch* in a monophyletic group, an alternative salmonid evolutionary relationship than those previously published. The diverse phylogenies of salmonids presented in Figure 5 as well as Figure 6 are demonstrative of the differential evolutionary rates of the visual opsins as compared to the more random mutation that occurs in non-coding DNA. By nature, the opsins are under direct environmental pressure to adapt. The replacements that are maintained in each are a direct result of differences in spectral constraints, be they habitat, mate choice, etc.

3.9 Conclusions

The phylogenetic tree shown in Figure 4 was in agreement with those previously reported (Yokoyama, 2000b). The low bootstrap values found for the internal branches of the *Oncorhynchus* sp. (bootstrap value less than 90) are attributed to the high degree of shared aa identity in the salmon opsin genes. Future MSP and *in vitro* expression-reconstitution-mutagenesis experiments should elaborate any differences in opsin sequence by relating their function to primary aa structure. Future research will be directed at examining opsin genes from other representatives of the genus *Salmo* sp. and the genus *Salvelinus* sp. as well as

Figure 6.
Salmonid phylogeny inferred by concatamer of opsin aa sequence using minimum evolution analysis. Concatamers were made by joining opsin aa sequence end to end in the order LWS-RH1-RH2-SWS1-SWS2. Amino acid sequences were corrected for multiple substitutions by a Poisson correction. Values shown are bootstrap greater than 50% of 1000 replicates. Scale bar values are substitutions per 1000 amino acids.


O. tshawytscha
O. keta
O. gorbuscha
O. nerka
O. kisutch
O. mykiss
S. salar
65
78
83
85
0.005

whitefish and esocids to clarify the opsin evolution of salmonidae and the possible selective pressures acting on their visual systems.

4. Oncorhynchus mykiss Proximal Promoters and Chromatin Immunoprecipitation

4.1 Chapter Introduction

Transcription factor influence on photoreceptor differentiation and opsin expression has been demonstrated in a variety of systems. For instance, the TFs Chx10 and Pax6 facilitate neuronal differentiation into non-photoreceptor neurons but inhibit differentiation into photoreceptors (Belecky-Adams *et al.*, 1997; Toy *et al.*, 2002). Chx10 also appears to regulate retinal progenitor proliferation after upregulation by retinoid orphan B receptor (Chow *et al.*, 1998). Wu *et al.* (2001) has shown that regenerating cones in the goldfish retina express the TF Notch 1 and N-cadherin prior to differentiation.

Mutations in the photoreceptor nuclear receptor/transcription factor (PNR) result in S-cone differentiation suggesting a key role for PNR in S-cone determination (Milam *et al.*, 2002). Similarly, neural retina leucine zipper (NRL) is essential for rod photoreceptor development as the mouse NRL knockout phenotype is an S-cone dominated retina (Mears *et al.*, 2001; Rehemtulla *et al.*, 1996). Other experiments show that NRL and cone-rod homeobox (CRX) act synergistically and that CRX is required for rod opsin expression (Chen *et al.*, 1997; Mitton *et al.*, 2000). Activation of retinoic acid respecters results in an increase in rod photoreceptor differentiation in rat and zebrafish retinae while decreasing cone differentiation (Hyatt *et al.*, 1996; Kelley *et al.*, 1999; Perkins *et al.*, 2002). An earlier study illustrates induction of cone photoreceptor differentiation by TH in rat retinal cell culture (Kelley *et al.*, 1995a). An *in vivo* follow-up study showed that THR-β2 is essential for execution of the M-cone differentiation pathway (Ng *et al.*, 2001). More recent data suggests that THR-β2 and CRX regulate cone opsin expression while PNR suppresses progenitor cell proliferation (Yanagi *et al.*, 2002).

The purpose of the current study was to identify candidate transcription factors that play a role in regulating the SWS1 opsin gene as well as regulate UVS cone apoptosis. Genome-

walking PCR was used to isolate the proximal promoter regions of five retinal opsin genes from rainbow trout (*Oncorhynchus mykiss*). Genome walking is a PCR technique that results in acquisition of genomic fragments upstream of the gene of interest by using reverse primers against known 5' sequence and forward primers against known adapters ligated to blunt end restriction libraries. Putative TF binding sites within each promoter were identified using the web-based MatInspector program and Transfac database (http://transfac.gbf.de/cgi-bin/matSearch/matsearch.pl). Chromatin immunoprecipitation (ChIP) analysis revealed that c-jun and NF-κB bind specifically to the SWS1 opsin promoter *in vivo* in the rainbow trout retina and show no interaction towards the promoters of other opsin types. Chromatin immunoprecipitation is a relatively new protocol where cross-links between TF's bound to DNA are created by a formaldehyde wash of freshly dissected tissue. Following cross linkage the DNA-TF complexes are sonicated and DNA bound to the TF of interest is isolated by subsequent antibody/protein A immunoprecipitation. Reversal of cross links followed by phenol chloroform DNA extraction and promoter specific PCR proves TF binding to the promoter region of interest.

The data presented in this chapter appears in a submitted manuscript under review titled "NF-κB and c-jun exhibit exclusive binding to the SWS1 opsin proximal promoter in rainbow trout (*Oncorhynchus mykiss*)." This manuscript was submitted in May of 2003 and is currently being revised.

4.2 Genome Walking PCR

Genomic DNA was isolated from the liver of rainbow trout by incubation at 65°C overnight in digestion buffer (100mM NaCl/10mM Tris-Cl pH 8.0/25mM EDTA/0.5% SDS/0.1mg/ml Proteinase K) followed by phenol-chloroform extraction and ethanol precipitation (Gross-Bellard *et al.*, 1973). Genome walking PCR was completed using the Universal Genome Walker kit (Clontech-BD Biosciences) as per the manufacturer's instructions. Briefly, four

genomic libraries were created by blunt-end digestions of 2.5 μg genomic DNA (0.1 μg/μl) with four restriction enzymes, Dra I, EcoR V, Pvu II, and Stu I, at 37°C overnight in a total volume of 100 μl. Restriction digests were phenol-chloroform extracted, ethanol precipitated and re-suspended in TE (10mM Tris-Cl pH 8.0/1mM EDTA). An adapter oligonucleotide (48mer – Table 4) was ligated to each restriction library using T4 DNA ligase at 16°C overnight. Ligation reactions were diluted ten-fold in TE to a final volume of 80 μl.

Nested PCR reactions were carried out for each opsin promoter with primers shown in Table 4. Forward primers supplied by the manufacturer are complementary to the 48mer adapter ligated to the genomic libraries denoted AP1 and AP2. Reverse nested gene-specific primers were designed to the 5' end of the encoded transcript of rainbow trout opsin genes (SWS1, SWS2, RH1, RH2, and LWS). First round PCR was carried out using 1 μl of the diluted genomic library as follows, an initial 4 minute denaturation at 94°C followed by 30 cycles of denaturation at 94°C for 60s, primer annealing at 63°C for 45s and product extension at 72°C for 120s. Second round PCR used 1 μl of the first PCR reaction as template under identical conditions with the exception of an annealing temperature of 67°C and a final 5 minute incubation at 72°C to facilitate T/A cloning. PCR products were cloned using the pGem®-T Easy vector (Promega) and ElectroMAX DH10B™ *E. coli* (Gibco BRL®). Standard dideoxy sequencing methods employed IRDye™700 and IRDye™800 labeled primers and was performed on a NEN® Global IR2 DNA Sequencer System (Li-cor). Three clones for each promoter PCR fragment were picked and sequenced from independent PCR reactions to ensure a lack of PCR error in the sequence data. Putative TF binding sites within each promoter region were identified by searching the Transfac database using the MatInspector V2.2 program (http://transfac.gbf.de/cgi-bin/matSearch/matsearch.pl). TF binding sites were identified based on a Core similarity, 4-5 bp

Table 4.
PCR primer and adapter sequences used for genome walking and chromatin immunoprecipitation analysis.

Primer Name	Outer Primer Sequence (5' - 3')	Inner Primer sequence (5' - 3')
Genome walk adapter specific	GTAATACGACTCACTATAGGGC	ACTATAGGGCACGCGTGGT
SWS1 genome walk	ATTAACCCTCACTAAATGCTGGGGA	CATTATGCTGAGTGATATCTTTT
SWS2 genome walk	AATCCCCACTACCAAGGTTTCCCC	CGCGGATCCGAACACTGCGTTTGCTG
LWS genome walk	TCTCTCGTTGTATCTTGGTTTTGTC	TTGGACGCTTTTTCTCTGTCTTG
RH2 genome walk	GAACAAAGCAGATCAGGAAGAACG	TTGCTTCCTTCTGTGCCGTTCT
RH1 genome walk	CGTGAGCCTTTGTCGTTTCCGG	CTTGGAGGCAGTGGTGGAGGC
	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')
SWS1 NF-κB ChIP assay	GCAAGGCTCTCTATGTCGGCTC	CCTTACTTTGTCGGGCGCAG
SWS1 c-jun/AP-1 ChIP assay	CTGCGCCCGACAAAGTAAGG	CCTCAAATGGGCTGACCTTAG
SWS2 Chip assay	GTTTGTGCGATTCAGTCCTC	CACGGGACGAGCATTTGACC
LWS Chip assay	GAATTGTCTGGTAAAGCGG	TTGGACGCTTTTTCTCTGTCTTG
RH2 Chip assay	GTTTTGGGAATTTTCATGTCT	CAGTGGATGTCCAATGAATGC
RH1 Chip assay	GGTGGTGCAGCTATTCTACTTG	GTTGCGGTCTTGCGGTGGCG
	Genome walking blunt end adapter (5'-3')	
	GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT	

of sequence known to bind TF, and a Matrix similarity, variable region of sequence flanking the core of the binding site. The MatInspector searches performed herein employed a core similarity of 1.0 (exact match) and a matrix similarity of 0.96 as identification thresholds to increase the likelihood of recognizing binding sites relevant to an *in vivo* state. Although the MatInspector program is useful as a tool for predicting TF binding sites further experimental qualification of promoter characteristics must be completed to validate TF function.

4.3 Western Blot and Immunohistochemistry

Retinae were dissected from dark-adapted parr rainbow trout and homogenized in 500µl of ice-cold buffer containing 30mM Tris-Cl pH 7.5, 10mM EGTA, 5mM EDTA, 250 mM sucrose, 1% Triton X-100, supplemented with 15 µg/ml aprotinin, 5 µg/ml leupeptin, 5 µg/ml pepstatin, 1 mM sodium orthovanadate and 1 mM PMSF. The homogenate was vortexed for 10 sec and centrifuged 14,000g at 4°C for 10 min, after which the supernatant was collected and stored at -80°C. Protein quantification was performed using a DC Protein Assay Kit II and the manufacturer's protocol (BioRad).

Homogenate was diluted four-fold in Red Loading Buffer (New England Biolabs Inc.), boiled for 3 minutes and proteins were resolved on a 12.5% SDS-polyacrylamide gel. Proteins were electroblotted onto BioTrace™ NT Membrane (Pall Gelman Laboratory) at 100 volts for 1 hour.

Immunodetection of membrane-bound protein was performed at room temperature with gentle agitation. Membranes were pre-blocked in a solution of 5% BSA, PBS, and 0.05% Tween and washed in PBS/0.05% Tween. After blocking, membranes were incubated for 1hr with 0.2 µg/ml c-Jun rabbit polyclonal antibody diluted in 1% BSA, PBS, 0.05% Tween (Delta Biolabs). The membranes were then washed and bound primary antibodies detected by incubating for 1 hr

with goat anti-rabbit antibodies conjugated to alkaline phosphatase (1:2000 dilution Vector Laboratories Inc.). After a final wash, alkaline phosphatase activity was detected with BCIP/NBT according to the manufacturer's protocols (Invitrogen). C-jun immunoblots were repeated in triplicate to ensure constant detection of protein signal at a particular size.

Retinal cryosections (12μm) were taken on a Leica CM1850 cryostat from rainbow trout retina fixed in 4% formaldehyde/1x PBS. Sections were equilibrated in 1x PBS/0.05%Tween with inactivated horse serum (HS) for 30 minutes at room temperature. Primary polyclonal antibodies for NF-κB (Stressgen) and c-jun (Delta Biolabs) were added to a final concentration of 0.5 μg/ml and incubated overnight at 4°C. Sections were washed three times for 30 minutes in PBS/Tween-HS and AMCA conjugated mouse anti-rabbit secondary antibody (Vector Laboratories Inc.) applied at 1/1000 dilution for one hour at room temperature. Sections were washed three times for 15 minutes in PBS/Tween-HS and then labeled with lectin peanut agglutinin conjugated Alexa Fluor (diluted 1/1000 in PBS/Tween Molecular Probes). Immunoreactive regions were visualized on a Zeiss Axioskop 2 epifluorescent microscope. Immunodetection of NF-κB and c-jun on retinal sections was repeated in triplicate to ensure constant signal localization within the tissue.

4.4 Chromatin Immunoprecipitation (ChIP)

ChIP experiments were based on the protocols of Kuo and Allis (1999) and Weinmann and Farnham (2002) repeated in triplicate from independent tissue dissections. Retinae were dissected from twelve juvenile rainbow trout to form three independent tissue preparations (four fish per preparation, average wet mass of whole fish - 6.42g, 6.67g, and 7.21g). Retina were lightly dissociated in 2 ml 1x PBS using a tissue homogenizer. Formaldehyde was added to the homogenate to a final concentration of 1% v/v. The cellular suspension was incubated for 15 minutes at room temperature on a nutating platform. The crosslinking reaction was stopped by

adding glycine to 0.125 M with incubation at room temperature for 5 minutes. Cells were harvested via centrifugation 5000g for 5 minutes at 4 °C and then rinsed twice with cold 1x PBS. Centrifugation after rinsing left a cell pellet that was re-suspended in cell lysis buffer (5mM PIPES/ 85mM KCl/ 0.5% NP40 pH 8.0 supplemented with 10µl/ml PMSF, 1µl/ml leupeptin, and 1µl/ml aprotinin). Cells were incubated 15 minutes on ice and then centrifuged at 5,000*g* for 5 minutes at 4°C to pellet nuclei. Pelleted nuclei were re-suspended in nuclei lysis buffer (50mM Tris-Cl pH 8.1/1% SDS/10 mM EDTA + supplemented with 10µl/ml PMSF, 1µl/ml leupeptin, and 1µl/ml aprotinin) and incubated on ice 20 minutes. Glass beads (0.1 g, Sigma G-1277) were added to each sample and sonicated so that chromatin was sheared to an average length of 600 bp. Samples were centrifuged 14,000g for 10 minutes at 4°C and the supernatants transferred to new tubes and re-centrifuged. Each resulting supernatant was split into three aliquots, one for c-jun analysis, one for NF-κB analysis, and one for a whole-cell control. Each aliquot was pre-cleared with protein A agarose and incubated on a nutating mixer for 15 minutes. Samples were centrifuged for 10 minutes at 14,000g. Supernatants were transferred to new tubes and their final volumes adjusted to 500 µl with a dilution buffer (0.01% SDS/1.1% Triton-X 100/1.2 mM EDTA/16.7 mM Tris-Cl pH 8.1/167mM NaCl supplemented with 10µl/ml PMSF, 1µl/ml leupeptin, and 1µl/ml aprotinin). Antibody (1 µg) was added to the appropriate sample (none in the whole cell control) followed by incubation overnight at 4°C on a nutating platform. Antibodies for NF-κB and c-jun were identical to those used in immunoblots and immunohistochemistry.

Three aliquots of protein A agarose (1ml each) were pre-equilibrated 1:1 (v:v) with lysis buffer and collected by centrifugation at 14,000g for 15 s. Each chromatin sample was combined with 20 µg of calf thymus DNA, an aliquot of agarose A and incubated at room temperature with rocking for 10 minutes. Samples were centrifuged 14,000g for 15s and the supernatant discarded

from the c-jun and NF-κB samples and saved from the no antibody whole cell control. The saved whole cell control supernatant contents act as a positive control for the PCR assay of the ChIP protocol. The pellet from the whole cell control condition underwent processing identical to the experimental samples as the contents act as a negative control in the PCR assay of the ChIP protocol.

The protein A agarose pellet from each sample was washed twice in 1.4 ml dialysis buffer (2 mM EDTA/50 mM Tris-Cl pH 8.0/0.2% sarkosyl supplemented with 10μl/ml PMSF) and eight times in 1.4 ml of immunoprecipitation wash buffer (100mM Tris-Cl pH 9.0/500 mM LiCl/1% NP40/1% deoxycholic acid supplemented with 10μl/ml PMSF). Each wash was incubated at room temperature for 3 minutes with rocking and then centrifuged 3 minutes at 14,000g. After the last wash the antibody-chromatin complexes were eluted from the protein A agarose pellet with the addition of 300 μl of elution buffer (50 mM $NaHCO_3$/1% SDS) followed by a 15-minute vortex and centrifugation at 14,000g for 3 minutes. Reversal of formaldehyde cross-links was accomplished by transferring supernatants to new tubes and treating with 1 μg of DNase-free RNase and NaCl to a final concentration of 0.3 M for 10 minutes at 67°C. Previously saved supernatant from the whole cell control incubation was treated in a similar manner. Genomic DNA within each sample was then ethanol precipitated at -20°C overnight and re-suspended in 100 μl TE buffer. De-proteination of the DNA samples was performed using proteinase K digestion at 45°C for 2 hours followed by phenol/chloroform extraction. Samples were ethanol precipitated a second time and re-suspended in TE buffer. Each experimental sample as well as the whole cell positive control was diluted to a final concentration of 5 ng/μl as indicated by spectrophotometric absorbance of the sample at 260 nm. Whole cell negative control was re-suspended in 100 μl of TE as spectrophotometry readings from this sample were below detection.

The chromatin immunoprecipitation protocol described above was repeated in triplicate on liver and brain tissue (pineal removed) from dissections of rainbow trout of similar size (four fish per preparation, average wet mass of whole fish - 6.59g, 6.33g, and 6.98g). This extra-retinal tissue control was necessary to test whether the TF's selected for investigation would bind the promoter of retinal opsins as inhibitors of transcription in non-retinal cells.

4.5 PCR analysis of ChIP

Primers complementary to the SWS1 opsin proximal promoter were created to produce 250 bp DNA fragments covering the putative AP-1 and NF-κB binding sites within the promoter. PCR analysis was carried out on DNA template prepared from each specifically immunoprecipitated chromatin sample (retina, liver and brain) using the corresponding primer set as well as for the whole cell sample positive controls and total input negative controls. Whole cell sample positive control (clean sonicated DNA) and total input negative control (samples with no primary antibody added) were also used as template for PCR. The forward primer from the AP-1-specific PCR and the reverse primer from the NF-κB-specific PCR were also used to ensure that the length of sonicated DNA within each sample was at least 600 bp. Primers complementary to the proximal promoters of other opsins were created to produce DNA fragments of approximately 550 bp, upstream of the ATG start codon. Each PCR reaction used 1 μl of template from the final ChIP DNA suspensions. Promoter-specific PCR was carried out under the following conditions: initial denaturation of 94°C for 4 minutes followed by 30 cycles of denaturation 94°C for 45s, primer annealing at 63°C and product extension at 72°C and ending with a 5 minute 72°C incubation. Regions of each opsin promoter targeted for amplification are shown in Figure 7.

A 350 bp fragment of the SWS1 opsin intragenic region was used as a control to ensure that the ChIP protocol did not selectively isolate the SWS1 opsin region. No putative AP-1 or

NF-κB binding sites were observed in the target SWS1 opsin coding region. PCR reactions were repeated in triplicate from each independent ChIP preparation to ensure signal strength in the PCR assay was constant for each promoter region analyzed. Amplified PCR products from the experimental conditions (SWS1 opsin promoter) and positive controls (remaining promoters) were cloned and sequenced as previously described to ensure that specific amplification of opsin promoter sequences occurred. All PCR primers and adapter sequences from genome walking and ChIP-PCR experiments are shown in Table 4. Procedures described herein were in accordance with the guidelines established by the Canadian Council on Animal Care. The University of Victoria Animal Care Committee approved all experimental protocols.

4.6 Results

Proximal promoter regions for each of the five photoreceptor opsins was acquired by genome walking PCR from rainbow trout genomic DNA. The identity of each promoter was confirmed by alignment of the 3' end of each PCR product sequence with the 5' coding region of the cDNA sequence reported in Allison *et al.* (2003) (Accession Nos. AF425072-AF425076). The region of sequence overlap between 3' promoter and 5' coding region where PCR primers were designed was an exact match for each opsin. The proximal promoter regions were submitted to Genbank (Accession numbers: AY305659-AY305663). A comparative search of the opsin promoter sequences with MatInspector revealed several putative TF binding sites. Positions of the putative binding sites are shown in Figure 7 and previously reported function of each TF is shown in Table 5. Two putative TF binding sites identified in the SWS1 opsin promoter were chosen for further analysis, AP-1 (c-jun) and NF-κB. These binding sites received matrix scores for the SWS1 promoter MatInspector analysis of 0.996 and 0.967 respectively. They have been previously described in other species as having a role in photoreceptor apoptosis. Although the AP-1 (c-jun) sites were also identified in the RH1 opsin promoter region, the matrix

Figure 7.
Putative transcription factor binding sites on rainbow trout opsin proximal promoter sequences (A) SWS1 Opsin promoter – 676 bp. Orange bar represents the NF- κB specific ChIP assay PCR product; the blue bar represents the AP-1/c-jun specific ChIP assay PCR product. (B) SWS2 Opsin promoter – 680 bp. (C) LWS Opsin promoter – 620 bp. (D) RH2 Opsin promoter – 790 bp. (E) RH1 Opsin promoter – 850 bp. Black bars above promoter blocks represent PCR products created in ChIP assay. The black bar PCR product above the SWS1 opsin promoter is created by the forward primer of the NF-κB specific PCR assay and the reverse primer of the AP-1/c-jun specific PCR assay. Scale bar in bottom right corner represents 100 bp.

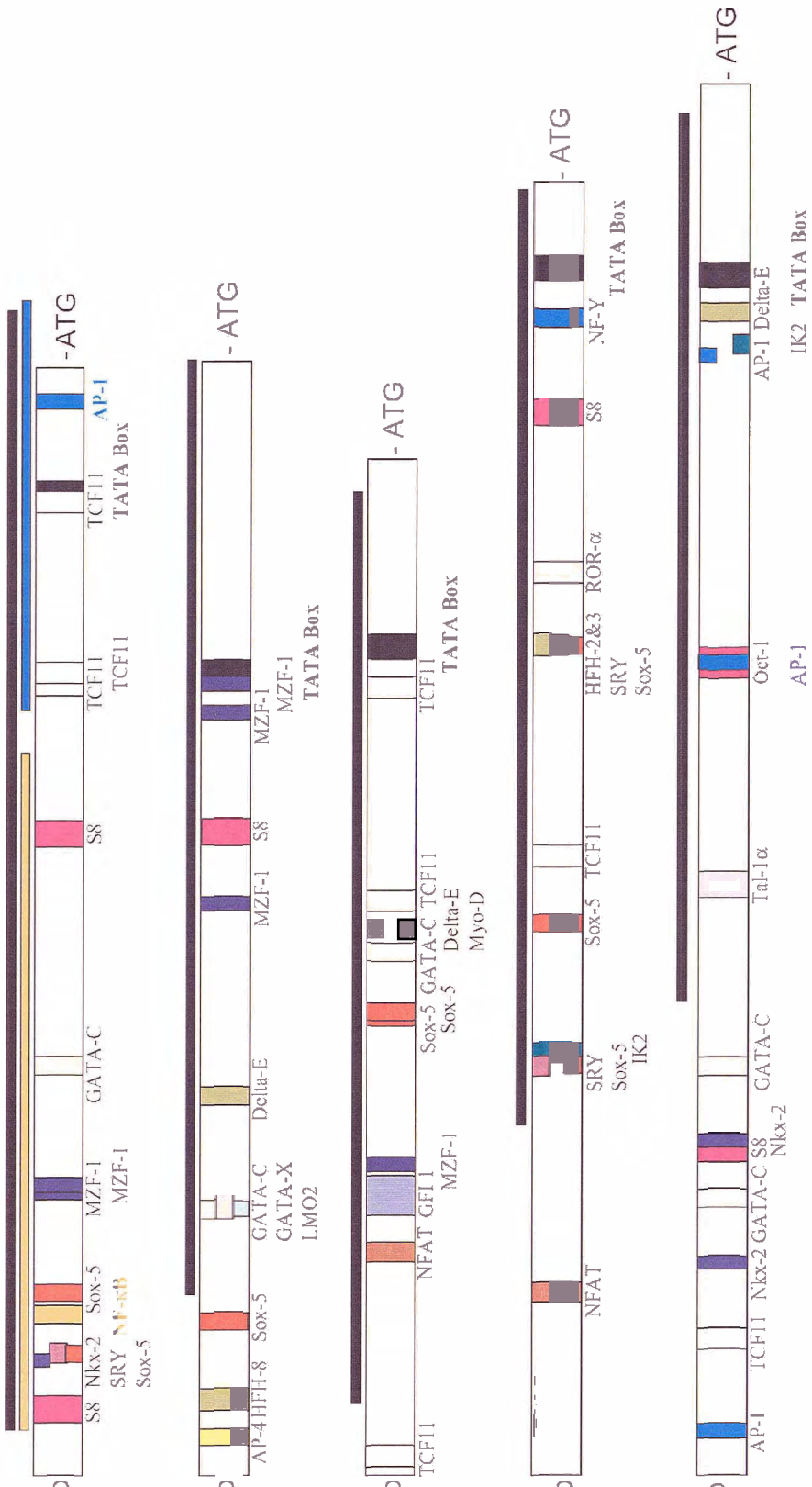

A -676bp
S8
Nkx-2
Sox-5
SRY
Sox-5
MZF-1
MZF-1
GATA-C
S8
TCF11
TCF11
TCF11
AP-1
TATA Box
- ATG
B -680bp
AP-4
HFH-8
Sox-5
GATA-C
GATA-X
LMO2
Delta-E
MZF-1
S8
MZF-1
TATA Box
- ATG
C -620bp
TCF11
NFAT
GFI1
MZF-1
Sox-5
Sox-5
GATA-C
TCF11
Delta-E
Myo-D
TCF11
TATA Box
- ATG
D -790bp
NFAT
SRY
Sox-5
IK2
Sox-5
TCF11
HFH-2&3
SRY
Sox-5
ROR-α
S8
NF-Y
TATA Box
- ATG
E -850bp
AP-1
TCF11
Nkx-2
GATA-C
S8
Nkx-2
Tal-1α
Oct-1
AP-1
AP-1
Delta-E
IK2
TATA Box
- ATG

Table 5.
Putative transcription factor binding site functions found within rainbow trout opsin proximal promoters.

Factor	LWS	RH2	SWS1	SWS2	RH1	Factor description
GATA Factor	+	+	+	+		Implicated in internal organ formation (Patient and McGhee, 2002)
SRY/Sox-5	+	+	+	+		Sex determination, pattern, and nervous system development (Prior and Walter, 1996)
S8 (Prx-2)		+	+	+	+	Implicated in crainiofacial/ teeth development (ten Berge *et al.*, 1998; ten Berge *et al.*, 2001)
TCF II	+	+	+		+	Downstream effectors of Wnt signaling essential for neural differentiation (Brantjes *et al.*, 2002)
Mzf-1	+		+	+		Protects cultured leukemia cells from retinoic acid induced apoptosis (Robertson *et al.*, 1998)
Delta-E	+			+	+	Implicated in the differentiation of the lens (Cvekl *et al.*, 1995)
AP-1			+		+	Important switch necessary for apoptosis in all cell types (Shaulian and Karin, 2002)
HFH		+		+		Studied in respiratory function and development (Warburton *et al.*, 2000)
Ik2		+			+	Ikaros zinc finger TF regulates lymphocyte differentiation (O'Riordan and Grosschedl, 2000)
NFAT	+	+				Nuclear factor of activated T cells exerts effects in several tissues synergistically with AP-1 (Horsley and Pavlath, 2002)
Nkx-2			+		+	Identified in cardiogenesis, expression also noted in the forebrain (Harvey, 1996)
Oct-1					+	Photo oxidative stress down regulates DNA binding of this TF (Hafezi *et al.*, 1999)
Tal-1β					+	Central in hematopoiesis, one of the stem cell leukemia transcription factors (Bloor *et al.*, 2002)
MyoD	+					Central TF in muscle differentiation (Brand-Saberi and Christ, 1999)
ROR - α		+				Retinoid-related receptor co-activated by THRs (Jetten *et al.*, 2001)
NF-κB			+			Photo-oxidative stress down regulates expression, results in photoreceptor apoptosis (Krishnamoorthy *et al.*, 1999)
Gfi-1	+					Zinc finger transcriptional repressor, regulates IL-2 expression (Zweidler-Mckay *et al.*, 1996)
Lmo2				+		Angiogenic TF complexes with GATA factors (Crispino and Orkin, 2002)
AP-4				+		Activator protein complex 4 binds Ig-κ E-box promoter elements (Aranburu *et al.*, 2001)

Table footnote: Presence of a given transcription factor binding site is indicated by + in the column specifying the opsin promoter type.

similarity score was lower (0.969 and 0.970) than the SWS1 AP-1 (c-jun). Therefore, we considered the SWS1 AP-1 (c-jun) site to be a more reliable report than the RH1 sites.

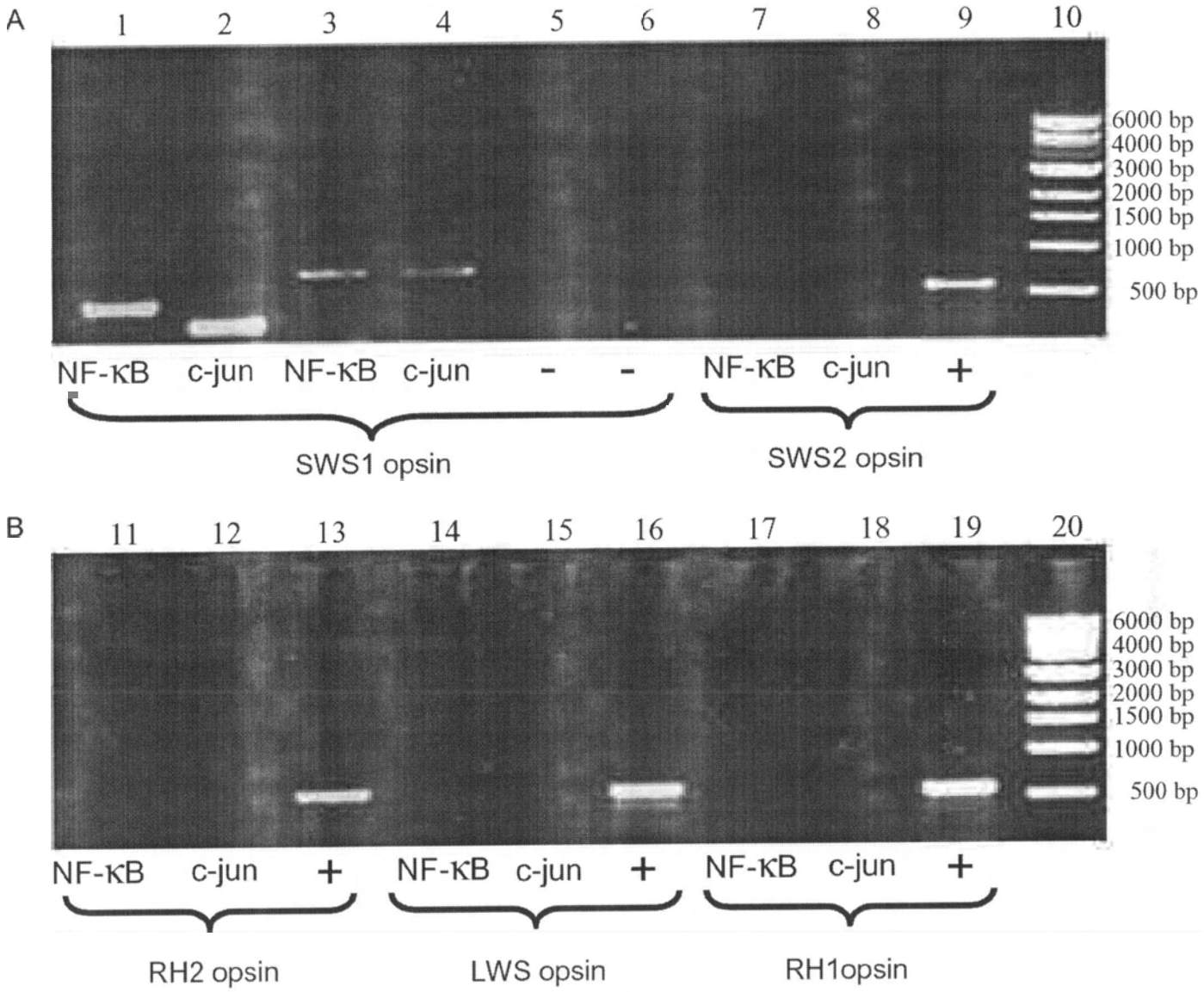
Chromatin immunoprecipitation and PCR analyses reveal that c-jun and NF-κB proteins bind specifically to the SWS1 opsin proximal promoter but not the SWS2, RH1, RH2, or LWS promoters (representative results presented in Figure 8). SWS1 opsin promoter ChIP analysis produced amplicon DNA bands of expected size for both c-jun (250 bp) and NF-κB (413 bp). SWS1 opsin promoter PCR was also performed using the NF-κB forward primer and the c-jun reverse primer in both the c-jun and NF- κB ChIP assays. This product (683 bp) ensured that sonication of genomic DNA had not resulted in DNA fragments below 500 bp and that efficient detection of TF-associated DNA by PCR would not be affected. Analyses of promoters from each of the remaining opsins showed no promoter-specific PCR product associated with c-jun or NF- κB. Sonicated genomic DNA used as PCR positive control with opsin specific promoters resulted amplicons of expected size (SWS2 – 572bp, LWS – 556bp, RH2 – 571 bp, RH1 – 543bp). The amplicon sequences acquired from the ChIP PCR procedure were identical to their respective opsin promoter sequences indicating that, although the PCR was successfully targeting opsin promoters, only SWS1 binds the TFs of interest. No product was observed in the intragenic control PCR when experimental template was used, although target sequence was amplified, cloned and sequenced when using the primer set on positive control template (data not shown).

Chromatin immunoprecipitation and PCR analysis of brain and liver tissue for both c-jun and NF-κB produced no PCR product in any of the experimental samples. Whole cell control yielded bands of expected size and sequence similar to the positive control shown in Figure 8 while the total input control yielded no identifiable PCR product.

Figure 8.
Visualization of PCR products resulting from c-jun and NF-κB ChIP-PCR assay

(A) Lanes 1-6 PCR products amplified from the UV opsin promoter. Lane 1) NF-κB specific assay SWS1 promoter PCR product (orange bar diagrammed in Figure 7a); Lane 2) C-jun specific assay SWS1 promoter PCR product (blue bar diagrammed in Figure 7a); Lane 3) NF-κB assay alternate primer set produces expected SWS1 PCR product (black bar diagrammed in Figure 7a); Lane 4) C-jun assay alternate primer set produces expected SWS1 PCR product (black bar diagrammed in Figure 7a); Lanes 5 & 6) Total input negative control for NF-κB and c-jun assays, respectively; Lanes 7 & 8) SWS2 opsin promoter PCR products from NF-κB and c-jun assays, respectively; Lane 9) Sonicated DNA positive control for the SWS2 specific PCR (whole cell control supernatant); Lane 10) 1 Kb DNA ladder (NEB).

(B) Lanes 11-12) LWS opsin promoter PCR products from NF-κB and c-jun assays, respectively; Lane 13) Sonicated DNA positive control for the LWS specific PCR; Lanes 14 & 15) RH2 opsin promoter PCR products from NF-κB and c-jun assays, respectively; Lane 16) Sonicated DNA positive control for the RH2 specific PCR; Lanes 17 & 18) RH1 opsin promoter PCR products from NF-κB and c-jun assays, respectively; Lane 19) Sonicated DNA positive control for the RH1 specific PCR; Lane 20) 1 Kb DNA ladder (NEB).


A
1 2 3 4 5 6 7 8 9 10
6000 bp
4000 bp
3000 bp
2000 bp
1500 bp
1000 bp
500 bp
NF-κB c-jun NF-κB c-jun - - NF-κB c-jun +
SWS1 opsin
SWS2 opsin
B
11 12 13 14 15 16 17 18 19 20
6000 bp
4000 bp
3000 bp
2000 bp
1500 bp
1000 bp
500 bp
NF-κB c-jun + NF-κB c-jun + NF-κB c-jun +
RH2 opsin
LWS opsin
RH1opsin

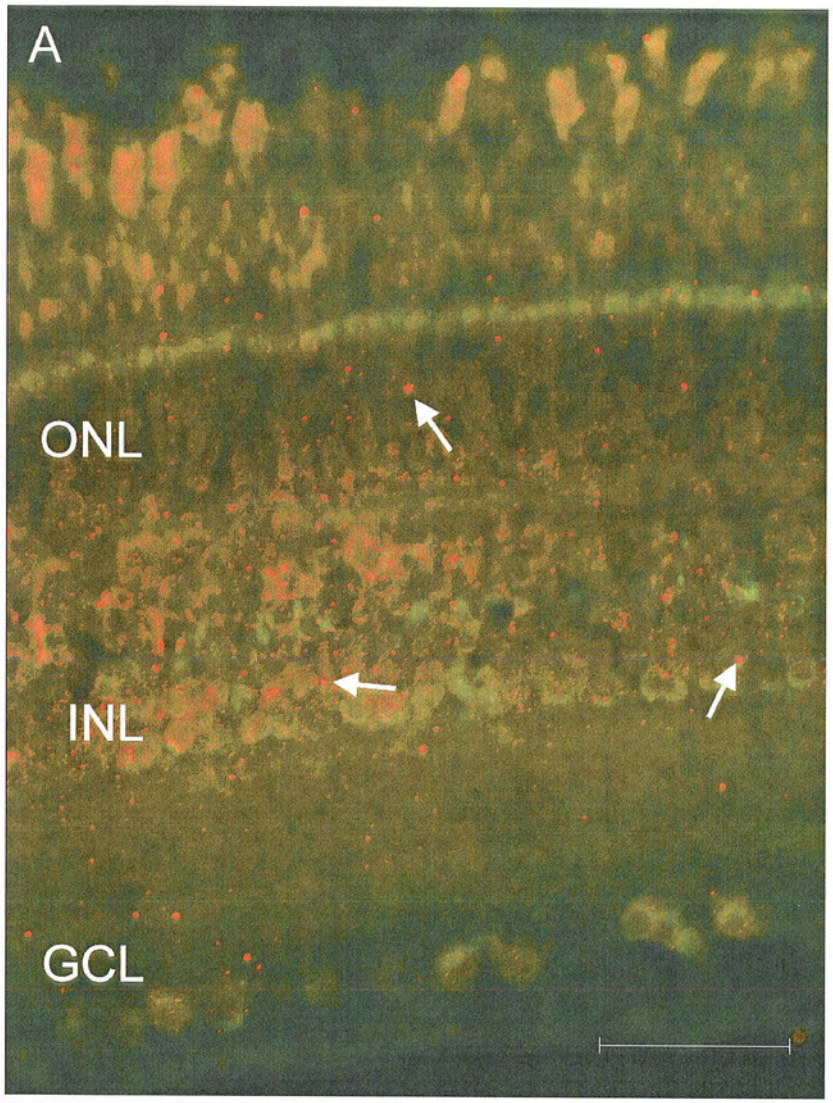
To further characterize trout proteins immunoreactive with the c-jun and NF- κB antibodies, immunoblot and immunohistochemistry analyses of trout retinal tissue were performed. C-jun antibody exhibited binding in all of the nuclear layers of the rainbow trout retina (Figure 9). Immunoblot of the c-jun antibody against a retinal protein homogenate revealed distinct bands at 24 and 26 kDa (Figure 10). Immunoblotting of the NF- κB antibody to rainbow trout retinal protein revealed no specific band (data not shown). Immunohistochemistry of NF-κB on normal rainbow trout retinal sections showed labeling in all layers of the retina (Figure 11).

4.7 Discussion

We have illustrated differential binding of two transcription factors, c-jun and NF-κB, in the promoter regions of the opsin genes of rainbow trout. In particular, we have demonstrated that of the opsin promoters in rainbow trout c-jun and NF-κB specifically bind the SWS1 opsin promoter only in the retina. To our knowledge, this represents the first report using the ChIP technique to identify chromatin-associated proteins within retinal tissue from any organism. We observe evidence of c-jun and NF-κB immunoreactivity in all of the nuclear layers of the rainbow trout retina. This includes the outer nuclear layer where photoreceptor nuclei are located. C-jun labeling that appeared near the rod outer segments is immunoreactive retinal pigmented epithelium tissue. Immunodetection of c-jun on retinal protein immunoblots are of the appropriate size. The detection of two bands is likely due to antibody cross-reactivity between c-jun and another jun protein (junD, junB). It is also possible that gene duplication of c-jun in salmonids resulted in two isoforms detected by immunoblot. Limited detection of NF-κB on immunoblots may be due to inability of the antibody to access the NF-κB epitope on membrane-bound denatured protein. This would result in detection of the NF-κB with ChIP and on retinal cryosections, where TFs are not denatured, but not immunoblot. C-jun and NF-κB detection not

Figure 9.
Immunohistochemistry of c-jun antibody on rainbow trout retinal cryosections

(A) Immunohistochemistry of c-jun antibody produces label in all nuclear layers of rainbow trout retina (arrows). (B) Negative control in the right panel shows lack of non-specific labeling by secondary antibody. Green label is lectin peanut agglutinin conjugated with Alexa Fluor. Scale bars indicate 50 μm.


A
ONL
INL
GCL

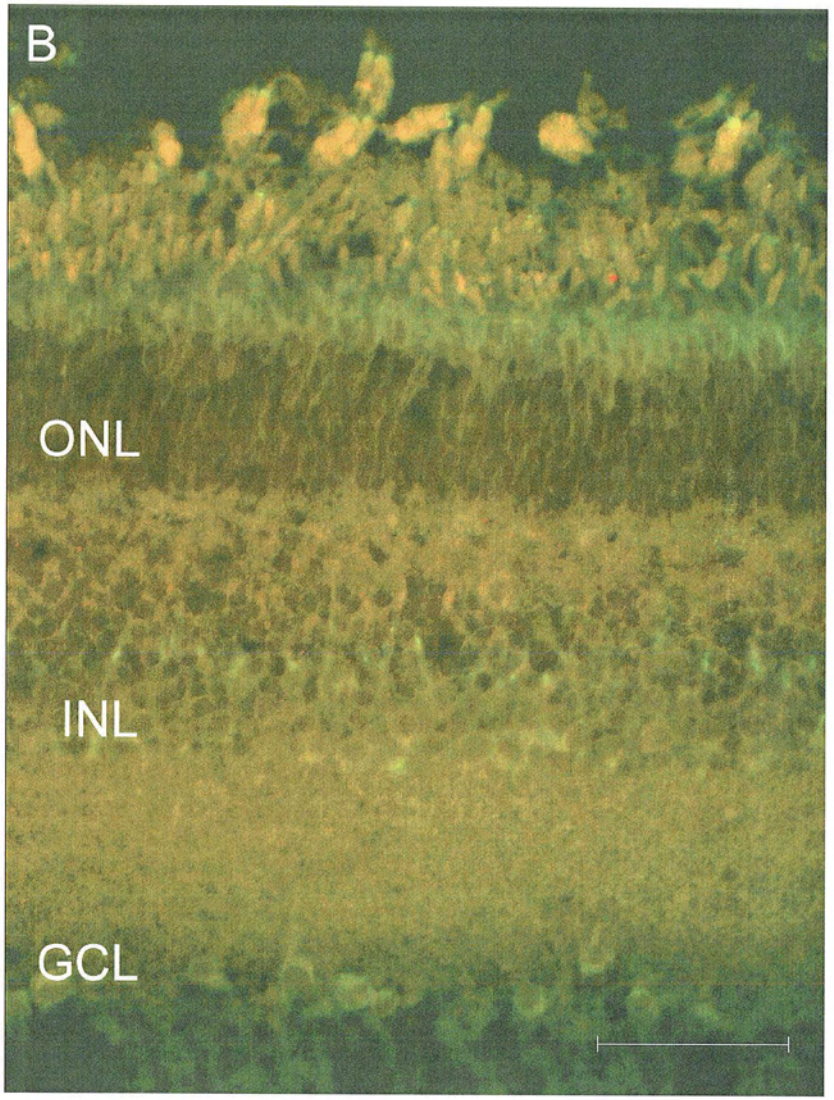

B
ONL
INL
GCL

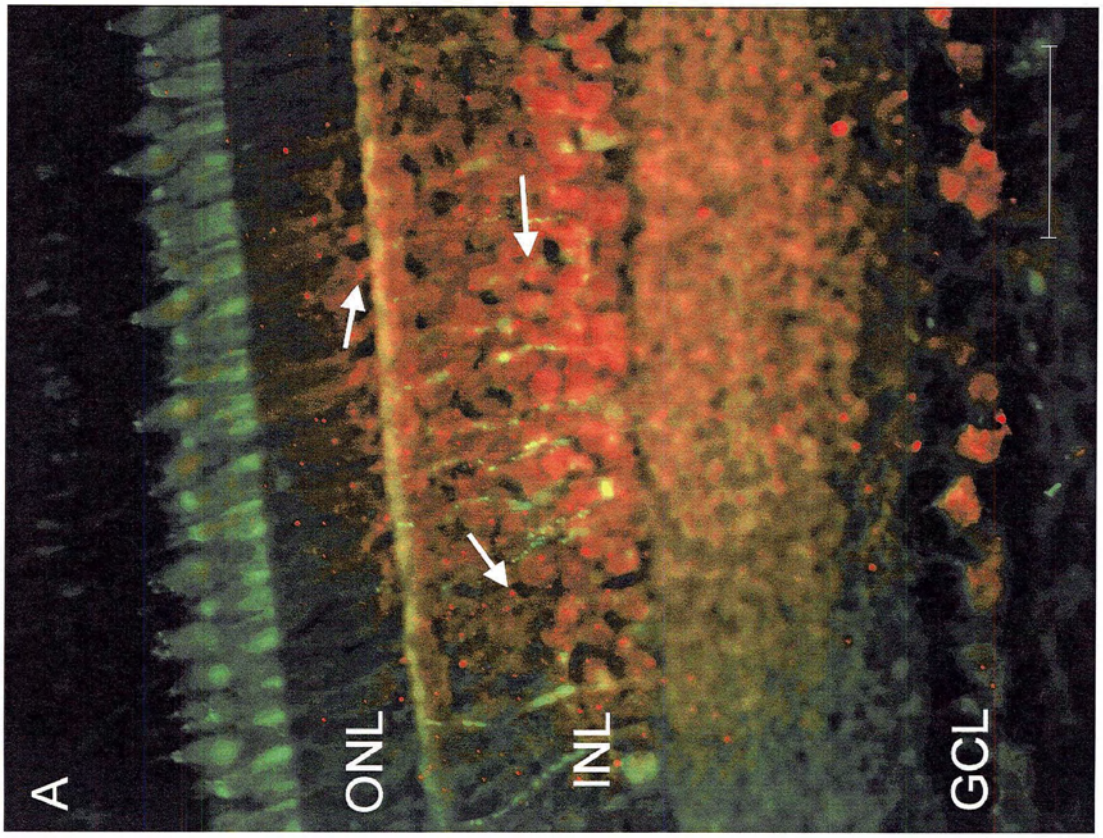
Figure 10.
Immunoblot of c-jun antibody to rainbow trout retinal protein.

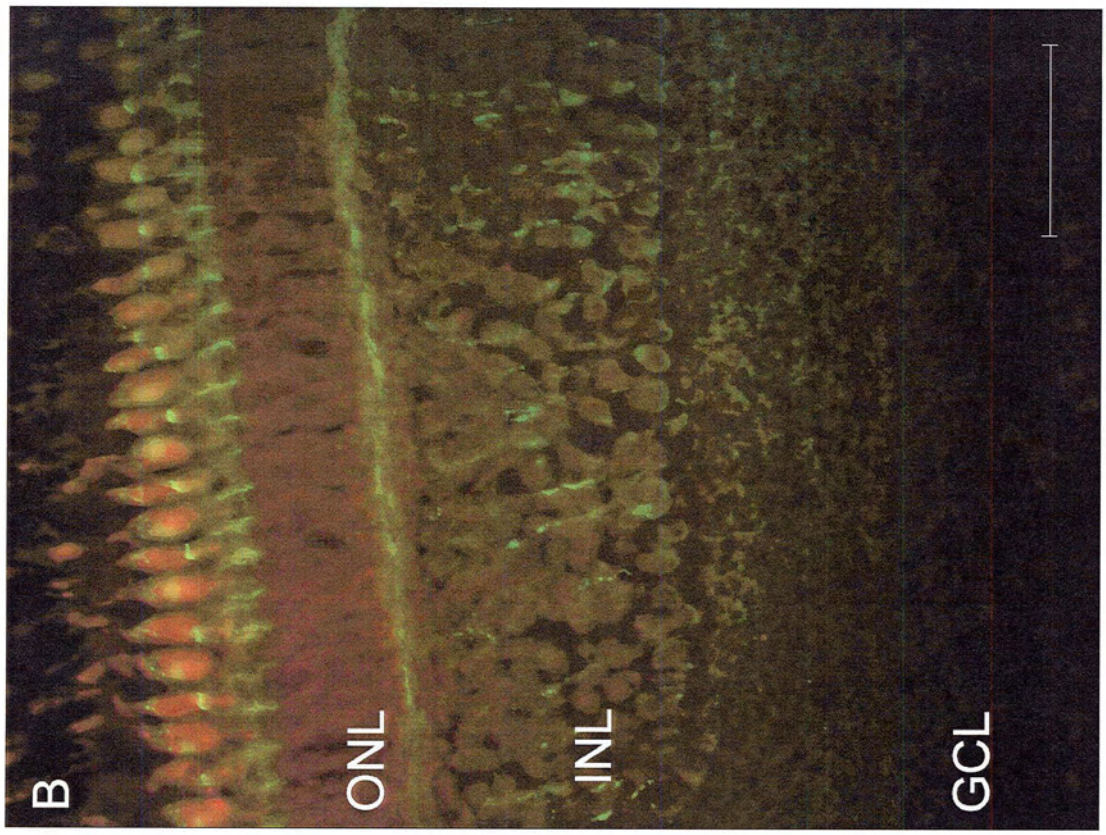
Immunoblot of c-jun antibody to rainbow trout retinal protein extract reveals two bands at 24 and 26 kDa.


47.5 kDa
32.5 kDa
25 kDa
16.5 kDa
6 kDa

Figure 11.
Immunohistochemistry of NF-κB antibody on rainbow trout retinal cryosections.

(A) Immunohistochemical labeling of NF-κB antibody (red) revealed labeling in all nuclear layers of rainbow trout retina (arrows). (B) Negative control in the right panel showed a lack of non-specific labeling by secondary antibody. Green label is peanut agglutinin conjugated with Alexa Fluor. Scale bars indicate 50 μm.


A
ONL
INL
GCL


B
ONL
INL
GCL

observed in the intragenic PCR control indicated that the genic region containing the SWS1 opsin gene was not preferentially isolated during the chromatin immunoprecipitation. The lack of opsin promoter amplification in the extra-retinal tissue ChIP assays indicated that c-jun and NF-κB are not acting as inhibitors of opsin transcription in other cell types. Even with this result we can not entirely conclude that non-photoreceptor cells are resulting in the ChIP assay results shown in Figure 8.

It should be noted that although the MatInspector program identified putative binding sites for AP-1 in the RH1 promoter the ChIP assay did not detect c-jun protein association with the promoter. The MatInspector program, while a useful algorithm for projecting possible TF binding sites, is often unreliable and results must be confirmed through experimental procedure. Thus, the AP-1 binding sites predicted in the RH1 promoter may reflect binding sites for NRL or other related factors, known to bind AP-1-like sites and influence RH1 expression in mammals (Mani *et al.*, 2001; Mitton *et al.*, 2000). Comparisons of SWS1 cone opsin proximal promoters from other species was not viable as genome annotations from closely related species such as *D. rerio* and *T. rubripes* are not complete and do not describe the contigs containing the SWS1 opsin promoter. The human SWS1 opsin proximal promoter (850 bp) shows no putative binding sites for AP-1 or NF-κB when using MatInspector V2.2 with the core and matrix similarity values defined in the methods.

The TFs assayed were chosen because of previous evidence for their role in photoreceptor apoptosis during photooxidative stress. For instance, Hafezi et al. (1999) illustrated differential DNA binding of AP-1 and Oct-1 in the retina after photooxidative stress using an electrophoresis mobility shift assay. Krishnamoorthy et al. (1999) show photooxidative stress causing cultured photoreceptor apoptosis also down-modulates NF-κB activity via caspase 1 activation. AP-1 is found in its active state as a homodimer of c-jun protein or as a heterodimer containing c-jun

associated with c-fos protein (Karin *et al.*, 1997; Shaulian and Karin, 2002). NF-κB is also a functionally a dimeric transcription factor containing monomeric Rel family DNA binding proteins (Karin and Lin, 2002). Previous research indicated that NF-κB and AP-1 factors can be coordinately activated in a number of different cell types (Mathas *et al.*, 2002; Stein *et al.*, 1993; Thomas *et al.*, 1997). Thus, it is possible that AP-1 and NF- κB act in concert to modulate the expression of the SWS1 opsin in trout retinal tissue.

Retinal dystrophies resulting in the loss of rod photoreceptors through apoptosis (retinitis pigmentosa, RP) are often caused by mutation of proteins involved in phototransduction or factors regulating their expression (Humphries *et al.*, 1997; Lem *et al.*, 1999; Nir *et al.*, 1990). RP models have been created in non-primate mammals through mutagenesis of the rhodopsin gene that result in misfolded mature protein that is not properly localized to the rod outer segments or protein that is not properly modified post-translation (Garriga and Manyosa, 2002; Rivolta *et al.*, 2001; Rivolta *et al.*, 2002). It is our contention that the specific loss of the SWS1 cone from the retina of salmonids at smoltification may result from alteration of SWS1 opsin transcription and involve c-jun and NF-κB activity. This contention is supported by the fact that both c-jun and NF-κB bind the SWS1 proximal promoter but fail to interact with promoters of other opsins. C-jun and NF-κB activity can be repressed by TH related factors including THRs or SMRT (Silencing Mediator of Retinoic acid and Thyroid hormone) (Lee *et al.*, 2000; Liden *et al.*, 1997; Perez *et al.*, 1993). TH is activated in the rainbow trout retina by deiodinases during development of the eye (Plate *et al.*, 2002). Up-regulation of SMRT during the TH-dependent metamorphic transition of rainbow trout could reduce SWS1 opsin expression and normal phototransduction leading to UVS cone apoptosis via AP-1 and NF-κB.

Considering the collective role of NF-κB and c-jun in photoreceptor apoptosis, their relation to thyroid hormone activity through SMRT, and the selective binding of these

transcription factors to the rainbow trout SWS1 opsin promoter, these TFs represent excellent candidates for further study of salmonid UVS cone degeneration. Reversal of protein-DNA formaldehyde induced cross-links could be problematic in ChIP assays although this was not the case in the present study. Other possible DNA-promoter interaction assays can be used in the case of problematic ChIP assays such as electrophoretic mobility shift or super-shift assays. Future study will include quantification of c-jun and NF-κB expression within the retina of rainbow trout during exogenous TH driven metamorphosis. Investigations attempting to specifically inhibit the activity of these TFs *in vivo* will also be pursued in an attempt to determine the effect on UVS cone fate during metamorphosis. Although the current study illustrates the selective binding of TFs to proximal promoters several regulatory elements can occure much further upstream of a gene's coding sequence. In these cases the twists in the chromosomal DNA are such that regulatory TFs bound to DNA at distal promoters (up to 20 Kb upstream) are brought in proximity to the RNA polymerase holoenzyme.

5. Examination of Gene Expression in Parr and Smolt Retina with DDRT-PCR

5.1 Chapter Introduction

Previous studies have shown that differential expression occurs in retina undergoing photoreceptor apoptosis through the study of retinitis pigmentosa and cDNA screening techniques. Differentially expressed genes in RP include clusterin, secreted frizzled-related protein and TIMP-3 (Jones *et al.*, 2000; Jones *et al.*, 1994; Jones *et al.*, 1992). Clusterin likely has a cytoprotective effect in degenerating photoreceptor cells and is not causally involved in apoptosis (Jomary *et al.*, 1999a). A differential cDNA screening approach illustrated TIMP-3 expression levels increase in patients with simplex retinitis pigmentosa (Jones *et al.*, 1994). This study was followed by one that localized TIMP-3 expression to the photoreceptor inner segments and the ganglion cell layer of retinitis pigmentosa affected retina (Jomary *et al.*, 1995). TIMP-3 is an inhibitor of metalloproteinases responsible for remodeling of the extra cellular matrix. TIMP-3 was also implicated in the homoestasis of the retina as well as neovascularization in the diseased state (Jomary *et al.*, 1997).

Few molecular markers are known that reflect the parr-smolt transition of the salmonids. Previous research has implicated genes enriched in the gill and liver including NA^+/K^+ ATPase, cortisol receptor, insulin-like growth factor-1 and transferrin (Hardiman and Gannon, 1996; Mizuno *et al.*, 2001; Sakamoto *et al.*, 1995; Singer *et al.*, 2002). Intracerebral levels of gonadotropin releasing hormone and growth hormone releasing hormone levels have been shown to peak at smoltification in salmonids (Parhar and Iwata, 1996; Parhar *et al.*, 1996). Identification of genes regulated in the retina at smoltification, however, is limited to the SWS1 opsin in apoptotic UVS cones. Thus, our objective was to resolve differences in retinal gene expression before and after smoltification in salmonid retina. To accomplish this we employed

the Differential Display Reverse Transcription-Polymerase Chain Reaction (DDRT-PCR) created by Liang and Pardee (1992).

The general strategy of DDRT-PCR is to amplify and isolate partial cDNA sequences from subsets of mRNAs by reverse transcription and the polymerase chain reaction with sets of arbitrary primers. DDRT-PCR is a suitable approach for the determination of differences in the transcriptome of animal models which lack significant genetic information. We have used DDRT-PCR to successfully identify differential expression in *O. mykiss* retina of a novel transcript, *rtp12.5*, which may define a unique state of retinal development. We have compared *rtp12.5*'s developmentally dependent transcription to the *O. mykiss* homologue of human *Sep15*, a false positive result identified in the same DDRT-PCR experiment.

The data presented in this chapter appears in the manuscript submitted and accepted to the journal *Comparative Biochemistry and Physiology- Part B: Biochemistry and Molecular Biology under* the title "Identification of a unique transcript down-regulated in the retina of rainbow trout (*Oncorhynchus mykiss*) at smoltification." This manuscript was submitted in May of 2003 and was accepted for publication in July of 2003. I am first author while Craig Hawryshyn is the senior author. Although I performed the experimental procedures and collected the data for this chapter, the remaining authors (W. T. Allison, and D.B Levin) made significant intellectual or technical contributions that warranted authorship.

5.2 RNA Isolation

O. mykiss retinal tissue was dissected from 6 parr (whole fish average wet mass 3.42g) and 6 smolt (whole fish average wet mass 68.34g). Total RNA was isolated using a TRIzol® LS Reagent (Invitrogen) extraction, a protocol modified from Chomczynski and Sacchi (1987). Poly-adenylated mRNA was subsequently isolated using the PolyAttract® (Promega) mRNA isolation system as per the manufacturer's protocol. First and second strand cDNA synthesis was

performed with SuperScript™ II reverse transcriptase (Life Technologies, Inc.) using 500 ng of mRNA and 1 µl of poly-T primer (1 µM primer concentration, 42°C for 1 hour). The synthesized cDNA was used as template for subsequent DDRT-PCR experiments.

5.3 DDRT-PCR

DDRT-PCR experiments were carried out using the Delta™ Differential Display kit (Clontech) with a slightly modified protocol; PCR conditions and primers are shown in Table 6. Twenty µl DDRT-PCR reactions were combined with 95% formalin gel loading buffer, denatured at 94°C for 5 minutes, and loaded on a denaturing 5% polyacrylamide/8 M urea gel in 0.5X TBE buffer. Polyacrylamide gels were run on a Bio-Rad protean II cooled slab gel system at 50 volts for 6 hours. The gel was stained with 1:1000 SYBR® green I stain in 0.5x TBE and visualized on a Molecular Dynamics Storm Phosphoimaging system to identify differentially displayed bands. The gel was stained with ethidium bromide, destained and visualized with a UV transilluminator. Using the phosphoimager picture, differentially displayed bands were identified and excised from the gel with a sterile razor blade while on the transilluminator.

Denatured PCR products (i.e. single stranded DNA) were eluted in 50 µl of TE buffer and incubated for 5 minutes at 100°C. The eluted DNA products were re-amplified using the same PCR conditions and primers used for the initial DDRT-PCR. Re-amplified bands were cleaned using Wizard® PCR preps (Promega) as per the manufacturer's protocol. PCR products were T-A cloned into the vector pBluescript® (Stratagene) by pre-treating EcoRV digested vector with Taq polymerase and dTTP, and ligating DNA amplicons with T4 DNA ligase (Promega) according to the manufacturer's protocol. Ligation reactions were used to transform electrocompetent *E. coli* (ElectroMAX DH10B™ Cells - Invitrogen) via electroporation. Putative positive colonies were screened by PCR using primers designed to the vector's M13 binding

sites. Five colonies containing cloned plasmid with insert of expected size were used to characterize transcripts from the isolated DDRT-PCR bands. Standard dideoxy sequencing method on positive colony plasmids employed IRDye700 and IRDye800 labeled primers performed on a NEN Global IR2 DNA Sequencer System (Li-cor).

5.4 RACE-PCR for full-length sequence

5' and 3' RACE was used to isolate the full-length sequence of the putative differentially expressed genes. The FirstChoice® RLM Race Kit (Ambion) was used on *O. mykiss* retinal mRNA according to the manufacturer's protocol, a brief description follows. Template for 5'-RACE PCR was created by first using calf intestinal phosphatase to remove free phosphates from the 5'-end of degraded or incomplete mRNAs, rRNAs or tRNAs. Full length capped mRNAs were digested by tobacco acid pyrophosphatase leaving the 5'-monophosphate necessary for adapter ligation by RNA ligase. A 45 base RNA adapter was ligated to 5'-end full length mRNA by RNA ligase. First strand cDNA synthesis was performed on the treated *O. mykiss* retinal mRNA by MMLV reverse transcriptase primed by random hexamers. Primers complementary to the 5'-adapter supplied with the kit were used as the forward primers in the subsequent nested PCR. 3'-RACE was accomplished by first synthesizing cDNA from *O. mykiss* retinal mRNA primed by a 46 bp adapter at 42°C for one hour with MMLV reverse transcriptase. 5' RACE forward and 3'RACE reverse primers were supplied with the kit. Gene specific primers were designed based on the gene fragments previously cloned and sequenced. 5' RACE reverse primers were created from the 3' end of the DDRT-PCR acquired fragment while 3'RACE forward primers were created from the 5' end of the DDRT-PCR acquired fragments. In order to confirm a single contiguous sequence for each gene, PCR was performed using primers complementary to 5' and 3' UTR sequence. RACE-PCR and full-length PCR products were

cloned and sequenced as described above in triplicate to insure sequence fidelity. All RACE-PCR and full-length PCR conditions and primers are specified in Table 6.

5.5 Confirmation of differential expression by hybridization

PCR products were confirmed to be differentially expressed by hybridizing cDNA fragments obtained from the DDRT-PCR experiments described above (*O. mykiss sep15* and *rtp12.5*) with parr and smolt *O. mykiss* retinal mRNAs. New mRNA fractions for each hybridization experiment (6 fish from each developmental stage per hybridization) were isolated using the protocol described above. The average wet mass of fish used for hybridization experiments was 3.67g for parr (18 experimental animals) and 67.69g for smolt (18 experimental animals). mRNA dot blot hybridization protocol was based on Christian (1992). Briefly, 500ng *O. mykiss* retinal mRNA from both parr and smolt stages was vacuum transferred to Hybond™ nylon membrane (Amersham Biosciences) and cross-linked using a UV transilluminator. Blots were hybridized with a DNA probe for *rtp12.5* and *O. mykiss sep15* acquired from cloning of DDRT-PCR amplicons. Probes were labeled with α-P^{32} dCTP (NEN) using the Random primers DNA labeling system (Invitrogen). Hybridizations were carried out overnight at 60°C /2x SSC/1% SDS buffer. Hybridized blots were exposed overnight to a Kodak phosphor screen and visual data collected on a Molecular Dynamics Storm Phosphoimager.

Similarly, the presence of *rtp12.5* and *O. mykiss sep 15* was examined in other tissues by northern hybridization following the protocol of Alwine *et al.* (1977). Five hundred (500) ng of brain and liver mRNA, isolated from the same parr and smolt *O. mykiss* used for the dot blot hybridizations, was electrophoretically separated on a denaturing formaldehyde agarose gel. mRNA was capillary transferred to a nylon membrane using 20xSSC and hybridized under the same conditions as the mRNA dot blots. Radiolabeled probes used for northern hybridization were identical to those described above for mRNA dot blot hybridizations. All membrane

Table 6.
DDRT-PCR, RACE-PCR and Full-length PCR primer sequences for differentially expressed candidates.

Nested Primer Name	Annealing Temp (°C)	Forward Primer Sequence (5' to 3')	Reverse Primer sequence (5' to 3')
DDRT -PCR	58	ATTAACCCTCACTAAATGCTGGGGA	CATTATGCTGAGTGATATCTTTTTTTTTAA
Rtp12.5 5' RACE 1st round	60	GTTGCTGCCCTTTGACTCGAG	GCTGATGGCGATGAATGAACACTG
Rtp12.5 5' RACE 2nd round	65	GGGTCTCTCCAGGATGTTCAGG	CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG
Rtp12.5 3'RACE 1st round	60	GATGGAGAAGGTCAAAGGGCTACA	GCGAGCACAGAATTAATACGACT
Rtp12.5 3'RACE 2nd round	65	GAGACCCAAAAAGTGAAGACCC	CGCGGATCCGAATTAATACGACTCACTATAGG
O. mykiss Sep 15 5' RACE 1st round	60	TATACTTCCAACTTCTCGCTCAG	GCTGATGGCGATGAATGAACACTG
O. mykiss Sep 15 5' RACE 2nd round	65	CCCCCAGCAGGTCACAGGAG	CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG
O. mykiss Sep 15 3' RACE 1st round	60	GTATCTCCTGTGGCTTCTTTCTC	GCGAGCACAGAATTAATACGACT
O. mykiss Sep 15 3' RACE 2nd round	65	GCTCCTGTGACCTGCTGGGG	CGCGGATCCGAATTAATACGACTCACTATAGG
Rtp12.5 Full length	59	GATGAAAGAAAGATGGCGTC	TCCATAGATGCACACTGAGGG
O. mykiss Sep 15 Full length	59	TCATCATACAGGAGCGGAACT	CCGAACACCATCACCACCAC
		5' RAC E Adapter	3' RACE adapter
		GCUGAUGGCGAUGAAUGAACACUGCGUU UGCUGGCUUUGAUGAAA	GAGCACAGAATTAATACGACTCACTATAGGTTTTT TTTTTTTVN

Table Footnote: PCR was carried out using an initial denaturation of 94°C for 4 minutes, followed by 28 cycles of 94°C denaturation for 45 seconds, annealing temperature (see table) for 45 seconds, 72°C elongation for 60°C seconds and 1 cycle of 72°C for 5 minutes.

hybridizations were carried out in triplicate; representative results are shown. Procedures described herein were in accordance with the guidelines established by the Canadian Council on Animal Care. The University of Victoria Animal Care Committee approved all experimental protocols.

5.6 Localization and confirmation of differential expression by in situ hybridization

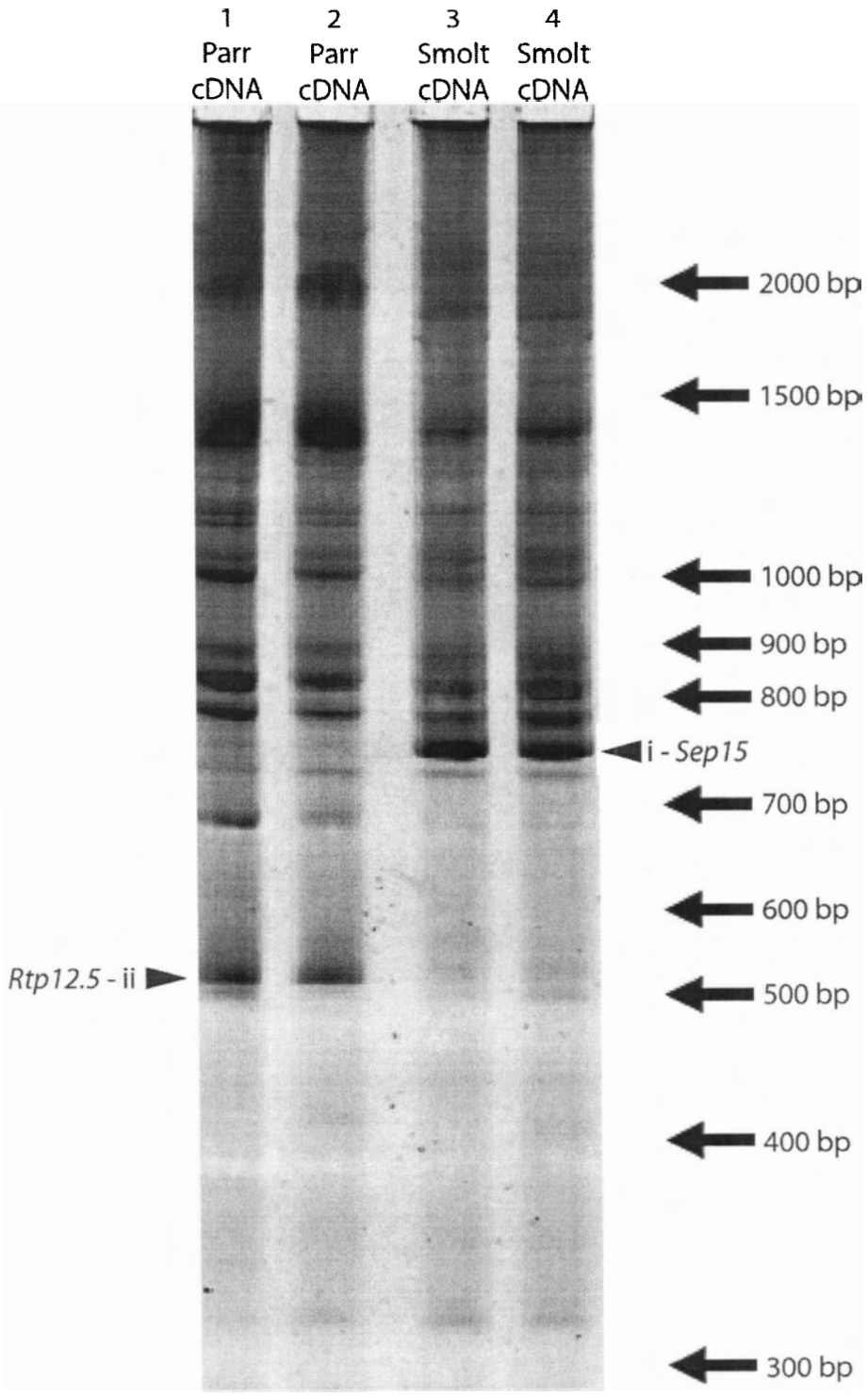
In situ hybridization was performed following the protocol of Braissant and Wahli (1998). Fresh retina were dissected from 3 parr (whole fish average wet mass – 3.32g) and 3 smolt (whole fish average wet mass 73.65g) *O. mykiss* and fixed overnight at 4°C in 4% paraformaldehyde in PBS. Retina were cryoprotected in 25% OCT in PBS overnight at 4°C and flash frozen in liquid nitrogen for crysectioning. Radial cryosections (12 μm) were taken from retina of parr and smolt *O. mykiss* on a Leica CM1850 cryostat and post fixed in 4% paraformaldehyde/PBS for 10 minutes at room temperature. Sections were treated twice with 0.1% active DEPC in PBS for 15 minutes at room temperature to inactivate endogenous RNases. Sections were washed in PBS (15 minutes) and equilibrated in 5x SSC (5 minutes, 3x). Sections were prehybridized for 4 hours at 59°C covered with hybridization buffer (50% deionized/5xSSC/40μg/ml sonicated calf thymus DNA). Sections were hybridized overnight at 59°C in the fresh hybridization buffer with the addition of fluorescein labeled riboprobes to antisense *rtp12.5,* antisense *O. mykiss sep15* (probe length and sequence same as RNA-membrane hybridizations) sense *rtp12.5* or sense *O. mykiss sep15* (sense controls are of sequence complementary to antisense probes). Riboprobes were created using T3 (sense probe) and T7 (anti sense probe) RNA polymerase with incorporation of fluorescein labeled rUTP (Roche Biochemical). Hybridized sections were washed with 2x SSC/60 minutes/room temperature, 2xSSC/60 minutes/65°C and 0.1X SSC/1 hour/65°C.

Sections were equilibrated in 100mM Tris-HCl/150mM NaCl, pH7.5 and treated with RNase H (30 minutes at 37°C). The sections were then incubated for 120 minutes at room temperature with alkaline phosphatase-coupled anti-digoxigenin antibody (Roche Biochemicals) diluted 1:5000 in the same buffer, supplemented with 0.5% blocking reagent (Roche Biochemicals). Excess antibody was washed twice with fresh buffer for 15 minutes and then equilibrated with a 100mM Tris-HCl/100mM NaCl/50mM $MgCl_2$ pH 9.5 buffer and developed with an NBT-BCIP (Gibco-BRL) colorimetric reaction in the same buffer. Color reactions were stopped by incubation of sections in 10mM Tris/0.1mM EDTA pH 8.0. Photo-microscopy of *in situ* hybridization was performed on a Zeiss Axioskop 2 under bright field with a 40x objective. *In situ* hybridization experiments were repeated in triplicate to ensure expression patterns shown in the results section are accurate.

5.7 Results

DDRT-PCR reactions from parr and smolt *O. mykiss* retinal cDNA were analyzed on a denaturing polyacrylamide gel (Figure 12). Several differentially expressed transcripts were observed, two of which were isolated for further analysis due to qualitative difference in band intensity. One differentially displayed band was isolated in the juvenile retinal RNA condition (*rtp12.5* at 536 bp) and another in mature retinal RNA condition (*O. mykiss sep15* at 750 bp). Both amplicons were isolated and characterized by sequence analysis. Subsequent RACE-PCR produced overlapping 3' and 5' sequence used to generate full-length cDNAs of *O. mykiss sep15* and *rtp12.5*. PCR products for full-length cDNA were cloned, sequenced, and compared to the RACE results confirming their identity. The full-length clone sequences were submitted to Genbank (Genbank Accession Nos. AY255832 (*rtp12.5*) and AY255833 (*O. mykiss sep15*)) and used to interrogate the translating Blastx database at NCBI (http://www.ncbi.nlm.nih.gov:80/BLAST/). Analysis of the full length *O. mykiss sep15* cDNA

Figure 12.
Analysis of differentially expressed transcripts isolated from retinal cDNA of parr and smolt *O. mykiss*. Differential Display RT-PCR performed on *O. mykiss* retina mRNA. Lanes 1 & 2) Amplified parr *O. mykiss* retina cDNA. Lanes 3 and 4) Amplified smolt *O. mykiss* retina cDNA. Differentially expressed transcripts are identified by arrowheads. Arrowhead **i** denotes *O. mykiss sep15* and arrowhead **ii** denotes *rtp12.5*.


1
Parr
cDNA
2
Parr
cDNA
3
Smolt
cDNA
4
Smolt
cDNA
2000 bp
1500 bp
1000 bp
900 bp
800 bp
i - Sep15
700 bp
600 bp
Rtp12.5 - ii
500 bp
400 bp
300 bp

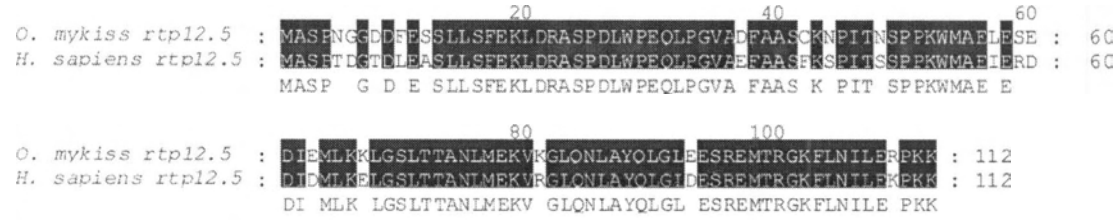
sequence revealed a homologue to the human selenoprotein *sep15* (Genbank Accession No. NP_004252 109/162 aa identities). Similar analysis of *rtp12.5* sequence shows homologues for a hypothetical gene product in human (Genbank Accession No. XP_040376 95/112 aa identities), mouse (Genbank Accession No. XP_126985 95/116 aa identities), and *Drosophila* (Genbank Accession No. AAF46076 37/157 aa identities). Hidden Markov Model (HMM) search through the swiss-prot database (http://cbi.labri.fr/outils/Pise/hmmsearch.html) reveals some homology to a protein in *C. elegans* (Genbank Accession No. NP_499179 20/112 aa identities (Eddy, 1998). Alignments of the *O. mykiss sep15* and *rtp12.5* cDNAs to their human homologues are displayed in Figure 13 (a & b). Figure 14a illustrates the level of expression of *rtp12.5* and *O. mykiss sep15* to parr and smolt *O. mykiss* retinal RNA. Developmental stage-specific expression was confirmed in the case of *rtp12.5* and not supported in the case of *O. mykiss sep15*. Expression of *rtp12.5* transcript is elevated in parr retina but absent from the retinal mRNA of smolt retina in *O. mykiss*. Expression of *O. mykiss sep15* appears the same in both parr and smolt retina, contrary to results from DDRT-PCR. The result from the DDRT-PCR in the case of *O. mykiss sep15* appears to be a false positive based on the more reliable mRNA dot blot hybridizations. False positive results in DDRT-PCR are common, necessitating the use of hybridization experiments to confirm the results (Sompayrac *et al.*, 1995). In contrast to observations from retinal tissue, *rtp12.5* and *O. mykiss sep15* expression in brain and liver are not stage dependent (Figure 14 b and c). Northern blot analysis indicates that the size of the *O. mykiss sep15* and *rtp12.5*. hybridized transcripts correspond to the size of the full-length cDNA acquired from retinal tissue. The single hybridized transcript signal in the multiple tissue blots for *rtp12.5* and *O. mykiss sep15* obviated the need to perform retinal mRNA northern hybridizations and instead allowed us to perform the more sensitive mRNA dot blot hybridizations described.

Figure 13.
Amino acid alignment of presented *O. mykiss sep15* and *rtp12.5* protein sequences and predicted SECIS structure.

A) Amino acid sequence alignment of *O. mykiss sep15* to human *Sep15* (Genbank accession no. NP_004252). Bold, italicized X in each sequence (at position 94 in human) represents the selenocysteine residue encoded by the UGA codon in the mRNA sequence. B) Amino acid sequence alignment of O. *mykiss rtp12.5* to human hypothetical protein C13ORF46 (Genbank accession no. XM_040376). C) Selenocysteine insertion sequence stem-hairpin loop structure as predicted by RNA structure v3.7.

A - *O. mykiss Sep 15* alignment to human *Sep15*

B - *O. mykiss rtp12.5* alignment to human homologue



C - Putative SECIS element in *O. mykiss Sep15* 3' UTR

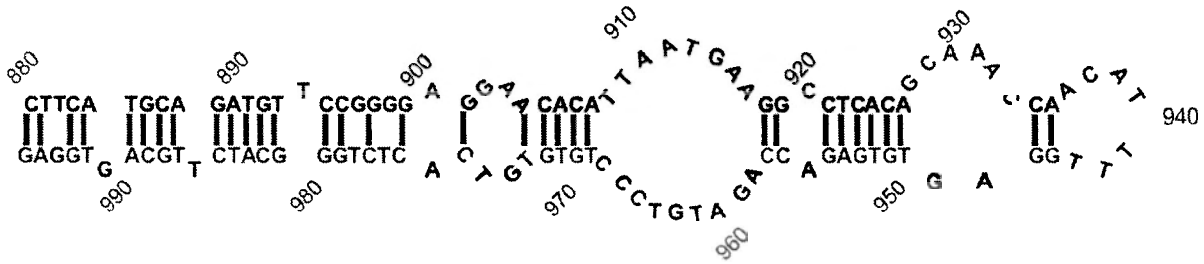
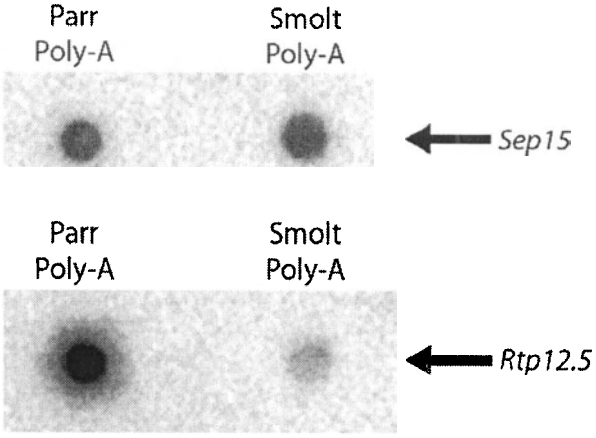


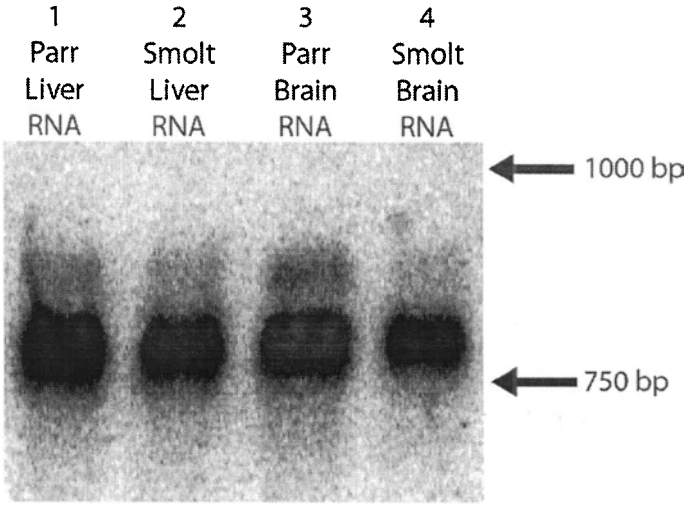
Figure 14.
O. mykiss retinal mRNA hybridizations to *rtp12.5* and *O. mykiss sep15* probes

A) Dot Blot hybridization of *O. mykiss sep15 (top)* and *rtp12.5* (bottom) to parr and smolt *O. mykiss* retina mRNA. *O. mykiss sep15* hybridization shows constitutive expression between the parr and smolt stages. *Rtp12.5* hybridizes a transcript showing high expression in the parr *O. mykiss* retina, and low in the smolt stage. B) Multiple Tissue Northern Hybridization of *O. mykiss sep15*; Lanes 1 and 2) Parr and smolt *O. mykiss* liver mRNA, respectively; Lanes 3 and 4) are parr and smolt *O. mykiss* brain mRNA, respectively. C) Multiple Tissue Northern Hybridization of *rtp12.5*. Lanes as per B.

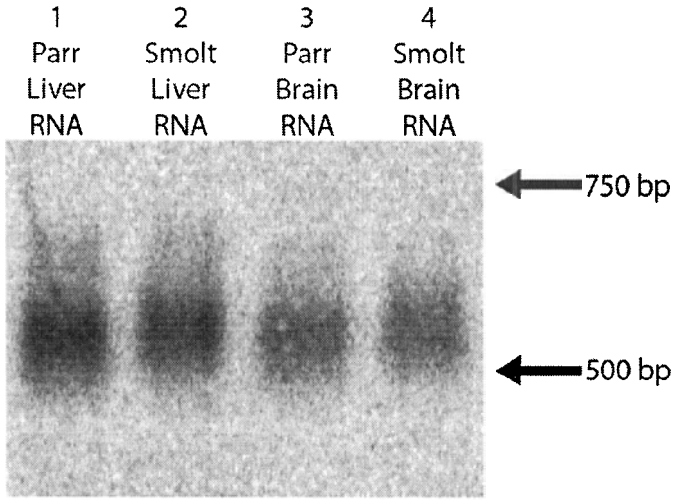
A - Retinal mRNA dot blots



B - *Sep15* Multiple Tissue Northern Blot



C - *Rtp12.5* Multiple Tissue Northern Blot



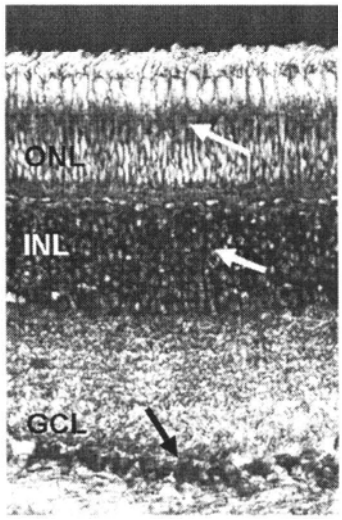
Localization of transcript expression to cellular structures within parr and smolt *O. mykiss* retina was determined by *in situ* hybridization (Figure 15 a through f). The *O. mykiss sep15* transcripts showed expression in the ganglion cell layer, the inner nuclear layer and the outer nuclear layer of both parr and smolt retina while *rtp12.5* appears to be confined to the ganglion cell layer and the entire inner nuclear layer of parr retina (Figure 5a, b, d, and f). *In situ* hybridization of smolt *O. mykiss* retinal sections with *rtp12.5* shows no labeling by the antisense probe (Figure 15b). Background staining of retinal tissue was assessed by using hybridization of the sense riboprobe to parr *O. mykiss* retina (Figure 15 c and f). Differential expression of the *rtp12.5* transcript in the retina has been described through DDRT-PCR analysis, mRNA dot blot hybridization and *in situ* hybridization. Although the *O. mykiss sep15* appears differentially displayed in the original PCR experiment, we could not confirm differential expression by RNA hybridization experiments. The lack of differential expression of *O. mykiss sep15*, however, makes it a suitable internal control for the stage specific *rtp12.5* transcript.

5.8 Discussion

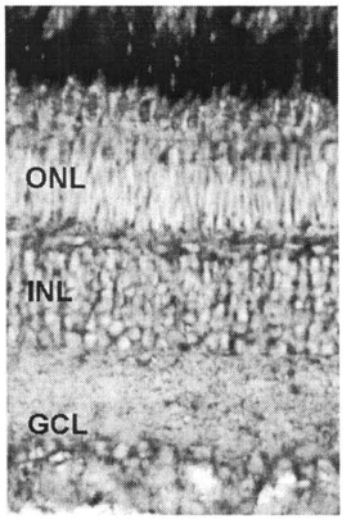
We have isolated two genes previously uncharacterized in teleosts, or in the retina of any species, from the transcriptome of *O. mykiss* retina. Expression of the *Sep15* gene has been found in prostate, liver, kidney, testis, and brain of humans and rodents (Gladyshev *et al.*, 1998). The *Sep15* gene has been implicated in cancer etiology and is associated with UDP-glucose:glycoprotein glucosyltransferase that is responsible for protein folding in the endoplasmic reticulum (Korotkov *et al.*, 2001; Kumaraswamy *et al.*, 2002; Kumaraswamy *et al.*, 2000). This is the first description of a *Sep15* transcript localized in a vertebrate retina and the first description of *Sep15* in a non-mammalian organism. We could not confirm differential expression of *O. mykiss Sep15* in the retina of rainbow trout. This was likely due to a false positive result, common in DDRT-PCR

Figure 15.
In situ hybridization of *O. mykiss sep15* and *rtp12.5* to parr and smolt *O. mykiss* retinal tissue cryosections. A) Antisense RNA probe *in situ* hybridization of *O. mykiss rtp12.5* to parr retinal tissue. B) Antisense RNA probe *in situ* hybridization of *O. mykiss rtp12.5* to smolt retinal tissue. C) Sense *in situ* hybridization of sense *O. mykiss rtp12.5* probe to parr retinal tissue. D) Antisense RNA probe *in situ* hybridization of *O. mykiss sep15* to parr retinal tissue. E) Antisense RNA probe *in situ* hybridization of *O. mykiss sep15* to smolt retinal tissue. F) Sense RNA probe *in situ* hybridization of *O. mykiss sep15* to parr retinal tissue. Hybridization signal is the black precipitate (developed by an NBT/BCIP alkaline phosphatase mediated reaction) in the micrographs as indicated by arrows.

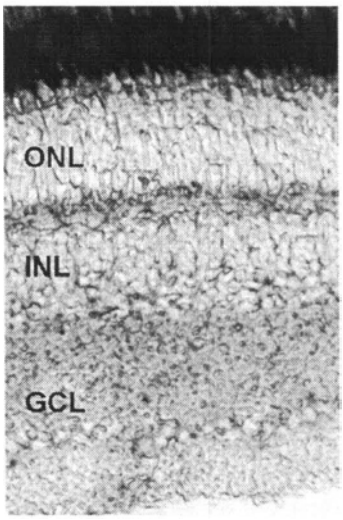
A - *Rtp12.5* antisense
in situ on parr retina



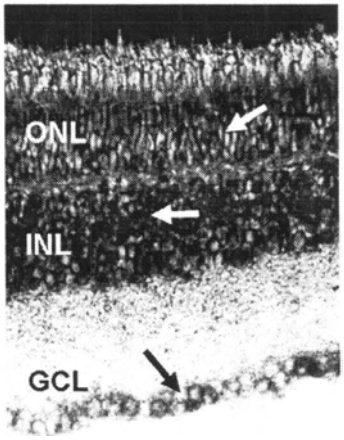
B - *Rtp12.5* antisense
in situ on smolt retina



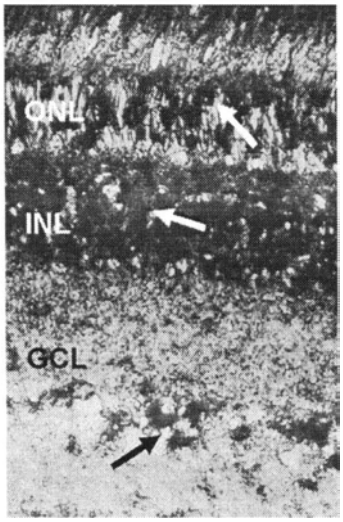
C - *Rtp12.5* sense
in situ on parr retina



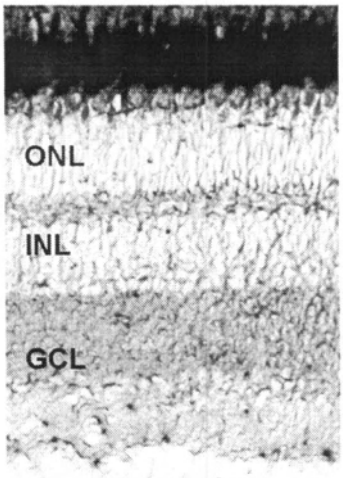
D - *Sep15* antisense
in situ on parr retina

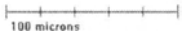


E - *Sep15* antisense
in situ on smolt retina



F - *Sep15* sense
in situ on parr retina





screens. Considering the importance of selenoproteins in strong redox reactions of cellular metabolism and the proposed role of *Sep15* in protein folding it is not surprising to see a lack of differential expression between parr and smolt trout.

A selenocysteine insertion sequence (SECIS) element in the 3' UTR identified in the human *Sep15* also appears to be present in the *O. mykiss* mRNA sequence as predicted by RNA structure v3.7 (Mathews *et al.*, 1999) (Figure 13c). The SECIS in human selenoproteins produces a stem-loop structure responsible for signaling selenocysteine tRNA insertion at the UGA codon as opposed to the truncation of the mature peptide at what is normally a stop codon (Shen *et al.*, 1993; Walczak *et al.*, 1997). We predict that the SECIS like element in the 3'UTR of the *O. mykiss* sequence also directs this modification during translation. If so, the 1043 bp cDNA will encode a mature protein of 157 aa with a selenocysteine residue located at position 84. Follow up studies using ^{75}Se tagged protein fractions that show a 15 kDa Se labeled protein could confirm this prediction.

Rtp12.5 is differentially expressed in the *O. mykiss* retina between parr and smolt developmental stages (smoltification). The novel cDNA sequence of *rtp12.5* in *O. mykiss* is 911 bp and encodes a putative protein of 112 aa in length. The predicted protein mass of *rtp12.5* is approximately 12.5 kDa with a pI of 4.80 (rainbow trout protein 12.5 kDa). PHD prediction was carried out (http://www.embl-heidelberg.de/predictprotein/predictprotein.html) to determine the likely secondary structure of *rtp12.5* (Rost and Sander, 1993; Rost and Sander, 1994). This analysis reveals a secondary structure of almost entirely alpha helix interrupted only by loops located at proline residues. Three sites in the protein sequence reveal two types of possible post-translation modification. A serine residue, at position 41, is a candidate for protein kinase C phosphorylation. Glycine residues at position 69 and 91 are probable sites for acetylation by covalent amide linkage of myristate via N-myristoyl transferase.

We have used the genome database users guide to navigate the human genome surrounding this previously uncharacterized gene product (Wolfsberg *et al.*, 2002). The human genome map reveals a hypothetical gene (Genbank Accession No. XM_040376) on chromosome 14 at site 14.Q.24 with the official name Chromosome 14 open reading frame 46 (C14ORF46). Based on amino acid sequence and Hidden Markov Model search, this gene may be similar to a nuclear protein identified by *in vivo* RNAi screening of expression in *C. elegans* (Genbank Accession No. NP_499179) (Maeda *et al.*, 2001). Knockdown of the expression of this gene resulted in deficiencies in egg laying abilities, abnormal oogenesis and reduced fertility in *C. elegans*. There is no identified homology of *rtp12.5* to any zebrafish mRNA or genomic contig assembled in the NCBI zebrafish database. Blast search for short nearly exact matches, at either the protein or the nucleotide level, results in hypothetical proteins from insects (*Anopheles gambiae* Genbank Accession No. EAA07566), bacteria (*Thermobifida fusca* Genbank Accession No. ZP_00057569) and plants (*Arabidopsis thaliana* Genbank Accession No. NP_176681).

The expression level of *rtp12.5* transcripts may be coupled to the loss of UV sensitivity and UVS cones through apoptosis based on the stage-specific disappearance of *rtp12.5* from the retinal mRNA (Allison *et al.*, 2003; Deutschlander *et al.*, 2001). Based on the expression pattern of the *rtp12.5* transcript throughout the inner nuclear and ganglion cell layers of the *O. mykiss* retina *rtp12.5* may be involved in neural remodeling during UVS photoreceptor apoptosis. This implies that at least a subset of the higher-order retinal neurons are changing their gene expression depending on the compliment of photoreceptor inputs they are processing. Such inputs might include chromatic information (especially in the UV range) and/or polarization sensitivity, both of which are known to be lost after smoltification (Hawryshyn, 2000; Hawryshyn *et al.*, 1990). We are currently examining neuronal processing in the outer plexiform layer of the retina and some of the changes that may occur subsequent to smoltification. The

expression pattern of this gene implies that at least a subset of amacrine, bipolar and ganglion cells could play a role in this complex neural processing.

We have shown that gene expression within the retina of the *O. mykiss* changes depending on the stage of the organism's life history. Differential display RT-PCR revealed two genes putatively differentially expressed between parr and smolt retina. A homologue of the human *Sep15* appears to be constitutively expressed through dot blot and *in situ* hybridization. *Rtp12.5*, a gene previously characterized only as a hypothetical genome annotation in human, mouse, and *Drosophila*, appears to be tightly regulated in the retina of *O. mykiss* in a developmental stage specific manner. A putative nuclear protein involved in oogenesis from *C. elegans* also shows some homology to *rtp12.5*. Retinal development in the salmonids, as well as amphibians, is TH dependent and result in extensive changes in gene expression. This gene may be TH responsive and involved in the plasticity of the neuronal connections during the disappearance of UVS cones from the retina. Further study will attempt to identify a protein product of *rtp12.5* and determine its function in the parr retina. Knockdown studies of *rtp12.5* in the retina of *O. mykiss* via siRNA may also provide functional information regarding its differential expression in the retina.

6. Summary

The preceding dissertation describes some fundamental aspects of opsin and retinal genomics in the salmonid visual system as they pertain to the function and development of a dynamic vertebrate retina. One of each of the photoreceptor opsin genes was successfully cloned and sequenced from six *Oncorhynchus* species and from one *Salmo* species. This information has led to identification of an alternate evolutionary relationship among the salmonids as well as unique species-level relationships between the cichlid and cyprinid fishes. Occurrences of statistically reliable positive selection in the RH1 and RH2 opsin make these genes good candidates for future study using site directed mutagenesis and *in vitro* expression systems to identify unique mechanisms of opsin molecular tuning. Similarly, specific aa substitutions in the salmonid SWS2 gene may also represent a notable spectral tuning system.

Acquisition of the salmonid opsin gene sequences allowed for the subsequent isolation of proximal promoter regions of these genes from rainbow trout genomic DNA. Using a web-based interrogative database, AP-1 and NF-κB binding sites exclusive to the SWS1 opsin proximal promoter were identified. Further analysis by a chromatin immunoprecipitation-PCR coupled assay resulted in the identification of these transcription factors binding the SWS1 opsin proximal promoter *in vivo*. These transcription factors in particular have been identified in previous research as important in photoreceptor specific apoptosis. The parallels found between mice models of retinal degeneration and the model presented here is certainly striking and should be pursued using functional assays of AP-1 and NF-κB expression in the salmonid retina.

Finally, using a transcriptome level PCR based analysis of retinal expression between two post-natal developmental stages of the salmonid we identified a novel transcript that is tightly regulated in the rainbow trout retina. This transcript, *rtp12.5*, is expressed in parr retina INL and GCL but not the ONL and is absent from the smolt retina transcriptome. *Rtp12.5* has no known

function or conserved domains when compared to other gene sequences in the Genbank database. *Rtp12.5* may play a role in creation or destruction of neural connections in photoreceptors, expressed in the parr retina before the UVS cones degenerate in order to remodel the neural connections of the horizontal, bipolar and ganglion cells that would otherwise process UVS cone signals. During this analysis, another transcript homologous to the human *Sep15* gene was identified from the rainbow trout retina as differentially expressed. This differential expression could not be confirmed by hybridization experiments where it appeared to be constitutively expressed in the retina of both parr and smolt rainbow trout. The identification of this transcript, however, served as an adequate internal control for hybridization analysis of *rtp12.5*.

This research represents some of the first molecular and genetics based analysis performed on the salmonid visual system. Previous work by Veldhoen *et al.* (1999) resulted in an SWS2 opsin anti-sera that reliably labels SWS cones in whole mount and on sections. More recent work by Allison *et al.* (2003) has resulted in the acquisition of riboprobes for labeling of each photoreceptor type with RNA duplexes. These publications, as well as those produced from the work herein, lay the groundwork for exploring the novel model of retinal development and photoreceptor degeneration found in salmonids. Future work regarding the molecular genetics of the opsin genes in salmonids should focus on the molecular tuning of the opsins in an A_1 restricted *in vitro* expression system. This will facilitate the discovery of molecular tuning mechanisms in the salmonid opsins as they relate to each as well as other organisms while removing confounds introduced by a paired pigment system. Discovery of duplicated opsin genes in salmonids will be possible using genomic analysis i.e. Southern hybridization, genomic library screening, etc. as well as the ongoing salmon genome project. Any duplication may also reveal interesting tuning mechanisms in the opsins.

To fully characterize the opsin promoters and their transcriptional control GFP reporter constructs should be created. These constructs can be packaged into adeno-associated virus-2 (AAV) via human HEK293 cell culture transfection. AAV viral stocks can mediate localized gene delivery of the packaged construct to the UVS photoreceptor via intraocular injection. Since construct delivery to the retina is virally mediated, integration into the genome occurs in each infected cell of the host organism. This allows a stable gene delivery as opposed to a transient one mediated by plasmid delivery with lipofection, electroporation or other *in vivo* transfection reagents. Site-directed mutagenesis of the opsin promoter's putative regulatory elements would allow us to measure the effects of specific TF binding on GFP expression, and therefore opsin gene expression. Similarly, these constructs allow us to produce a GFP reporter whose expression is confined to UVS photoreceptors, specifically, when the SWS1 opsin promoter is used to drive GFP expression. For instance, as the transcription factors AP-1 or NF-κB are found to bind to the SWS1 promoter, deletion of that TF binding site from the SWS1 promoter-GFP construct may result in significant differences in GFP expression within the UVS cones. An increase in GFP expression will be interpreted as a transcriptional activator alternatively, if GFP appears in other retinal cell types then the TF is a repressor – i.e. loss of TF binding results in a loss of UVS cone specificity. TF's of interest can also be upregulated in specific photoreceptors if their ORFs are cloned into the construct between the opsin promoter and the GFP reporter. These TFs can also be upregulated throughout the retina through use of the cytomegalovirus (CMV) promoter that can be shuttled into the GFP construct in place of the opsin specific promoter.

RNA interference could be used to knockdown translation of candidate TF genes in the retina or in specific photoreceptors using the constructs and AAV vectors described above. RNA interference is a relatively new technique that employs small double stranded RNA sequences

(siRNA 20-25mers) that are identical to coding regions of the gene of interest. Double stranded RNAs introduced to a host cell by our cell-specific promoters, are cleaved into siRNAs by the enzyme dicer. These create a triplex with target mRNA through the RNA inducing silencing complex (RISC) which cleaves the native mRNA thereby eliminating translation of the gene of interest (McManus *et al.*, 2002; Scherr *et al.*, 2003). Some recent studies have employed short hairpin RNA (shRNA) constructs whose expression is driven by the RNA polymerase III promoter, H1, to knockdown translation of a gene (Brummelkamp *et al.*, 2002; Sui *et al.*, 2002). ShRNA constructs produce a 50-60 bp transcript that results in perfectly matched 20-25mer stem hairpin loop secondary structure. The stem hairpin loop structures knockdown expression as efficiently as the synthetic siRNAs used in cell culture experiments. Delivery of these constructs via viral vectors *in vitro* and *in vivo* results in a stable, long-term expression of the siRNA (Abbas-Terki *et al.*, 2002; Xia *et al.*, 2002). siRNA-coding constructs could be created under control of an opsin promoter with the opsin promoter-GFP vector. Oligonucleotides (50-60mer) could be designed complementary to candidate genes; duplexes created by hybridizing complementary oligos (custom designed and ordered from a commercial vendor) could be cloned into the opsin promoter-GFP vector and packaged into AAV viral particles. Transcription of shRNAs would be quantified by the amount of GFP expression visualized in the retina.

Opsin promoter-GFP constructs would be able to knockdown or up-regulate multiple genes simultaneously, or selectively up-regulate and knockdown translation of different genes. This technology could be used to investigate the function of *rtp12.5* both *in vitro* and *in vivo*. Cloning of the *rtp12.5* promoter into a GFP construct could describe the genetic regulation of this gene similar to the process described above. Also, *rtp12.5* could be up or down regulated through the use of a CMV driven expression system or RNA interference respectively. Use of knock-up and knock-down technology in tissue-specific, even cell-specific, systems is the next

logical step in determining how salmonid opsin genes are regulated, what role opsin specific TF's play in the salmonid retina and how candidate genes in the salmonid retina contribute to the post-natal degeneration of UVS cones triggered by thyroid hormone.

Literature Cited

Abbas-Terki T, Blanco-Bose W, Deglon N, Pralong W, Aebischer P. 2002. Lentiviral-mediated RNA interference. Human Gene Therapy. 13:2197-201.

Abney WdW. 1913. Researches in colour vision. Longmans, Green, London.

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. 195:185-97.

Ali MA, Wagner HJ. 1974. Visual pigments: Phylogeny and ecology. In: Ali MA (ed) Vision in Fishes: New Approaches. Plenam Press, New York

Allen DM, Macfarland W, Munz FW, Poston HA. 1973. Changes in the visual pigments of trout. Canadian Journal of Zoology. 51:901-914.

Allison WT, Dann SG, Helvik V, Bradley C, Moyer HD, Hawryshyn CW. 2003. Ontogeny of ultraviolet-sensitive cones in the retina of rainbow trout (*Oncorhynchus mykiss*). Journal of Comparative Neurology. 461:294-306.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. Journal of Molecular Biology. 215:403-10.

Alwine JC, Kemp DJ, Stark GR. 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. Proceedings of the National Academy of Sciences USA. 74:5350-4.

Aranburu A, Carlsson R, Persson C, Leanderson T. 2001. Transcription factor AP-4 is a ligand for immunoglobulin-kappa promoter E-box elements. Biochemical Journal. 354:431-8.

Baniahmad A. 2002. Introduction to thyroid hormone receptors. Methods in Molecular Biology. 202:1-12.

Beatty DD. 1984. Visual pigments and the labile scotopic visual system of fish. Vision Research. 24:1563-73.

Beaudet L, Browman HI, Hawryshyn CW. 1993. Optic nerve response and retinal structure in rainbow trout of different sizes. Vision Research. 33:1739-46.

Beaudet L, Novales Flamarique I, Hawryshyn CW. 1997. Cone photoreceptor topography in the retina of sexually mature Pacific salmonid fishes. Journal of Comparative Neurology. 383:49-59.

Belecky-Adams T, Tomarev S, Li HS, Ploder L, McInnes RR, Sundin O, Adler R. 1997. Pax-6, Prox 1, and Chx10 homeobox gene expression correlates with phenotypic fate of retinal precursor cells. Investigative Ophthalmology and Visual Science. 38:1293-303.

Bowmaker JK. 1998. Evolution of colour vision in vertebrates. Eye. 12:541-7.

Braissant O, Wahli W. 1998. A Simplified In Situ Hybridization Protocol Using Non-radioactively Labeled Probes to Detect Abundant and Rare mRNAs on Tissue Sections. Biochemica.10-17.

Brantjes H, Barker N, van Es J, Clevers H. 2002. TCF: Lady Justice casting the final verdict on the outcome of Wnt signalling. Biological Chemistry. 383:255-61.

Browman HI, Hawryshyn CW. 1992. Thyroxine induces a precocial loss of ultraviolet photosensitivity in rainbow trout (*Oncorhynchus mykiss*, Teleostei). Vision Research. 32:2303-12.

Browman HI, Hawryshyn CW. 1994. The developmental trajectory of ultraviolet photosensitivity in rainbow trout is altered by thyroxine. Vision Research. 34:1397-406.

Brummelkamp TR, Bernards R, Agami R. 2002. A system for stable expression of short interfering RNAs in mammalian cells. Science. 296:550-3.

Cajal SR. 1893. La rétine des vertébrés. La Cellule. 9:17-257.

Cameron DA, Powers MK. 2000. Morphology and visual pigment content of photoreceptors from injured goldfish retina. Visual Neuroscience. 17:623-30.

Carleton KL, Harosi FI, Kocher TD. 2000. Visual pigments of African cichlid fishes: evidence for ultraviolet vision from microspectrophotometry and DNA sequences. Vision Research. 40:879-90.

Carleton KL, Kocher TD. 2001. Cone opsin genes of african cichlid fishes: tuning spectral sensitivity by differential gene expression. Molecular Biology and Evolution. 18:1540-50.

Chang BS, Crandall KA, Carulli JP, Hartl DL. 1995. Opsin phylogeny and evolution: a model for blue shifts in wavelength regulation. Molecular Phylogenetics and Evolution. 4:31-43.

Chen S, Wang QL, Nie Z, Sun H, Lennon G, Copeland NG, Gilbert DJ, Jenkins NA, Zack DJ. 1997. Crx, a novel Otx-like paired-homeodomain protein, binds to and transactivates photoreceptor cell-specific genes. Neuron. 19:1017-30.

Chinen A, Hamaoka T, Yamada Y, Kawamura S. 2003. Gene duplication and spectral diversification of cone visual pigments of zebrafish. Genetics. 163:663-675.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry. 162:156-9.

Chow L, Levine EM, Reh TA. 1998. The nuclear receptor transcription factor, retinoid-related orphan receptor beta, regulates retinal progenitor proliferation. Mechanisms of Development. 77:149-64.

Christian PD. 1992. A simple vacuum dot-blot hybridisation assay for the detection of *Drosophila* A and C viruses in single *Drosophila*. Journal of Virology Methods. 38:153-65.

Clarke WC, Groot C, Margolis L. 1995. Physiological ecology of Pacific salmon. University of British Columbia Press, Vancouver, p 510

Cowing JA, Poopalasundaram S, Wilkie SE, Bowmaker JK, Hunt DM. 2002a. Spectral tuning and evolution of short wave-sensitive cone pigments in cottoid fish from Lake Baikal. Biochemistry. 41:6019-25.

Cowing JA, Poopalasundaram S, Wilkie SE, Robinson PR, Bowmaker JK, Hunt DM. 2002b. The molecular mechanism for the spectral shifts between vertebrate ultraviolet- and violet-sensitive cone visual pigments. Biochemistry Journal. 367:129-35.

Creevey CJ, McInerney JO. 2002. An algorithm for detecting directional and non-directional positive selection, neutrality and negative selection in protein coding DNA sequences. Gene. 300:43-51.

Crispino JD, Orkin SH. 2002. The use of altered specificity mutants to probe specific protein-protein interactions involved in the activation of GATA-1 target genes. Methods. 26:84-92.

Cvekl A, Sax CM, Li X, McDermott JB, Piatigorsky J. 1995. Pax-6 and lens-specific transcription of the chicken delta 1-crystallin gene. Proceedings of the National Academy of Sciences USA. 92:4681-5.

de Gaudemar B. 1998. Sexual selection and breeding patterns: Insights from salmonids (*Salmonidae*). Acta Biotheoretica. 46:235-251.

Deutschlander ME, Greaves DK, Haimberger TJ, Hawryshyn CW. 2001. Functional mapping of ultraviolet photosensitivity during metamorphic transitions in a salmonid fish, *Oncorhynchus mykiss*. Journal of Experimental Biology. 204:2401-13.

Dowling JE. 1987. The Retina: An Approachable Part of the Brain. The Belknap Press of Harvard University, Cambridge.

Ebrey T, Koutalos Y. 2001. Vertebrate photoreceptors. Progress in Retinal Eye Research. 20:49-94.

Eddy SR. 1998. Profile hidden Markov models. Bioinformatics. 14:755-63.

Epand RM. 1998. Lipid polymorphism and protein-lipid interactions. Biochimica Biophysica Acta. 1376:353-68.

Fasick JI, Robsinson PR. 1998. Mechanism of spectral tuning in the dolphin visual pigments. Biochemistry. 37:433-8.

Foote CJ, Brown GS, Hawryshyn CW. In Press-Animal Behavior. Female colour and male choice in sockeye salmon: can a pre-existing bias explain convergent evolution between anadromous and non-anadromous morphs. Animal Behavior.

Garriga P, Manyosa J. 2002. The eye photoreceptor protein rhodopsin. Structural implications for retinal disease. FEBS Letters. 528:17-22.

Gladyshev VN, Jeang KT, Wootton JC, Hatfield DL. 1998. A new human selenium-containing protein. Purification, characterization, and cDNA sequence. Journal of Biological Chemistry. 273:8910-5.

Graham CH, Hartline HK. 1935. The response of single visual sense cells to lights of different wave lengths. Journal of General Physiology. 18:917-931.

Groot C, Margolis L. 1991. Pacific salmon life histories. University of British Columbia Press, Vancouver, p 564

Gross-Bellard M, Oudet P, Chambon P. 1973. Isolation of high-molecular-weight DNA from mammalian cells. European Journal of Biochemistry. 36:32-8.

Hafezi F, Marti A, Grimm C, Wenzel A, Reme CE. 1999. Differential DNA binding activities of the transcription factors AP-1 and Oct-1 during light-induced apoptosis of photoreceptors. Vision Research. 39:2511-8.

Hardiman G, Gannon F. 1996. Differential transferrin gene expression in Atlantic salmon (Salmo salar L) freshwater parr and seawater smolts. Journal of Applied Ichthyology-Zeitschrift Fur Angewandte Ichthyologie. 12:43-47.

Harvey CB, Williams GR. 2002. Mechanism of thyroid hormone action. Thyroid. 12:441-6.

Harvey RP. 1996. NK-2 homeobox genes and heart development. Developmental Biology. 178:203-16.

Hawryshyn CW. 2000. Ultraviolet polarization vision in fishes: possible mechanisms for coding e-vector. Philosphical Transactions of the Royal Society of London, Part B Biological Sciences. 355:1187-90.

Hawryshyn CW. 2003. Mechanisms of Ultraviolet Polarisation Vision in Fishes. In: Collin S, Marshall J (eds) Sensory Processing in the Aquatic Environment. Springer-Verlag, New York

Hawryshyn CW, Arnold MG, Bowering E, Cole RL. 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. 166:565-574.

Hawryshyn CW, Arnold MG, Chaisson DJ, Martin PC. 1989. The ontogeny of ultraviolet photosensitivity in rainbow trout (*Salmo gairdneri*). Visual Neuroscience. 2:247-54.

Hawryshyn CW, Haimberger TJ, Deutschlander ME. 2001. Microspectrophotometric measurements of vertebrate photoreceptors using CCD-based detection technology. Journal of Experimental Biology. 204:2431-8.

Hawryshyn CW, Harosi FI. 1994. Spectral characteristics of visual pigments in rainbow trout (*Oncorhynchus mykiss*). Vision Research. 34:1385-92.

Hawryshyn CW, Martens G, Allison WT, Anholt BR. 2003. Regeneration of ultraviolet-sensitive cones in the retinal cone mosaic of thyroxin-challenged post-juvenile rainbow trout (*Oncorhynchus mykiss*). Journal of Experimental Biology. 206:2665-73.

Hecht S. 1937. Rods, cones, and the chemical basis of vision. Physiological Reviews. 17:239-290.

Helvik JV, Drivenes O, Naess TH, Fjose A, Seo HC. 2001. Molecular cloning and characterization of five opsin genes from the marine flatfish Atlantic halibut (*Hippoglossus hippoglossus*). Visual Neuroscience. 18:767-80.

Hisatomi O, Kayada S, Aoki Y, Iwasa T, Tokunaga F. 1994. Phylogenetic relationships among vertebrate visual pigments. Vision Research. 34:3097-102.

Horsley V, Pavlath GK. 2002. NFAT: ubiquitous regulator of cell differentiation and adaptation. Journal of Cell Biology. 156:771-4.

Hubel DH. 1988. Eye, Brain, and Vision. W.H. Freeman, New York.

Humphries MM, Rancourt D, Farrar GJ, Kenna P, Hazel M, Bush RA, Sieving PA, Sheils DM, McNally N, Creighton P, Erven A, Boros A, Gulya K, Capecchi MR, Humphries P. 1997. Retinopathy induced in mice by targeted disruption of the rhodopsin gene. Nature Genetics. 15:216-9.

Hyatt GA, Schmitt EA, Fadool JM, Dowling JE. 1996. Retinoic acid alters photoreceptor development in vivo. Proceedings of the National Academy of Sciences USA. 93:13298-303.

Jetten AM, Kurebayashi S, Ueda E. 2001. The ROR nuclear orphan receptor subfamily: critical regulators of multiple biological processes. Progress in Nucleic Acid Research: Molecular Biology. 69:205-47.

Johnson RL, Grant KB, Zankel TC, Boehm MF, Merbs SL, Nathans J, Nakanishi K. 1993. Cloning and expression of goldfish opsin sequences. Biochemistry. 32:208-14.

Jomary C, Chatelain G, Michel D, Weston A, Neal MJ, Jones SE. 1999a. Effect of targeted expression of clusterin in photoreceptor cells on retinal development and differentiation. Journal of Cellular Science. 112:1455-64.

Jomary C, Darrow RM, Wong P, Organisciak DT, Neal MJ, Jones SE. 1999b. Lack of causal relationship between clusterin expression and photoreceptor apoptosis in light-induced retinal degeneration. Journal of Neurochemistry. 72:1923-9.

Jomary C, Neal MJ, Iwata K, Jones SE. 1997. Localization of tissue inhibitor of metalloproteinases-3 in neurodegenerative retinal disease. Neuroreport. 8:2169-72.

Jomary C, Neal MJ, Jones SE. 1995. Increased expression of retinal TIMP3 mRNA in simplex retinitis pigmentosa is localized to photoreceptor-retaining regions. Journal of Neurochemistry. 64:2370-3.

Jones SE, Jomary C, Grist J, Stewart HJ, Neal MJ. 2000. Altered expression of secreted frizzled-related protein-2 in retinitis pigmentosa retinas. Investigative Ophthalmology and Visual Science. 41:1297-301.

Jones SE, Jomary C, Neal MJ. 1994. Expression of TIMP3 mRNA is elevated in retinas affected by simplex retinitis pigmentosa. FEBS Letters. 352:171-4.

Jones SE, Meerabux JM, Yeats DA, Neal MJ. 1992. Analysis of differentially expressed genes in retinitis pigmentosa retinas. Altered expression of clusterin mRNA. FEBS Letters. 300:279-82.

Kamermans M, van Dijk BW, Spekreijse H. 1991. Color opponency in cone-driven horizontal cells in carp retina. Aspecific pathways between cones and horizontal cells. Journal of General Physiology. 97:819-43.

Karin M, Lin A. 2002. NF-kappaB at the crossroads of life and death. Nature Immunology. 3:221-7.

Karin M, Liu Z, Zandi E. 1997. AP-1 function and regulation. Current Opinions in Cell Biology. 9:240-6.

Kawamura S, Yokoyama S. 1996. Phylogenetic relationships among short wavelength-sensitive opsins of American chameleon (*Anolis carolinensis*) and other vertebrates. Vision Research. 36:2797-804.

Kelley MW, Turner JK, Reh TA. 1995a. Ligands of steroid/thyroid receptors induce cone photoreceptors in vertebrate retina. Development. 121:3777-85.

Kelley MW, Turner JK, Reh TA. 1995b. Regulation of proliferation and photoreceptor differentiation in fetal human retinal cell cultures. Investigative Ophthalmology and Visual Science. 36:1280-9.

Kelley MW, Williams RC, Turner JK, Creech-Kraft JM, Reh TA. 1999. Retinoic acid promotes rod photoreceptor differentiation in rat retina in vivo. Neuroreport. 10:2389-94.

Kennedy MJ, Lee KA, Niemi GA, Craven KB, Garwin GG, Saari JC, Hurley JB. 2001. Multiple phosphorylation of rhodopsin and the in vivo chemistry underlying rod photoreceptor dark adaptation. Neuron. 31:87-101.

Kepler. 1604. Ad Vitellionem Paralipomena, Quibus Astronomiae Pars Optica Traditur.

Kolb H, Fernandez E, Nelson R, Jones BW. 2003. Webvision: The Organization of the Retina and Visual System. University of Utah

Korotkov KV, Kumaraswamy E, Zhou Y, Hatfield DL, Gladyshev VN. 2001. Association between the 15-kDa selenoprotein and UDP-glucose:glycoprotein glucosyltransferase in the endoplasmic reticulum of mammalian cells. Journal of Biological Chemistry. 276:15330-6.

Krishnamoorthy RR, Crawford MJ, Chaturvedi MM, Jain SK, Aggarwal BB, Al-Ubaidi MR, 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. 274:3734-43.

Kumar S, Tamura K, Jakobsen IB, Nei M. 2001. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics. 17:1244-5.

Kumaraswamy E, Korotkov KV, Diamond AM, Gladyshev VN, Hatfield DL. 2002. Genetic and functional analysis of mammalian Sep15 selenoprotein. Methods in Enzymology. 347:187-97.

Kumaraswamy E, Malykh A, Korotkov KV, Kozyavkin S, Hu Y, Kwon SY, Moustafa ME, Carlson BA, Berry MJ, Lee BJ, Hatfield DL, Diamond AM, Gladyshev VN. 2000. Structure-expression relationships of the 15-kDa selenoprotein gene. Possible role of the protein in cancer etiology. Journal of Biological Chemistry. 275:35540-7.

Kunz YW, Wildenburg G, Goodrich L, Callaghan E. 1994. The fate of ultraviolet receptors in the retina of the Atlantic salmon (*Salmo salar*). Vision Research. 34:1375-83.

Kuo MH, Allis CD. 1999. In vivo cross-linking and immunoprecipitation for studying dynamic Protein:DNA associations in a chromatin environment. Methods. 19:425-33.

Lee SK, Kim JH, Lee YC, Cheong J, Lee JW. 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. 275:12470-4.

Lem J, Krasnoperova NV, Calvert PD, Kosaras B, Cameron DA, Nicolo M, Makino CL, Sidman RL. 1999. Morphological, physiological, and biochemical changes in rhodopsin knockout mice. Proceedings of the National Academy of Sciences USA. 96:736-41.

Liang P, Pardee AB. 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science. 257:967-71.

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. 272:21467-72.

Lipkin VM, Obukhov AN. 2000. Structure and mechanisms of function of visual system proteins. Membrance and Cell Biology. 13:165-93.

Ma JX, Kono M, Xu L, Das J, Ryan JC, Hazard ES, 3rd, Oprian DD, Crouch RK. 2001. Salamander UV cone pigment: sequence, expression, and spectral properties. Visual Neuroscience. 18:393-9.

Maeda I, Kohara Y, Yamamoto M, Sugimoto A. 2001. Large-scale analysis of gene function in Caenorhabditis elegans by high-throughput RNAi. Current Biology. 11:171-6.

Mani SS, Batni S, Whitaker L, Chen S, Engbretson G, Knox BE. 2001. Xenopus rhodopsin promoter. Identification of immediate upstream sequences necessary for high level, rod-specific transcription. Journal of Biological Chemistry. 276:36557-65.

Mathas S, Hinz M, Anagnostopoulos I, Krappmann D, Lietz A, Jundt F, Bommert K, Mechta-Grigoriou F, Stein H, Dorken B, Scheidereit C. 2002. Aberrantly expressed c-Jun and JunB are a hallmark of Hodgkin lymphoma cells, stimulate proliferation and synergize with NF-kappa B. European Molecular Biology Organization Journal. 21:4104-13.

Mathews DH, Sabina J, Zuker M, Turner DH. 1999. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. Journal of Molecular Biology. 288:911-40.

McArthur AG, Hegelund T, Cox RL, Stegeman JJ, Liljenberg M, Olsson U, Sundberg P, Celander MC. 2003. Phylogenetic analysis of the cytochrome P450 3 (CYP3) gene family. The Journal of Molecular Evolution. 57:200-211.

McManus MT, Petersen CP, Haines BB, Chen J, Sharp PA. 2002. Gene silencing using micro-RNA designed hairpins. RNA. 8:842-50.

Mears AJ, Kondo M, Swain PK, Takada Y, Bush RA, Saunders TL, Sieving PA, Swaroop A. 2001. Nrl is required for rod photoreceptor development. Nature Genetics. 29:447-52.

Merbs SL, Nathans J. 1992. Absorption spectra of human cone pigments. Nature. 356:433-5.

Meyer A, Kocher TD, Basasibwaki P, Wilson AC. 1990. Monophyletic origin of Lake Victoria cichlid fishes suggested by mitochondrial DNA sequences. Nature. 347:550-3.

Milam AH, Rose L, Cideciyan AV, Barakat MR, Tang WX, Gupta N, Aleman TS, Wright AF, Stone EM, Sheffield VC, Jacobson SG. 2002. The nuclear receptor NR2E3 plays a role in human retinal photoreceptor differentiation and degeneration. Proceedings of the National Academy of Sciences USA. 99:473-8.

Minamoto T, Shimizu I. 2002. A novel isoform of vertebrate ancient opsin in a smelt fish, *Plecoglossus altivelis*. Biochemistry and Biophysical Research Communications. 290:280-6.

Mitton KP, Swain PK, Chen S, Xu S, Zack DJ, Swaroop A. 2000. The leucine zipper of NRL interacts with the CRX homeodomain. A possible mechanism of transcriptional synergy in rhodopsin regulation. Journal of Biological Chemistry. 275:29794-9.

Mizuno S, Ura K, Onodera Y, Fukada H, Misaka N, Hara A, Adachi S, Yamauchi K. 2001. Changes in transcript levels of gill cortisol receptor during smoltification in wild masu salmon, *Oncorhynchus masou*. Zoological Science. 18:853-860.

Munz FW, Beatty DD. 1965. A critical analysis of the visual pigments of salmon and trout. Vision research. 5:1-17.

Murata S, Takasaki N, Saitoh M, Okada N. 1993. Determination of the phylogenetic relationships among Pacific salmonids by using short interspersed elements (SINEs) as temporal landmarks of evolution. Proceedings of the National Academy of Sciences USA. 90:6995-9.

Murata S, Takasaki N, Saitoh M, Tachida H, Okada N. 1996. Details of retropositional genome dynamics that provide a rationale for a generic division: the distinct branching of all the pacific salmon and trout (*Oncorhynchus*) from the Atlantic salmon and trout (*Salmo*). Genetics. 142:915-26.

Nei M, Kumar S. 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.

Neitz M, Neitz J. 1995. Numbers and ratios of visual pigment genes for normal red-green color vision. Science. 267:1013-6.

Ng L, Hurley JB, Dierks B, Srinivas M, Salto C, Vennstrom B, Reh TA, Forrest D. 2001. A thyroid hormone receptor that is required for the development of green cone photoreceptors. Nature Genetics. 27:94-8.

Nir I, Agarwal N, Papermaster DS. 1990. Opsin gene expression during early and late phases of retinal degeneration in rds mice. Experimental Eye Research. 51:257-67.

Novales Flamarique I, Hawryshyn CW. 1993. Spectral Characteristics of Salmonid Migratory Routes from Southern Vancouver-Island (British-Columbia). Canadian Journal of Fisheries and Aquatic Sciences. 50:1706-1716.

Novales Flamarique I, Hawryshyn CW. 1997. Is the use of underwater polarized light by fish restricted to crepuscular time periods? Vision Research. 37:975-89.

Novales Flamarique I, Hendry A, Hawryshyn CW. 1992. The Photic Environment of a Salmonid Nursery Lake. Journal of Experimental Biology. 169:121-141.

Oakley TH, Phillips RB. 1999. Phylogeny of salmonine fishes based on growth hormone introns: Atlantic (*Salmo*) and Pacific (*Oncorhynchus*) salmon are not sister taxa. Molecular Phylogenetics and Evolution. 11:381-393.

Olsen RE, Mortensen A. 1997. The influence of dietary astaxanthin and temperature on flesh colour in Arctic charr *Salvelinus alpinus* L. Aquaculture Research. 28:51-58.

O'Riordan M, Grosschedl R. 2000. Transcriptional regulation of early B-lymphocyte differentiation. Immunological Reviews. 175:94-103.

Osinov AG, Lebedev VS. 2000. Genetic divergence and phylogeny of the Salmoninae based on allozyme data. Journal of Fish Biology. 57:354-381.

Osorio D, Vorobyev M. 1996. Colour vision as an adaptation to frugivory in primates. Proceedings of the Royal Society of London Part B: Biological Sciences. 263:593-9.

Palacios AG, Varela FJ, Srivastava R, Goldsmith TH. 1998. Spectral sensitivity of cones in the goldfish, Carassius auratus. Vision Research. 38:2135-46.

Parhar IS, Iwata M. 1996. Intracerebral expression of gonadotropin-releasing hormone and growth hormone-releasing hormone is delayed until smoltification in the salmon. Neuroscience Research. 26:299-308.

Parhar IS, Pfaff DW, Schwanzel-Fukuda M. 1996. Gonadotropin-releasing hormone gene expression in teleosts. Brain Research: Molecular Brain Research. 41:216-27.

Parkyn DC, Hawryshyn CW. 2000. Spectral and ultraviolet-polarisation sensitivity in juvenile salmonids: a comparative analysis using electrophysiology. Journal of Experimental Biology. 203:1173-91.

Patient RK, McGhee JD. 2002. The GATA family (vertebrates and invertebrates). Current Opinion in Genetics and Development. 12:416-22.

Perez P, Schonthal A, Aranda A. 1993. Repression of c-fos gene expression by thyroid hormone and retinoic acid receptors. Journal of Biological Chemistry. 268:23538-43.

Perkins BD, Kainz PM, O'Malley DM, Dowling JE. 2002. Transgenic expression of a GFP-rhodopsin COOH-terminal fusion protein in zebrafish rod photoreceptors. Visual Neuroscience. 19:257R-264R.

Philp AR, Bellingham J, Garcia-Fernandez J, Foster RG. 2000. A novel rod-like opsin isolated from the extra-retinal photoreceptors of teleost fish. FEBS Letters. 468:181-8.

Plate EM, Adams BA, Allison WT, Martens G, Hawryshyn CW, Eales JG. 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 comparative Endocrinology. 127:59-65.

Polyak SL. 1941. The Retina. University of Chicago Press, Chicago.

Prior HM, Walter MA. 1996. SOX genes: architects of development. Molecular Medicine. 2:405-12.

Provencio I, Loew ER, Foster RG. 1992. Vitamin A2-based visual pigments in fully terrestrial vertebrates. Vision Research. 32:2201-8.

Rehemtulla A, Warwar R, Kumar R, Ji X, Zack DJ, Swaroop A. 1996. The basic motif-leucine zipper transcription factor Nrl can positively regulate rhodopsin gene expression. Proceedings of the National Academy of Sciences USA. 93:191-5.

Rivolta C, Berson EL, Dryja TP. 2001. Dominant Leber congenital amaurosis, cone-rod degeneration, and retinitis pigmentosa caused by mutant versions of the transcription factor CRX. Human Mutation. 18:488-98.

Rivolta C, Sharon D, DeAngelis MM, Dryja TP. 2002. Retinitis pigmentosa and allied diseases: numerous diseases, genes, and inheritance patterns. Human Molecular Genetics. 11:1219-27.

Robertson KA, Hill DP, Kelley MR, Tritt R, Crum B, Van Epps S, Srour E, Rice S, Hromas R. 1998. The myeloid zinc finger gene (MZF-1) delays retinoic acid-induced apoptosis and differentiation in myeloid leukemia cells. Leukemia. 12:690-8.

Rodieck RW. 1973. The Vertebrate Retina: Principles of Structure and Function. W.H. Freeman, San Fransico.

Rodieck RW. 1998. The First Steps in Seeing. Sinauer Associates, Inc., Sunderland, MA.

Rost B, Sander C. 1993. Prediction of protein secondary structure at better than 70% accuracy. Journal of Molecular Biology. 232:584-99.

Rost B, Sander C. 1994. Combining evolutionary information and neural networks to predict protein secondary structure. Proteins. 19:55-72.

Rzhetsky A, Nei M. 1993. Theoretical Foundation of the Minimum-Evolution Method of Phylogenetic Inference. Molecular Biology and Evolution. 10:1073-1095.

Sakamoto T, Hirano T, Madsen SS, Nishioka RS, Bern HA. 1995. Insulin-Like Growth-Factor-I Gene-Expression During Parr-Smolt Transformation of Coho Salmon. Zoological Science. 12:249-252.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Scherr M, Morgan MA, Eder M. 2003. Gene Silencing Mediated by Small Interfering RNAs in Mammalian Cells. Current Medical Chemistry. 10:245-56.

Schuman BN, Meyers MK. 1969. The Human Eye. Atheneum, New York.

Seehausen O, Mayhew PJ, Van Alphen JJM. 1999a. Evolution of colour patterns in East African cichlid fish. Journal of Evolutionary Biology. 12:514-534.

Seehausen O, Van Alpen JJM, Witte F. 1999b. Can ancient colour polymorphisms explain why some cichlid lineages speciate rapidly under disruptive sexual selection? Belgian Journal of Zoology. 129:43-60.

Seehausen O, van Alphen JJM. 1998. The effect of male coloration on female mate choice in closely related Lake Victoria cichlids (*Haplochromis nyererei* complex). Behavioral Ecology and Sociobiology. 42:1-8.

Seehausen O, van Alphen JJM, Lande R. 1999c. Color polymorphism and sex ratio distortion in a cichlid fish as an incipient stage in sympatric speciation by sexual selection. Ecology Letters. 2:367-378.

Shaulian E, Karin M. 2002. AP-1 as a regulator of cell life and death. Nature Cell Biology. 4:E131-6.

Shedlock AM, Parker JD, Crispin DA, Pietsch TW, Burmer GC. 1992. Evolution of the salmonid mitochondrial control region. Molecular Phylogenetics and Evolution. 1:179-92.

Shen Q, Chu FF, Newburger PE. 1993. Sequences in the 3'-untranslated region of the human cellular glutathione peroxidase gene are necessary and sufficient for selenocysteine incorporation at the UGA codon. Journal of Biological Chemistry. 268:11463-9.

Shi Y, Radlwimmer FB, Yokoyama S. 2001. Molecular genetics and the evolution of ultraviolet vision in vertebrates. Proceedings of the National Academy of Sciences USA. 98:11731-6.

Shi Y, Yokoyama S. 2003. Molecular analysis of the evolutionary significance of ultraviolet vision in vertebrates. Proceedings of the National Academy of Sciences USA.

Singer TD, Clements KM, Semple JW, Schulte PM, Bystriansky JS, Finstad B, Fleming IA, McKinley RS. 2002. Seawater tolerance and gene expression in two strains of Atlantic salmon smolts. Canadian Journal of Fisheries and Aquatic Sciences. 59:125-135.

Sompayrac L, Jane S, Burn TC, Tenen DG, Danna KJ. 1995. Overcoming limitations of the mRNA differential display technique. Nucleic Acids Research. 23:4738-9.

Staley KB, Ewing RD. 1992. Purine levels in the skin of juvenile coho salmon (*Oncorhynchus kisutch*) during parr-smolt transformation and adaptation to seawater. Comparative Biochemistry and Physiology B. 101:447-52.

Stein B, Baldwin AS, Jr., Ballard DW, Greene WC, Angel P, Herrlich P. 1993. Cross-coupling of the NF-kappa B p65 and Fos/Jun transcription factors produces potentiated biological function. European Molecular Biology Organization Journal. 12:3879-91.

Stell WK, Lightfood DO, Wheeler TG, Leeper HF. 1975. Goldfish retina: functional polarization of cone horizontal cell dendrites and synapses. Science. 190:989-90.

Stenkamp DL, Hisatomi O, Barthel LK, Tokunaga F, Raymond PA. 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. 37:363-76.

Steven DM. 1947. Carotenoid pigmentation in trout. Nature. 160:540-541.

Sugawara T, Terai Y, Okada N. 2002. Natural selection of the rhodopsin gene during the adaptive radiation of East African Great Lakes cichlid fishes. Molecular Biology and Evolution. 19:1807-11.

Sui G, Soohoo C, Affar el B, Gay F, Shi Y, Forrester WC. 2002. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. Proceedings of the National Academy of Sciences USA. 99:5515-20.

Takahashi Y, Ebrey TG. 2003. Molecular basis of spectral tuning in the newt short wavelength sensitive visual pigment. Biochemistry. 42:6025-34.

ten Berge D, Brouwer A, Korving J, Martin JF, Meijlink F. 1998. Prx1 and Prx2 in skeletogenesis: roles in the craniofacial region, inner ear and limbs. Development. 125:3831-42.

ten Berge D, Brouwer A, Korving J, Reijnen MJ, van Raaij EJ, Verbeek F, Gaffield W, Meijlink F. 2001. Prx1 and Prx2 are upstream regulators of sonic hedgehog and control cell proliferation during mandibular arch morphogenesis. Development. 128:2929-38.

Terai Y, Mayer WE, Klein J, Tichy H, Okada N. 2002. The effect of selection on a long wavelength-sensitive (LWS) opsin gene of Lake Victoria cichlid fishes. Proceedings of the National Academy of Sciences USA. 99:15501-6.

Thomas RS, Tymms MJ, McKinlay LH, Shannon MF, Seth A, Kola I. 1997. ETS1, NFkappaB and AP1 synergistically transactivate the human GM-CSF promoter. Oncogene. 14:2845-55.

Toy J, Norton JS, Jibodh SR, Adler R. 2002. Effects of homeobox genes on the differentiation of photoreceptor and nonphotoreceptor neurons. Investigative Ophthalmology and Visual Science. 43:3522-9.

Twig G, Levy H, Perlman I. 2003. Color opponency in horizontal cells of the vertebrate retina. Progress in Retinal Eye Research. 22:31-68.

Valberg A, Lee BB. 1991. From Pigments to Perception. Plenum Press, London.

Veldhoen K, Hawryshyn CW, Beaudet L, Runions J, Sharma J. 1999. Antibody labelling of blue-sensitive cones in the retina of teleost fishes. Canadian Journal of Zoology. 77:1733-9.

Vihtelic TS, Doro CJ, Hyde DR. 1999. Cloning and characterization of six zebrafish photoreceptor opsin cDNAs and immunolocalization of their corresponding proteins. Visual Neuroscience. 16:571-85.

Walczak R, Hubert N, Carbon P, Krol A. 1997. Solution structure of SECIS, the mRNA element required for eukaryotic selenocysteine insertion--interaction studies with the SECIS-binding protein SBP. Biomedical and Environmental Science. 10:177-81.

Warburton D, Schwarz M, Tefft D, Flores-Delgado G, Anderson KD, Cardoso WV. 2000. The molecular basis of lung morphogenesis. Mechanisms of Development. 92:55-81.

Weinmann AS, Farnham PJ. 2002. Identification of unknown target genes of human transcription factors using chromatin immunoprecipitation. Methods. 26:37-47.

Wilson MF. 1997. Variation in salmonid life histories: patterns and perspectives. Res. Pap. PNW-RP-498. Department of Agriculture, Forest Service, Pacific Northwest Research Station, Portland, p 50

Wolff E, Last RJ. 1968. Anatomy of the Eye and Orbit, Including the Central Connections, Development, and Comparative Anatomy of the Visual Apparatus. W. B. Saunders Co., Philadelphia.

Wolfsberg TG, Wetterstrand KA, Guyer MS, Collins FS, Baxevanis AD. 2002. A user's guide to the human genome. Nature Genetics. 32:1-79.

Wu DM, Schneiderman T, Burgett J, Gokhale P, Barthel L, Raymond PA. 2001. Cones regenerate from retinal stem cells sequestered in the inner nuclear layer of adult goldfish retina. Investigative Ophthalmology and Visual Science. 42:2115-24.

Xia H, Mao Q, Paulson HL, Davidson BL. 2002. siRNA-mediated gene silencing in vitro and in vivo. Nature Biotechnology. 20:1006-10.

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. 43:3489-94.

Yen PM. 2001. Physiological and molecular basis of thyroid hormone action. Physiological Reviews. 81:1097-142.

Yokoyama S. 1995. Amino acid replacements and wavelength absorption of visual pigments in vertebrates. Molecular Biology and Evolution. 12:53-61.

Yokoyama S. 1996. Molecular evolution of retinal and nonretinal opsins. Genes Cells. 1:787-94.

Yokoyama S. 2000a. Color vision of the coelacanth (*Latimeria chalumnae*) and adaptive evolution of rhodopsin (RH1) and rhodopsin-like (RH2) pigments. Journal of Heredity. 91:215-20.

Yokoyama S. 2000b. Molecular evolution of vertebrate visual pigments. Progress in Retinal Eye Research. 19:385-419.

Yokoyama S. 2000c. Phylogenetic analysis and experimental approaches to study color vision in vertebrates. Methods in Enzymology. 315:312-25.

Yokoyama S, Blow NS, Radlwimmer FB. 2000. Molecular evolution of color vision of zebra finch. Gene. 259:17-24.

Yokoyama S, Radlwimmer FB. 1999. The molecular genetics of red and green color vision in mammals. Genetics. 153:919-32.

Yokoyama S, Radlwimmer FB. 2001. The molecular genetics and evolution of red and green color vision in vertebrates. Genetics. 158:1697-710.

Yokoyama S, Shi Y. 2000. Genetics and evolution of ultraviolet vision in vertebrates. FEBS Letters. 486:167-72.

Yokoyama S, Tada T. 2000. Adaptive evolution of the African and Indonesian coelacanths to deep-sea environments. Gene. 261:35-42.

Yokoyama S, Tada T. 2003. The spectral tuning in the short wavelength-sensitive type 2 pigments. Gene. 306:91-8.

Yokoyama S, Zhang H, Radlwimmer FB, Blow NS. 1999. Adaptive evolution of color vision of the Comoran coelacanth (*Latimeria chalumnae*). Proceedings of the National Academy of Sciences USA. 96:6279-84.

Zweidler-Mckay PA, Grimes HL, Flubacher MM, Tsichlis PN. 1996. Gfi-1 encodes a nuclear zinc finger protein that binds DNA and functions as a transcriptional repressor. Molecular Cellular Biology. 16:4024-34.

Appendix 1 – Nucleotide Sequences Submitted to GenBank

Oncorhynchus mykiss RH1 Open Reading Frame GenBank Accession # AF425072
ATGAACGGCACAGAGGGACCAGATTTCTACGTCCCTATGTCCAATGCTACTGGCATTGTTAGGAACCCC
TATGAATACCCCCAGTACTACCTTGTCAGCCCGGCGGCGTACTCACTCATGGCTGCCTACATGTTCTTC
CTCATCCTCACCGGATTCCCCATCAACTTCCTCACACTCTACGTCACCATTGAGCACAAAAAGCTGAGG
ACCGCCCTGAACTACATCCTGCTGAACCTGGCTGTGGCCGATCTCTTCATGGTAATCGGAGGCTTCACC
ACTACGATGTACACCTCCATGCATGGCTATTTCGTCTTTGGAAGAACGGGCTGCAACATCGAGGGATTC
TTTGCTACCCATGGTGGTGAGATTGCCCTGTGGTCCCTGGTTGTCCTGGCTATCGAGAGGTGGTTGGTC
GTCTGCGAACCTATTAGCAACTTCCGCTTCAGTGAGACCCATGCCATCATGGGCGTGGCCTTTACCTGG
GTCATGGCTGCTGCTTGCTCCGTGCCCCCTCTGCTTGGCTGGTCCCGCTATATCCCCGAAGGCATGCAG
TGCTCATGTGGAATTGACTACTACACACGCGCCCCTGACATCAACAATGAGTCCTTTGTCATCTACATG
TTCGTTGTCCACTTTATGATTCCCCTGTTCATCATCTCCTTCTGCTACGGCAACCTGCTCTGCGCTGTCA
AGGCAGCTGCCGCCGCCCAGCAGGAGTCTGAGACCACCCAGAGGGCTGAGAGGGAAGTGACCCGAAT
GGTCATCATGATGGTCGTCTCCTTCCTAGTGTGCTGGGTGCCCTACGCCAGCGTGGCCTGGTATATCTTC
TGCAACCAGGGATCAGAGTTCGGCCCTGTCTTCATGACAATTCCGGCATTCTTTGCCAAGAGTTCGTCC
CTGTACAACCCTCTCATCTACGTGTTGATGAACAAGCAGTTCCGCAACTGCATGATCACCACCCTGTGC
TGTGGGAAGAACCCCTTCGAGGAGGAGGAGGGAGCCTCCACCACTGCCTCCAAGACCGAGGCCTCCTC
CGTGTCCTCCAGCTCCGTGGCTCCTGCATAA

Oncorhynchus mykiss RH2 Open Reading Frame GenBank Accession # AF425076
ATGCAGAACGGCACAGAAGGAAGCAACTTCTACATTCCCATGTCCAACCGGACTGGGCTTGTAAGGAG
TCCTTTTCTATACCAACAGTACTACTTGGCGCCTCCATGGCAATACTATTGTCTTGCCGTCTATACGTTC
TTCCTGATCTGCTTTGGATTCCCAATCAACGGTCTGACCCTGTATGTCACAGCCACAAACAAGAAGCTT
CGGCAACCCCTTAACTTCATCCTGGTCAACTTGGCTGCAGCTGGAATGATAATGGTCCTCTTTGGATTC
ACCATCACCATCACATCTGCTGTCAATGGTTACTACATCTGGGGACCATTGGGCTGTGCTATTGAGGGC
TTCATGGCTACACTTGGAGGTGAGGTTGCCCTGTGGTCTCTGGTAGTGCTGGCTGTCGAGAGATACATT
GTGGTCTGCAAGCCCATGGGCAGCTTCACGTTCACTTCCACCCATGCTGGTGCTGGTGTGGCATTCACC
TGGATAGCTGCTATGGCTTGTGCTGCTCCCCCACTGCTTGGCTGGTCCAGGTACATCCCAGAGGGCATG
CAGTGCTCATGTGGACCTGACTACTACACCTTGGCCGAAGGCTTCAACAATGAATCATATGTGATCTAC
ATGTTTACCTGCCACTTCATCATCCCAGTCTGCTTGATCGCCTTCACTTATGGAAGTCTTGTCCTGACAG
TCAAGGCGGCTGCAGCTCCCCAGCAGGACTCAGCATCTACCCAGAAAGCTGAGAAGGAAGTGACACGT
ATGTGCATCCTGATGGTTAGGGGCTTCATGATCGCCTGGACCCCCTATGCCAGCCTTGCTGCCTACATC
TTCTTCAACAAAGGAATTGCCTTCAGTGCCCAGTCCATGGCTATACCTGCCTTCTTCTCCAAGAGCTCA
GCCTTGTTCAACCCCATTATCTATGTGATGATGAACAAACAGTTCCGTGGATGCATGCTGGCCACTGTA
GGGATGAAAGCAGAAGAAGATGAAACTTCTGTGTCAACAAGCAAGACAGAAGTGTCTTCTGTGGGCCC
TGCATAG

Oncorhynchus mykiss LWS Open Reading Frame GenBank Accession # AF425073
ATGGCAGAAAGCTGGGGAAGTGCTGCTTATGCAGCCAGGCGACAAAACCAAGATACAACGAGAGAAT
CTTCCTTTACCTTCACCAACAGCAATAACACCAAAGATCCCTTTGAGGGCCCCAACTACCACATTGCTC
CAAGATGGGTGTACATTGTTTCAACACTATGGATGATCATAGTGGTCATCCTCTCAGTCTTCACCAATG
GCCTGGTACTGGTGGCCACTGCAAAATTCAAGAAGCTCCAACACCCTCTGAACTGGATCTTGGTCAACC
TTGCTATTGCTGACATTGGAGAAACACTTTTGGCAAGCACCATCAGCGTTTGCAACCAGTTTTTTGGCT
ACTTCATTCTGGGACATCCAATGTGTGTCTTTGAGGGATACACTGTCTCCACTTGCGGAATTGCTGCTCT
GTGGTCCCTGGCTGTCATCTCTTGGGAGAGATGGGTGGTGGTGTGCAAGCCCTTTGGAAGTGTCAAGTT
TGATGCCAAATGGGCCATGGGAGGCATTATCTTCTCCTGGGTCTGGTCTGCTTTCTGGTGTGCCCCCCC
CATCTTTGGCTGGAGCAGGTACTGGCCTCACGGCCTGAAGACCTCCTGCGGACCTGATGTGTTCGGAGG
CAATGAGGATCCTGGAGTCAAATCCTACATGATTACTCTCATGATTACATGCTGCTTCTTCCCCCTGTTC
GTGATCATCTTCTGCTACATTTTCGTGTGGCTAGCCATTCGTGCTGTTGCTCGCCAAACAAAAGACTCTG
AGTCAACACAGAAGGCCGAGAAGGAAGTGTCCAGGATGGTTGTTGTCGTGATTATAGCTTACTGTGTA
TGCTGGGGACCTTATACCTGCTTTGCCTGCTTTGCTGCGGCTAACCCTGGGTATGCTTTCCACCCTCTGG
CAGCCGCAATTCCTGCCTACTTTGCCAAGAGCGCCACCATCTACAATCCAGTTATATATGTCTTCATGA
ACAAACAGTTCCGTACTTGCATCATGCAGCTCTTTGGAAAGGCAGAAGATGATGGCACTGAAGTGTCT
ACATCAAAAACAGAGGTTTCCTCTGTGGCACCTGCATAA

Oncorhynchus mykiss SWS1 Open Reading Frame GenBank Accession # AF425074
ATGGGAAAGGACTTCCATCTGTACGAGAACATCTCTAAGGTCAGCCCATTTGAGGGGCCACAGTATCA
CCTGGCCCCAATATGGGCTTTCTACCTACAGACTGCTTTCATGGGCTTTGTGTTCTTTGCTGGAACACCT
CTAAACTTTATCATTCTCGTGGTGACAGTGAAGTACAAGAAGCTGAGACAACCGCTGAACTACATCCT
GGTCAACGTCTCGTTAGCAGGTTTCATCTTTGTGACGTTTTCTGTGAGTCAAGTGTTTGTTTCTAGCGCG
AGAGGATACTACTTCCTGGGTTACACCTTGTGTGCAATGGAAGCTTGCATGGGTTCAATAGCAGGGTTG
GTGTCAGCTTGGTCCCTGGCTGTCCTTGCCTTTGAGAGATATGTGGTCATCTGCAAACCCTTCGGCACCT
TCAAATTTGACAACAACCAGGCTCTGGCGGCTGTTGGCTTCACCTGGGTCATGGGGATCGGATGTGCCA
CCCCACCGTTCTTCGGCTGGAGCAGGTATATCCCTGAGGGTCTGGGCTGCTCCTGTGGACCTGACTGGT
ACACAAACAATGAGGAGTACCACTGTGCCAGCTACACCAAATTCCTCATTGTTACCTGTTTCCTCATGC
CCATGTCCATCATCTTCTTCTCCTACTCCCAACTACTGGGAGCACTACGGGCTGTGGCAGCTGCACAGG
CTGAGTCAGCTTCCACCCAGAAGGCAGAAAAGGAAGTATCCAGGATGGTGATTGTGATGGTGTGCTCC
TTCATTCTGTGTTACGGCCCCTACGCCTTGGCAGGCTTGTACTTCGCCTATACAACAAGCGAGAACAAA
GACTACCGCCTGGTCACCATCCCTGCTTTCTTCCCTAAAAGCTCCTGTGTATACAACCCTCTCATTTACG
CCTTCATGAACAAACAGTTCAACGCCTGCATCATGGAGACAGTGTTGGGAAAGCAGATTGAGGAGACT
TCAGTTTCAGCCTCCAAGACAGAGGTCTCCACAGCCTAA

Oncorhynchus mykiss SWS2 Open Reading Frame GenBank Accession # AF425075
ATGAACACAATGAGGTCAAATGCTCGTCCCGTGGAGCTCCAGGAGGGATTCTACATCCCTATCGCGCT
GGATACCAACAACATCACTTCACTCAGCCCCTTCCTGGTTCCTCAGGACCACCTGGCGGGCAGTGCTAT
CTTCTATGGCATGTCATTTTTCATGTTCTTCCTATTTGTTGCCGGCACTGCTATCAACGTCCTTACCATCG
TGTGTACTATCCAGTTCAAGAAGCTGCGATCCCATCTCAACTACATTCTGGTGAACTTGGCAATAGCTA
ACCTGCTGGTGTCCATGTTTGGATCCTCCACCGCAAGCTTGTCCTTTGCCTACAGATACTTTATCATGGG
ATCGGTTGCATGCCAGATTGAGGGATTTACAGCTACTCTTGGCGGTATGATGAGCTTATGGTCTCTCTC
AGTAGTGGCGTTTGAAAGATGGTTGGTTATTTGTAAGCCAGTCGGTAACTTTCAATTCAAGAGCACACA
TGCAGTAATCGGCTGTGCAGTCACTTGGGTGTTTGGATTGGCTGCCAGTCTTCCCCCTCTGTTTGGCTGG
AGTAGATACATTCCAGAAGGTCTCCAGTGCTCCTGTGGACCAGACTGGTACACCACAAACAACAAATA
CAACAATGAGTCCTATGTGATGTTCCTCTTCTTCTTCTGCTTCGGAGTCCCATTCAGTGTCATTGTTTTCT
GCTATGCCCAGCTGCTCTTCATGATGAAGGCGGCTGCCGCGGCACAGGCAGACTCTGCCTCCACCCAG
AAGGCAGAGAAGGAGGTCACCAAGATGGTGGTGGTGATGGTGGTCGGCTTCCTAGTGTGCTGGATGCC
CTATGCCTCCTTTGCTGTCTGGGTTGTACAAAACCGTGGTGCACCCTTTGATCTCCGATTGGCCACCATA
CCTTCCTGCTTCTCCAAGGCCTCCACAGTCTACAACCCTCTCATCTATGTCTTCATGAATAAGCTGTTCC
GCTCATGCATGATGAATTTGCTGGGATTAAAGTCCGGGGATGATGAGGAAGCATCATCAACATCCTCG
GTCACTCAAGTGTCTTCTGCTGGTTAA

Oncorhynchus keta RH1 Open Reading Frame GenBank Accession # AY214141
ATGAACGGCACAGAGGGACCAGATTTCTACGTCCCTATGTCCAATGCTACTGGCATTGTTAGGAACCCC
TATGAATACCCCCAGTACTACCTTGTCAGCCCAGCGGCGTACTCACTCATGGCTGCCTACATGTTCTTC
CTCATCCTCACCGGCTTCCCCATCAACTTCCTCACACTCTACGTCACCATCGAGCACAAAAAGCTGAGG
ACCGCCCTGAACTACATCCTGCTGAACCTGGCTGTGGCCGATCTCTTCATGGTAATCGGAGGCTTTACC
ACTACGATGTACACCTCCATGCATGGCTATTTCGTCTTTGGAAGAACGGGCTGCAACATCGAGGGATTC
TTTGCTACCCATGGTGGTGAGATTGCCCTGTGGTCCCTGGTTGTCCTGGCTATTGAGAGGTGGTTGGTC
GTCTGCAAACCTATTAGCAACTTCCGCTTCACTGAGACCCATGCCATCATAGGCGTGGCCTTTACCTGG
GTCATGGCTGCTGCTTGCTCCGTGCCCCCTCTGCTTGGCTGGTCCCGCTATATCCCCGAAGGCATGCAG
TGCTCATGTGGAATTGACTACTACACACGCGCCCCTGACATCAACAATGAGTCCTTTGTCATCTACATG
TTCGTTGTCCACTTTATGATTCCCCTGTTCATCATCTCCTTCTGCTACGGCAACCTGCTCTGCGCTGTCA
AGGCAGCTGCCGCCGCCCAGCAGGAGTCTGAGACCACCCAGAGGGCTGAGAGGGAAGTGACCCGCAT
GGTCATCATGATGGTCGTCTCCTTCCTAGTGTGCTGGGTGCCCTACGCCAGCGTGGCCTGGTATATCTTC
TGCAACCAGGGATCAGAGTTCGGCCCCGTCTTCATGACAATTCCGGCATTCTTTGCCAAGAGTTCGTCC
CTGTACAACCCTCTCATCTACGTGTTGATGAACAAGCAGTTCCGTAACTGCATGATCACCACCCTGTGC
TGTGGGAAGAACCCCTTCGAGGAGGAGGAGGGAGCCTCCACCACTGCCTCCAAGACCGAGGCCTCCTC
CGTGTCCTCCAGCTCCGTGGCTCCTGCATAA

Oncorhynchus keta RH2 Open Reading Frame GenBank Accession # AY214142
ATGCAGAACGGCACAGAAGGAAGCAACTTCTACATTCCCATGTCCAATCGGACTGGGCTTGTAAGGGG
TCCTTTTCTATACCAACAGTACTACTTGGCGCCTCCATGGCAATTCTATGGTCTTGCTGTCTATATGTTC

TTCCTGATCTGCTTAGGATTCCCCATCAACGGGCTGACCTTGTATGTCACAGCCACAAACAAGAAGCTT
CGGCAACCCCTTAACTTCATCCTGGTCAACTTGGCTGCAGCTGGAATGATCATGGTCCTCTTTGGATTC
ACCATCACCATCACATCTGCTGTCAATGGTTACTTCATCTGGGGACCATTGGGCAGTGCCATTGAGGGC
TTCATGGCTACACTTGGAGGTGAGGTTGCCCTGTGGTCTCTGGTAGTGCTGGCTGTCGAGAGATACATT
GTGGTCTGCAAGCCCATGGGCAGCTTCACGCTCACTGCCTCCCACGCTGGTGCTGGTGTTGCATTCACC
TGGATAGCTGCTATGGCTTGTGCTGCTCCCCCACTGGTTGGCTGGTCCAGGTACATCCCAGAGGGCATG
CAATGCTCATGTGGACCTGACTACTACACCTTGGCCGAAGGCTTCAACAATGAATCATATGTGATCTAC
ATGTTCAGCTGCCACTTCATCATCCCAGTCTGTTTGATCGCCTTCACTTATGGAAGTCTTGTCCTCACAG
TCAAGGCGGCTGCAGCTTCCCAGCAGGACTCAGCATCTACCCAGAAAGCTGAGAAGGAAGTGACACGT
ATGTGCATCCTGATGGTTTGTGGTTTCATGATCGCCTGGACCCCCTATGCCACCCTCGCTGCCTACATCT
TCTTCAACAAAGGAATTGCCTTCAGTGCCCAGTCCATGGCTATACCTGCCTTCTTCTCCAAGAGCTCAG
CTTTGTTCAACCCCATTATCTATGTTCTGATGAACAAACAGTTCCGTGGCTGCATGCTGGCCACTGTAG
GGATGAAAGCAGAAGAAGATGAAACTTCTGTGTCAACAAGCAAGACAGAAGTGTCTTCTGTGGGACCT
GCATAG

Oncorhynchus keta LWS Open Reading Frame GenBank Accession # AY214140
ATGGCAGAAAGCTGGGGTAGTGCTGCTTATGCAGCCAGGCGACAAAACCAAGATACAACGAGAGAAT
CTTCCTTTACCTTCACCAACAGCAATAACACCAAAGATCCCTTTGAGGGCCCTAACTACCACATTGCTC
CAAGATGGGTGTACAATGTTTCAACACTTTGGATGATCATAGTGGTCATCCTCTCAGTCTTCACCAATG
GCCTGGTACTGGGGGCCACTGCAAAATTCAAGAAGCTCCAACACCCTCTGAACTGGATCTTGGTCAAC
CTTGCTATTGCTGACATTGGAGAAACACTTTTGGCAAGCACCATCAGCGTTTGCAACCAGTTTTTTGGC
TACTTCATTCTGGGACATCCAATGTGTGTCTTTGAGGGATACACTGTCTCCACTTGCGGAATTGCTGCTC
TGTGGTCCCTGGCTGTCATCTCTTGGGAGAGATGGGTGGTGGTGTGCAAGCCCTTTGGAAGTGTCAAGT
TTGATGCCAAATGGGCCATGGGAGGCATTATCTTCTCCTGGGTCTGGTCTGCTTTCTGGTGTGCCCCTCC
CATCTTTGGCTGGAGCAGGTACTGGCCTCACGGCCTGAAGACTTCCTGCGGACCTGATGTGTTCGGAGG
CAATGAGGATCCTGGAGTCAAGTCCTACATGATTACTCTCATGATTACATGCTGCTTCTTCCCCCTGGTC
GTCATCATCTTCTGCTACATTTTCGTGTGGCTAGCCATTCGTGGTGCTGCGCAACAGAAAAAAGACTCT
GAGTCAACACAGAAGCCCGAGAAGGAAGTGTCCAGGATGGTTGTTGTCATGATTATAGCTTACTGTGT
ATGCTGGGGACCTTATACCTGCTTTGCCTGCTTTGCTGCGGCTAACCCTGGGTATGCTTTCCACCCTCTG
GCAGCCGCAATTCCTGCCTACTTTGCCAAGAGCGCCACCATCTACAATCCAGTTATATATGTCTTCATG
AACAAACAGTTCCGTACTTGCATCATGCAGCTCTTTGGAAAGGCAGAAGATGATGGCACTGAAGTGTC
TACATCAAAAACAGAGGTTTCCTCTGTGGCACCTGCATAA

Oncorhynchus keta SWS1 Open Reading Frame GenBank Accession # AY214143
ATGGGAAAGGACTTCCATCTGTACGAGAACATCTCTAAGGTCAGCCCATTTGAGGGGCCACAGTATCA
CCTGGCCCCAATATGGGCTTTCTACCTACAGACTGCTTTCATGGGCTTTGTGTTCTTTGCTGGAACACCT
CTAAACTTTATCATTCTCGTGGTGACAGTGAAGTACAAGAAGCTGAGACAACCGCTGAACTACATCCT
GGTCAACGTCTCGTTAGCAGGTTTCATCTTTGTGACGTTTTCTGTGAGTCAAGTGTTTGTTTCTAGCGCG
AGAGGATACTACTTCCTGGGTTACACCTTGTGTGCAATGGAAGCTTGCATGGGTTCAATAGCAGGGTTG
GTGTCAGCTTGGTCCCTGGCTGTCCTTGCCTTTGAGAGATATGTGGTCATCTGCAAACCCTTCGGCACCT
TCAAATTTGACAACAACCAGGCTCTGGCGGCTGTTGGCTTCACCTGGGTCATGGGGATCGGATGTGCCA
CCCCACCGTTCTTCGGCTGGAGCAGGTATATCCCTGAGGGTCTGGGCTGCTCCTGTGGACCTGACTGGT
ACACAAACAACGAGGAGTACCACTGTGCCAGCTACACCAAATTCCTTATTGTTACCTGTTTCCTCATGC
CCATGTCCATCATCTTCTTCTCCTACTCCCAGCTGCTGGGAGCACTACGGGCTGTGGCAGCCGCGCAGG
CTGAGTCAGCTTCCACCCAGAAGGCAGAGAAGGAAGTATCCAGGATGGTGATTGTGATGGTGTGCTCC
TTCATTCTGTGTTACGGCCCCTACGCCCTGGCAGGCTTGTACTTTGCCTACACAACAAGTGAGAACAAA
GACTACCGCCTGGTGACCATCCCTGCTTTCTTCTCTAAAAGCTCCTGCGTATACAATCCTCTTATTTACG
CCTTCATGAACAAACAGTTTAACGCCTGCATCATGGAGACTGTGTTCGGAAAGCAGATTGAAGAGACT
TCAGTTTCAGCCTCCAAGACTGAGGTCTCCACAGCATAA

Oncorhynchus keta SWS2 Open Reading Frame GenBank Accession # AY214144
ATGAACACAATGAGGTCGAATGCTCGCCCCGTGGAGCTCCAGGAGGGATTCTACATCCCTATCGCGCT
GGATACCAACAACATCACTTCACTCAGCCCCTTCCTGGTTCCTCAGGACCACCTGGCGGGCAGTGCTAT
CTTCTATGGCATGTCATTTTTCATGTTCTTCCTATTTGTTGCCGGCACTGCTATCAACGTCCTTACCATCG
TGTGTACTATCCAGTTCAAGAAGCTGCGATCCCATCTCAACTACATTCTGGTGAACTTGGCAATAGCTA
ACCTGCTGGTGTCCATGTTTGGATCCTCCACCGCCAGCTTGTCCTTTGCCTACAAATACTTTATCATGGG

ATCGGTTACATGCCAGATTGAGGGATTTACAGCTACTCTTGGCGGTATGGTGAGCTTATGGTCCCTCTC
AGTAGTGGCGTTTGAAAGATGGTTGGTTATTTGTAAGCCAGTCGGTAACTTTCAATTCAAGAGCACACA
TGCAATAATCGGCTGTGCAATCACTTGGGTGTTTGGGTTGGCTGCCAGTCTTCCCCCTCTGTTTGGCCGG
AGTAGATACATTCCAGAAGGTCTCCAGTGCTCCTGTGGACCAGACTGGTACACCACAAACAACAAATA
CAACAATGAGTCCTATGTGATGTTCCTCTTCTTCTTCTGCTTTGGAGTCCCATTCAGTGTCATTGTTTTCT
GCTATGCCCAGCTGCTCTTCATGATGAAGGCGGCTGCCGCGGCACAGGCAGACTCTGCCTCCACCCAG
AAGGCAGAGAAGGAGGTCACCAAGATGGTGGTAGTGATGGTGGTGGGCTTCCTAGTGTGCTGGATGCC
CTATGCCTCCTTTGCTGTCTGGGTTGTACAAAACCGCGGTGCACCCTTTGATCTCCGATTGGCCACCATA
CCTTCCTGCTTCTCCAAGGCCTCCACAGTCTACAACCCTCTCATCTATGTCTTCATGAATAAGCTGTTCC
GCTCATGCATGATTAATTTGCTGGGATTAAAGTCCGGGGATGATGAGGAAGCATCATCAACATCCTCG
GTCACTCAAGTGTCTTCTGCTGGTTAA

Oncorhynchus nerka RH1 Open Reading Frame GenBank Accession # AY214156
ATGAACGGCACAGAGGGACCAGATTTCTACGTCCCTATGTCCAATGCTACTGGCATTGTTAGGAACCCC
TATGAATACCCCCAGTACTACCTTGTCAGCCCAGCGGCGTACTCACTCATGGCTGCCTACATGTTCTTC
CTCATCCTCACCGGCTTCCCCATCAACTTCCTCACACTCTATGTCACCATCGAGCACAAAAAGCTGAGG
ACCGCCCTGAACTACATCCTGCTGAACCTGGCTGTGGCCGATCTCTTCATGGTAATCGGAGGCTTCACC
ACTACGATGTACACCTCCATGCATGGCTATTTCGTCTTTGGAAGAACGGGCTGCAACATCGAGGGATTC
TGTGCTACCCATGGTGGTGAGATTGCCCTATGGTCCCTGGTTGTCCTGGCTATTGAGAGGTGGTTGGTC
GTCTGCAAACCTATTAGCAACTTCCGCTTCAGTGAGACCCATGCCATCATAGGCGTGGCCTTTACCTGG
GTCATGGCTGCTGCTTGCTCCGTCCCCCCTCTGCTTGGGTGGTCCCGCTATATCCCCGAAGGCATGCAG
TGCTCATGTGGAATTGACTACTACACGCGCGCCCCTGACATCAACAATGAGTCCTTTGTCATCCACATG
TTCGTTGTCCACTTTATGATTCCCCTGTTCATCATCTCCTTCTGCTACGGCAACCTGCTCTGCGCTGTCA
AGGCAGCTGCCGCCGCCCAGCAGGAGTCTGAGACCACCCAGAGGGCTGAGAGGGAAGTGACCCGCAT
GGTCATCATGATGGTCGTCTCCTTCCTAGTGTGCTGGGTGCCCTACGCCAGCGTGGCCTGGTATATCTTC
TGCAACCAGGGAACAGAGTTCGGCCCCGTCTTCATGACAATTCCGGCATTCTTTGCCAAGAGTTCGTCC
CTGTACAACCCTCTCATCTACGTGTTGATGAACAAGCAGTTCCGCAACTGCATGATCACCACCCTGTGC
TGTGGGAAGAACCCCTTCGAGGAGGAGGAGGGAGCCTCCACCACTGCCTCCAAGACCGAGGCCTCCTC
CGTGTCCTCCAGCTCCGTGGCTCCTGCATAA

Oncorhynchus nerka RH2 Open Reading Frame GenBank Accession # AY214157
ATGCAGAACGGCACAGAAGGAAGCAACTTCTACATTCCCATGTCCAACAGGACTGGGCTTGTAAGGAG
TCCTTTTCTATACCAACAGTACTACTTGGCGCCTCCATGGCAATACTATGGTCTTGCTGTCTATATGTTC
TTCCTGATCTGCTTTGGATTCCCCATCAACGGGCTGACCTTGTATGTCACAGCCACAAACAAGAAGCTT
CGGCAACCCCTTAACTTCATCCTGGTCAACTTGGCTGCAGCTGGAATGATAATGGTCCTCTTTGGATTC
ACCATCACCATTACATCTGCTGTCAATGGTTACTTCATCTGGGGACCATTGGGCTGTGCCATTGAGGGA
TTCATGGCTACACTTGGAGGTGAGGTTGCCCTGTGGTCTCTGGTAGTGCTGGCTGTCGAGAGATACATT
GTGGTCTGCAAGCCTATGGGCAGCTTCACGTTCACTGCCTCCCACGCTGGTGCTGGTGTTGCATTCACC
TGGATAGCTGCTATGGCTTGTGCTGCTCCCCCACTGGTTGGCTGGTCCAGGTACATCCCAGAGGGCATG
CAATGCTCATGTGGACCTGACTACTACACCTTGGCCGAAGGCTTCAACAATGAATCATATGTGATCTAC
ATGTTTACCTGCCACTTCATCATCCCAGTCTGTTTGATCGCCTTCACTTATGGAAGTCTTGTCCTCACAG
TCAAGGCGGCTGCAGCTTCCCAGCAGGACTCAGCATCTACCCAGAAAGCTGAGAAGGAAGTGACACGT
ATGTGCATCCTGATGGTTTGTGGTTTCATGATCGCCTGGACCCCCTATGCCACCCTCGCTGCCTACATCT
TCTTCAACAAAGGAATTGCCTTCAGTGCCCAGTCCATGGCTATACCTGCCTTCTTCTCCAAGAGCTCAG
CCTTGTTCAACCCCATTATCTATGTTCTGATGAACAAACAGTTCCGTGGCTGCATGCTGGCCGCTGTAG
GGATGAAAGCAGAAGAAGATGAAACGTCTGTGTCAGCAAGCAAGACAGAAGTGTCTTCTGTGGGCCCT
GCATAG

Oncorhynchus nerka LWS Open Reading Frame GenBank Accession # AY214155
ATGGCAGAAAGCTGGGGAAGTGCTGCTTATGCAGCCAGGCGACAAAACCAAGATACAACGAGAGAAT
CTTCCTTTACCTTCACCAACAGCAATAACACCAAAGATCCCTTTGAGGGCCCCAACTACCACATTGCCC
CAAGATGGGTGTACAATGTTTCAACACTTTGGATGATCATTGTGGTAATCCTCTCAGTCTTCACCAATG
GCCTGGTACTGGTGGCCACTGCAAAATTCAAGAAGCTCCAACACCCTCTGAACTGGATCTTGGTCAACC
TTGCTATTGCTGACATTGGAGAAACACTTTTGGCAAGCACCATCAGCGTTTGCAACCAGTTTTTTGGCT
ACTTCATTCTGGGACATCCAATGTGTGTCTTTGAGGGATACACTGTCTCCACTTGCGGAATTGCTGCTCT
GTGGTCCCTGGCTGTCATCTCTTGGGAGAGATGGGTGGTGGTGTGCAAGCCCTTTGGAAGTGTCAAGTT

TGATGCCAAATGGGCCATGGGAGGCATTATCTTCTCCTGGGTCTGGTCTGCTTTCTGGTGTGCCCCCCC
CATCTTTGGCTGGAGCAGGTACTGGCCTCACGGCCTGAAGACTTCCTGCGGACCTGATGTGTTCGGAGG
CAATGAGGATCCTGGAGTCAAGTCCTACATGATTACTCTCATGATTACATGCTGCTTCTTCCCCCTGTTC
GTTATCATCTTCTGCTACATTTTCGTGTGGCTAGCCATTCGTGCTGTTGCTGCGCAACAGAAAGACTCTG
AGTCAACACAGAAGGCCGAGAAGGAAGTGTCCAGGATGGTTGTTGTCATGATTATAGCTTACTGTGTA
TGCTGGGGACCTTATACCTGCTTTGCCTGCTTTGCTGCGGCTAACCCTGGGTATGCTTTCCACCCTCTGG
CAGCCGCAATTCCTGCCTACTTTGCCAAGAGCGCCACCATCTACAATCCAGTTATATATGTCTTCATGA
ACAAACAGTTCCGTACTTGCATCATGCAGCTCTTTGGAAAGGCAGAAGATGATGGCACTGAAGTGTCT
ACGTCAAAAACAGAGGTTTCCTCTGTGGCACCTGCATAA

Oncorhynchus nerka SWS1 Open Reading Frame GenBank Accession # AY214158
ATGGGAAAGGACTTCCATCTGTACGAGAACATCTCTAAGGTCAGCCCATTTGAGGGGCCACAGTATCA
CCTGGCCCCAATATGGGCTTTCTACCTACAGACTGCTTTCATGGGCTTTGTGTTCTTTGCTGGAACACCC
CTAAACTTTATCATTCTCGTGGTGACAGTGAAGTACAAGAAGCTGAGACAACCGCTGAACTACATCCT
GGTCAACGTCTCGTTAGCAGGCTTCATCTTTGTGACGTTTTCTGTGAGTCAAGTGTTTGTTTCTAGCGCG
AGAGGATACTACTTCCTGGGTTACACCTTGTGTGCAATGGAAGCTTGCATGGGTTCAATAGCAGGGTTG
GTGTCAGCTTGGTCCCTGGCTGTCCTTGCCTTTGAGAGATATCTGGTCATCTGCAAACCCTTTGGCACCT
TCAAATTTGACAACAACCAGGCTCTGGCGGCTGTTGGCTTCACCTGGGTCATGGGGATCGGATGTGCCA
CCCCACCGTTCTTCGGCTGGAGCAGGTATATCCCTGAGGGTCTGGGCTGCTCCTGTGGACCTGACAGGT
ACACAAACAACGAGGAGTACCACTGTGCCAGCTACACCAAATTCCTTATTGTTACCTGTTTCCTCATGC
CCATGTCCATCATCTTCTTCTCCTACTCCCAGCTGCTGGGAGCACTACGGGCTGTGGCAGCTGCACAGG
CTGAGTCAGCTTCCACCCAGAAGGCAGAGAAGGAAGTATCCAGGATGGTGATTGTGATGGTGTGCTCC
TTCATTCTGTGTTACGGCCCCTACGCCCTGGCAGGCTTGTACTTTGCCTACACAACAAGCGAGAACAAA
GACTACCGCCTGGTGACCATCCCTGCTTTCTTCTCTAAAAGCTCCTGCGTATACAATCCTCTTATTTACG
CCTTCATGAACAAACAGTTTAACGCCTGCATCATGGAGACTGTGTTCGGAAAGCAGATTGAAGAGACT
TCAGTTTCAGCCTCCAAGACTGAGGTCTCCACAGCATAA

Oncorhynchus nerka SWS2 Open Reading Frame GenBank Accession # AY214159
ATGAACACAATGAGGTCGAATGCTCGCCCCGTGGAGCTCCAGGAGGGTTTCTACATCCCTATCGCGCT
GGATACCAACAACATCACTTCACTCAGCCCCTTCCTGGTTCCTCAGGACCACCTGGCGGGCAGTGCTAT
CTTCTATGGCATGTCATTTTTCATGTTCTTCCTATTTGTTGCCGGCACTGCTATCAACGTCCTTACCATCG
TGTGTACTATCCAGTTCAAGAAGCTGCGATCTCATCTCAACTACATTCTGGTGAACTTGGCAATAGCTA
ACCTGCTGGTGTCCATGTTTGGCTCCTCCACCGCCAGCTTGTCCTTTGCCTACAAATACTTTATCATGGG
ATCGGTTGCCTGCCTGATTGAGGGATTTACAGCTACTCTTGGCGGTATGGTGAGCTTATGGTCTCTCTC
AGTAGTGGCGTTTGAAAGATGGTTGGTTATTTGTAAGCCAGTCGGTAACTTTCAATTCAAGAGCACACA
TGCAATAATCGGCTGTGCAATCACTTGGGTGTTTGGGTTCGCTGCCAGTCTTCCCCCTCTGTTTGGCTGG
AGTAGATACATTCCAGAAGGTCTCCAGTGCTCCTGTGGACCAGACTGGTACACCACAAACAACAAATA
CAACAATGAGTCCTATGTGATGTTCCTCTTCTTCTTCTGCTTTGGAGTCCCATTCAGTGTCATTGTTTTCT
GCTATGCCCAGCTGCTCTTCATGATGAAGGCGGCTGCCGCGGCACAGGCAGACTCTGCCTCCACCCAG
AAGGCAGAGAAGGAGGTCACCAAGATGGTGGTGGTGATGGTGGTGGGCTTCCTAGTGTGCTGGATGCC
CTATGCCTCCTTTGCTGTCTGGGTTGTACAAAACCGCGGTGCACCCTTTGATCTCCGATTGGCCACCATA
CCTTCCTGCTTCTCCAAGGCCTCCACAGTCTACAACCCTCTCATCTATGTCTTCATGAATAAGCTGTTCC
GTTCATGCATGATGAATTTGCTGGGATTAAAGTCTGGGGATGATGAGGAAGCATCATCAACATCCTCG
GTCACTCAAGTGTCTTCTGCTGGTTAA

Oncorhynchus gorbuscha RH1 Open Reading Frame GenBank Accession # AY214151
ATGAACGGCACAGAGGGACCAGATTTCTACGTCCCTATGTCCAATGCTACTGGCATTGTTAGGAACCCC
TATGAATACCCCCAGTACTACCTTGTCAGCCCAGCGGCGTACTCACTCATGGCTGCCTACATGTTCTTC
CTCATCCTCACCGGCTTCCCCATCAACTTCCTCACACTCTACGTCACCATCGAGCACAAAAAGCTGAGG
ACCGCCCTGAACTACATCCTGCTGAACCTGGCTGTGGCCGATCTCTTCATGGTGATCGGAGGCTTCACC
ACTACGATGTACACCTCCATGCATGGCTATTTCGTCTTTGGAAGAACGGGCTGCAACATCGAGGGATTC
TTTGCTACCCATGGTGGTGAGATTGCCCTGTGGTCCCTGGTTGTCCTGGCTATTGAGAGGTGGTTGGTC
GTCTGCAAACCTATTAGCAACTTCCGCTTCACTGAGACCCATGCCATCATAGGCGTGGCCTTTACCTGG
GTCATGGCTGCTGCTTGCTCCGTGCCCCCACTGCTTGGCTGGTCCCGCTATATCCCCGAAGGCATGCAG
TGCTCATGTGGAATTGACTACTACACACGCGCCCCTGACATCAACAATGAGTCCTTTGTCATCTACATG
TTCGTTGTCCACTTTATGATTCCCCTGTTCATCATCTCCTTCTGCTACGGCAACCTGCTCTGCGCTGTCA

AGGCAGCTGCCGCCGCCCAGCAGGAGTCTGAGACCACCCAGAGGGCTGAGAGGGAAGTGACCCGCAT
GGTCATCATGATGGTTGTCTCCTTCCTAGTGTGCTGGGTGCCCTACGCCAGCGTGGCCTGGTATATCTTC
TGCAACCAGGGATCAGAGTTCGGCCCCGTCTTCATGACAATTCCAGCATTCTTTGCCAAGAGTTCGTCC
CTGTACAACCCTCTCATCTACGTGTTGATGAACAAGCAGTTCCGTAACTGCATGATCACCACCCTGTGC
TGTGGGAAGAACCCCTTCGAGGAGGAGGAGGGAGCCTCCACCACTGCCTCCAAGACCGAGGCCTCCTC
CGTGTCCTCCAGCTCTGTGGCTCCTGCATAA

Oncorhynchus gorbuscha RH2 Open Reading Frame GenBank Accession # AY214152
ATGCAGAACGGCACAGAAGGAAGCAACTTCTACATTCCCATGTCCAACCGGACTGGGCTTGTAAGGAG
TCCTTTTCTATACCAACAGTACTACTTGGCGCCTCCATGGCAATTCTATGGTCTTGTTTTCTACATGTTTT
TCCTGATCTGTTTTGGATTCCCCATCAATGGTCTGACCTTGTACGTCACAGCCACAAACAAGAAGCTCC
GGCAACCCCTCAACTTCATCCTGGTCAACTTGGCTGCAGCTGGAATGATCATGGTCCTCTTTGGATTCA
CCATCACCATCACATCTGCTGTCAATGGTTACTTCATCTGGGGACCAATGGGCTGTGCTATTGAGGGCT
TCATGGCTACACTTGGAGGTGAGGTTGCCCTGTGGTCTCTGGTAGTGCTGGCTGTCGAGAGATACATTG
TGGTCTGCAAGCCCATGGGCAGCTTCACGTTCACTGCCTCCCACGCTGGTGCTGGTGTTGCATTCACCT
GGATAGCTGCTATGGCTTGTGCTGCTCCCCCACTGATTGGCTGGTCCAGGTACATCCCAGAGGGCATGC
AATGCTCATGTGGACCTGACTACTACACCTTGGCCGAAGGCTTCAACAATGAATCATATGTGATATACA
TGTTCAGCTGCCACTTCATCATCCCAGTCTGTTTGATCGCCTTCACTTATGGAAGTCTTGTCCTCACAGT
CAAGGCGGCTGCAGCTTCCCAGCAGGACTCAGCATCTACCCAGAAAGCTGAGAAGGAAGTGACACGT
ATGTGCATCCTGATGGTTTGTGGTTTCATGATCGCCTGGACCCCCTATGCCACCCTCGCTGCCTACATCT
TCTTCAACAAAGGAATTGCCTTCAGTGCCCAGTCCATGGCTATACCTGCCTTCTTCTCCAAGAGCTCAG
CTTTGTTCAACCCCATTATCTATGTTCTGATGAACAAACAGTTCCGTGGCTGCACGCTGGCCACTGTAG
GGATGAAAGCAGAAGAAGATGAAACTTCTGTGTCAACAAGCAAGACAGAAGTGTCTTCTGTGGGACCT
GCATAG

Oncorhynchus gorbuscha LWS Open Reading Frame GenBank Accession # AY214150
ATGGCAGAAAGCTGGGGAAGTGCTGCTTATGCAGCCAGGCGACAAAACCAAGATACAACGAGAGAAT
CTTCCTTTACCTTCACCAACAGCAATAACACCAAAGATCCCTTTGAGGGCCCCAACTACCACATTGCTC
CAAGATGGGTGTACAATGTTTCAACACTTTGGATGATCATAGTGGTCATCCTCTCAGTCTTCACCAATG
GCCTGGTACTGGTGGCCACTGCAAAATTCAAGAAGCTCCAACACCCTCTGAACTGGATCTTGGTCAACC
TTGCTATTGCTGACATTGGAGAAACACTTTTGGCAAGCACCATCAGCGTTTGCAACCAGTTTTTTGGCT
ACTTCATTCTGGGACATCCAATGTGTGTCTTTGAGGGATACACTGTCTCCACTTGCGGAATTGCTGCTCT
GTGGTCCCTGGCTGTCATCTCTTGGGAGAGATGGGTGGTGGTGTGCAAGCCCTTTGGAAGTGTCAAGTT
TGATGCCAAATGGGCCATGGGAGGCATTATCTTCTCCTGGGTCTGGTCTGCTTTCTGGTGTGCCCCCCC
CATCTTTGGCTGGAGCAGGTACTGGCCTCACGGCCTGAAGACTTCCTGCGGACCTGATGTGTTCGGAGG
CAATGAGGATCCTGGAGTCAAGTCCTACATGATCACTCTCATGATTACATGCTGCTTCTTCCCCCTGTTC
GTTATCATCTTCTGCTACGTTTTCGTGTGGCTAGCCATTCGTGCTGTTGCTGCGCAACAGAAAAACTCTG
AGTCAACACAGAAGGCCGAGAAGGAAGTGTCCAGGATGGTTGTTGTCATGATTATAGCTTACTGTGTA
TGCTGGGGACCTTATACCTGCTTTGCCTGCTTTGCTGCAGCTAACCCTGGGTATGCTTTCCACCCTCTGG
CAGCCGCAATTCCTGCCTACTTTGCCAAGAGCGCCACCATCTACAATCCAGTTATATATGTCTTCATGA
ACAAACAGTTCCGTACTTGCATCATGCAGCTCTTTGGAAAGGCAGAAGATGATGGCACTGAAGTGTCT
ACATCAAAAACAGAGGTTTCCTCTGTGGCACCTGCATAA

Oncorhynchus gorbuscha SWS1 Open Reading Frame GenBank Accession # AY214153
ATGGGAAAGGACTTCCATCTGTACGAGAACATCTCTAAGGTCAGCCCATTTGAGGGGCCACAGTATCA
CCTGGCCCCAAAATGGGCTTTCTACCTACAGACTGCTTTCATGGGCTTTGTGTTCTTTGCTGGAACACCC
CTAAACTTTATCATTCTCCTGGTGACAGTGAAGTACAAGAAGCTGAGACAACCGCTGAACTACATCCTG
GTCGACGTCTCGCTAGCAGGCTTCATCTTTGTGACGTTTTCTGTGAGTCAAGTGTTCGTTTCCAGCGCGA
GAGGATACTACTTCCTGGGTTACACCTTGTGTGCAATGGAAGCGTGCATGGGTTCAATAGCAGGGTTG
GTGTCAGCTTGGTCCCTGGCTGTCCTTGCCTTTGAGAGATATGTGGTCATCTGCAAACCCTTTGGCACCT
TCAAATTTGACAACAACCAGGCTCTGGCGGCTGTCGGCTTCACCTGGGTCATGGGGATCGGATGTGCC
ACCCCGCCGTTCTTCGGCTGGAGCAGGTATATCCCTGAGGGTCTGGGCTGCTCCTGTGGACCTGACTGG
TACACAAACAACGAGGAGTACCACTGTGCCAGCTACACCAAATTCCTTATTGTTACCTGTTTCCTCATG
CCCATGTCCATCATCTTCTTCTCCTACTCCCAGCTGCTGGGAGCACTACGGGCTGTGGCAGCTGCGCAG
GCTGAGTCAGCTTCCACCCAGAAGGCAGAGAAGGAAGTATCCAGGATGGTGATTGTGATGGTGTGCTC
CTTCATTCTGTGTTACGGCCCCTACGCCCTGGCAGGCTTGTACTTCGCCTACACAACAAGCGAGAACAA

AGACTACCGCCTGGTGACCATCCCTGCTTTCTTCTCTAAAAGCTCCTGTGTATACAATCCTCTTATTTAC
GCCTTCATGAACAAACAGTTTAACGCCTGCATCATGGAGACTGTGTTCGGAAAGCAGATTGAAGAGAC
TTCAGTTTCAGCCTGCAAGACTGAGGTCTCCACAGCATAA

Oncorhynchus gorbuscha SWS2 Open Reading Frame GenBank Accession # AY214154
ATGAACACAATGAGGTCGAATGCTCGCCCCGTGGAGCTCCAGGAGGGATTCTACATCCCTATCGCGCT
GGATACCAACAACATCACTTCACTCAGCCCCTTCCTGGTTCCTCAGGACCACCTGGCGGGCAGTGCTAT
CTTCTATGGCATGTCATTTTTCATGTTCTTCCTATTTGTTGCCGGCACTGCTATCAACGTCCTTACCATCG
TGTGTACTATCCAGTTCAAGAAGCTGCGATCCCATCTCAACTACATTCTGGTGAACTTGGCAATAGCTA
ACCTGCTGGTGTCCATGTTTGGATCCTCCACCGCCAGCTTGTCCTTTGCCTACAAATACTTTATCATGGG
ATCGGTTACATGCCAGATTGAGGGATTTACAGCTACTCTTGGCGGTATGGTGAGCTTATGGTCTCTCTC
AGTAGTGGCGTTTGAAAGATGGTTGGTTATTTGTAAGCCAGTCGGTAACTTTCAATTCAAGAGCACACA
TGCAATAATCGGCTGTGCAATCACTTGGGTGTATGGGTTGGCTGCCAGTCTTCCCCCTCTGTTTGGCTG
GAGTAGATACATTCCAGAAGGTCTCCAGTGCTCCTGTGGACCAGACTGGTACACCACAAACAACAAAT
ACAACAATGAGTCCTATGTGATGTTCCTCTTCTTCTTCTGCTTTGGAGTCCCATTCAGTGTCATTGTTTTC
TGCTATGCCCAGCTGCTCTTCATGATGAAGGCGGCTGCCGCGGCACAGGCAGACTCTGCCTCCACCCAG
AAGGCAGAGAAGGAGGTCACCAAGATGGTGGTGGCGATGGTGGTGGGCTTCCTAGTGTGCTGGATGCC
CTATGCCTCCTTTGCTGTCTGGGTTGTACAAAACCGTGGTGCACCCTTTGATCTCCGATTGGCCACCATA
CCTTCCTGCTTCTCCAAGGCCTCCACAGTCTACAACCCTCTCATCTATGTCTTCATGAATAAGCTGTTCC
GCTCATGCATGATGAATTTGCTGGGATTAAAGTCCGGGGATGATGAGGAAGCATCATCAACATCCTCG
GTCACTCAAGTGTCTTCTGCTGGTTAA

Oncorhynchus tshawytscha RH1 Open Reading Frame GenBank Accession # AY214136
ATGAACGGCACAGAGGGACCAGATTTCTACGTCCCTATGTCCAATGCTACTGGCATTGTTAGGAACCCC
TATGAATACCCCCAGTACTACCTTGTCAGCCCAGCGGCGTACTCACTCATGGCTGCCTACATGTTCTTC
CTCATCCTCACCGGCTTCCCCATCAACTTCCTCACACTCTACGTCACCATCGAGCACAAAAAGCTGAGG
ACCGCCCTGAACTACATCCTGCTGAACCTGGCTGTGGCCGATCTCTTCATGGTAATCGGAGGCTTCACC
ACTACGATGTACACCTCCATGCATGGCTATTTCGTCTTTGGAAGAACGGGCTGCAACATCGAGGGATTC
TTTGCTACCCATGGTGGTGAGATTGCCCTGTGGTCCCTGGTTGTCCTGGCTATTGAGAGGTGGTTGGTC
GTCTGCAAACCTATTAGCAACTTCCGCTTCAGTGAGACCCATGCCATCATGGGCGTGGCCTTTACCTGG
GTCATGGCTGCTGCTTGCTCCGTGCCCCCTCTGCTTGGCTGGTCCCGCTATATCCCCGAAGGCATGCAG
TGCTCATGTGGAATTGACTACTACACACGCGCCCCTGACATCAACAATGAGTCCTTTGTCATCTACATG
TTCGTTGTCCACTTTATGATTCCCCTGTTCATCATCTCCTTCTGCTACGGCAACCTGCTCTGCGCTGTCA
AGGCAGCTGCCGCCGCCCAGCAGGAGTCTGAGACCACCCAGAGGGCTGAGAGGGAAGTGACCCGCAT
GGTCATCATGATGGTCGTCTCCTTCCTAGTGTGCTGGGTGCCCTACGCCAGCGTGGCCTGGTATATCTTC
TGCAACCAGGGATCAGAGTTCGGCCCCGTCTTCATGACAATTCCGGCATTCTTTGCCAAGAGTTCGTCC
CTGTACAACCCTCTCATCTACGTGTTGATGAACAAGCAGTTCCGCAACTGCATGATCACCACCCTGTGC
TGTGGGAAGAACCCCTTCGAGGAGGAGGAGGGAGCCTCCACCACTGCCTCCAAGACCGAGGCCTCCTC
CGTGTCCTCCAGCTCCGTGGCTCCTGCATAA

Oncorhynchus tshawytscha RH2 Open Reading Frame GenBank Accession # AY214137
ATGCAGAACGGCACAGAAGGAAGCAACTTCTACATTCCCATGTCCAACCGGACTGGGCTTGTAAGGAG
TCCTTTTCTATACCAACAGTACTACTTGGCGCCTCCATGGCAATTCTATGGTCTTGCTGTCTACATGTTT
TTCCTGATCTGTTTGGGATTCCCCATCAATGGTCTGACCTTGTACGTCACAGCCACAAACAAGAAGCTC
CGGCAACCCCTCAACTTCATCCTGGTCAACTTGGCTGCAGCTGGAATGATCATGGTCCTCTTTGGATTC
ACCATCACCATCACATCTGCTGTCAATGGTTACTTCATCTGGGGACCAATGGGCTGTGCTATTGAGGGC
TTCATGGCTACACTTGGAGGTGAGGTTGCCCTGTGGTCTCTGGTAGTGCTGGCTGTCGAGAGATACATT
GTGGTCTGCAAGCCCATGGGCAGCTTCACGTTCACTGCCTCCCACGCTGGTGCTGGTGTTGCATTCACC
TGGATAGCTGCTATGGCTTGTGCTGCTCCCCCACTGATTGGCTGGTCCAGGTACATCCCAGAGGGCATG
CAATGCTCATGTGGACCTGACTACTACACCTTGGCCGAAGGCTTCAACAATGAATCATATGTGATCTAC
ATGTTCAGCTGCCACTTCATCATCCCAGTCTGTTTGATCGCCTTCACTTATGGAAGTCTTGTCCTCACAG
TCAAGGCGGCTGCAGCTTCCCAGCAGGACTCAGCATCTACCCAGAAAGCTGAGAAGGAAGTGACACGT
ATGTGCATCCTGATGGTTTGTGGTTTCATGATCGCCTGGACCCCCTATGCCACCCTCGCTGCCTACATCT
TCTTCAACAAAGGAATTGCCTTCAGTGCCCAGTCCATGGCTATACCTGCCTTCTTCTCCAAGAGCTCAG
CTTTGTTCAACCCCATTATCTATGTTCTGATGAACAAACAGTTCCGTGGCTGCATGCTGGCCACTGTAG

GGATGAAAGCAGAAGAAGATGAAACTTCTGTGTCAACAAGCAAGACAGAAGTGTCTTCTGTGGGACCT
GCATAG

Oncorhynchus tshawytscha LWS Open Reading Frame GenBank Accession # AY214135
ATGGCAGAAAGCTGGGGAAGTGCTGCTTATGCAGCCAGGCGACAAAACCAAGATACAACGAGAGAAT
CTTCCTTTACCTTCACCAACAGCAATAACACCAAAGATCCCTTTGAGGGCCCCAACTACCACATTGCTC
CAAGATGGGTGTACAATGTTTCAACACTTTGGATGATCATAGTGGTCATCCTCTCAGTCTTCACCAATG
GCCTGGTACTGGTGGCCACTGCAAAATTCAAGGAGCTCCAACACCCTCTGAGCTGGATCTTGGTCAACC
TTGCTATTGCTGACATTGGAGAAACACTTTTGGCAAGCACCATCAGCGTTTGCAACCAGTTTTTTGGCT
ACTTCATCCTGGGACATCCAATGTGTGTCTTTGAGGGATACACTGTCTCCACTTGCGGAATTGCTGCTCT
GTGGTCCCTGGCTGTCATCTCTTGGGAGAGATGGGTGGTGGTGTGCAAGCCCTTTGGAAGTGTCAAGTT
TGATGCCAAATGGGCCATGGGAGGCATTATCTTCTCCTGGGTCTGGGCTGCTTTCTGGTGTGCCCCCCC
CATCTTTGGCTGGAGCAGGTACTGGCCTCACGGCCTGAAGACTTCCTGCGGACCTGATGTGTTCGGAGG
CAATGAGGATCCTGGAGTCAAGTCCTACATGATTACTCTCATGATTACATGCTGCTTCTTCCCCCTGGTC
GTCATCATCTTCTGCTACATTTTCGTGTGGCTAGCCATTCGTGGTGCTGCGGCGCAACAGAAAGACTCT
GAGTCAACACAGAAGCCCGAGAAGGAAGTGTCCAGGATGGTTGTTGTCATGATTATAGCTTACTGTGT
ATGCTGGGGACCTTATACCTGCTTTGCCTGCTTTGCTGCGGCTAACCCTGGGTATGCTTTCCACCCTCTG
GCAGCCGCAATTCCTGCCTACTTTGCCAAGAGCGCCACCATCTACAATCCAGTTATATATGTCTTCATG
AACAAACAGTTCCGTACTTGCATCATGCAGCTCTTTGGAAAGGCAGAAGATGATGGCACTGAAGTGTC
TACATCAAAAACAGAGGTTTCCTCTGTGGCACCTGCATAA

Oncorhynchus tshawytscha SWS1 Open Reading Frame GenBank Accession # AY214138
ATGGGAAAGGACTTCCATCTGTACGAGAACATCTCTAAGGTCAGCCCATTTGGGGGGCCACAGTATCA
CCTGGCCCCAATATGGGCTTTCTACCTACAGACTGCTTTCATGGGCTTTGTGTTCTTTGCTGGAACACCT
CTAAACTTTATCATTCTCGTGGAGACAGTGAAGTACAAGAAGCTGAGACAACCGCTGAACTACATCCT
GGTCAACGTCTCGTTAGCAGGTTTCATCTTTGTGACGTTTTCTGTGAGTCAAGTGTTTGTTTCTAGCGCG
AGAGGATACTACTTCCTGGGTTACACCTTGTGTGCAATGGAAGCTTGCATGGGTTCAATAGCAGGGTTG
GTGTCAGCTTGGTCCCTGGCTGTCCTTGCCTTTGAGAGATATGTGGTCATCTGCAAACCCTTCGGCACCT
TCAAATTTGACAACAACCAGGCTCTGGCGGCTGTTGGCTTCACCTGGGTCATGGGGATCGGATGTGCCA
CCCCACCGTTCTTCGGCTGGAGCAGGTATATCCCTGAGGGTCTGGGCTGCTCCTGTGGACCTGACTGGT
ACACAAACAATGAGGAGTACCACTGTGCCAGCTACACCAAATTCCTTATTGTTACCTGTTTCCTCATGC
CCATGTCCATCATCTTCTTCTCCTACTCCCAGCTGCTGGGAGCACTACGGGCTGTGGCAGCCGCGCAGG
CTGAGTCAGCTTCCACCCAGAAGGCAGAGAAGGAAGTATCCAGGATGGTGATTGTGATGGTGTGCTCC
TTCATTCTGTGTTACGGCCCCTACGCCCTGGCAGGCTTGTACTTTGCCTACACAACAAGCGAGGTCAAA
GACTACCGCCTTGTGACCATCCCTGCTTTCTTCTCTAAAAGCTCCTGTGTATACAATCCTCTTATTTACG
CCTTCATGAACAAACAGTTTAACGCCTGCATCATGGAGACTGTGTTCGGAAAGCAGATTGAAGAGACT
TCAGTTTCAGCCTCCAAGACTGAGGTCTCCACAGCATAA

Oncorhynchus tshawytscha SWS2 Open Reading Frame GenBank Accession # AY214139
ATGAACACAATGAGGTCGAATGCCCGCCCCGTGGAGCTCCAGGAGGGATTCTACATCCCTATCGCGCT
GGATACCAACAACATCACTTCACTCAGCCCCTTCCTGGTTCCTCAGGACCACCTGGCGGGCAGTGCTAT
CTTCTATGGCATCTCATTTTTCATGTTCTTCCTATTTGTTGCCGGCACTGCTATCAACGTCCTTACCATCG
TGTGTACTATCCAGTTCAAGAAGCTGCGATCCCATCTCAACTACATTCTGGTGAACTTGGCAATAGCTA
ACCTGCTGGTGTCCATGTTTGGATCCTCCACCGCCAGCTTGTCCTTTGCCTACAGATACTTTATCATGGG
ATCGGTTGCATGCCAGATTGAGGGATTTACAGCTACTCTTGGCGGTATGGTGAGCTTATGGTCTCTCTC
AGTAGTGGCGTTTGAAAGATGGTTGGTTATTTGTAAGCCAGTCGGTAACTTTCAATTCAAGAGCACACA
TGCAATAATCGGCTGTGCAATCACTTGGGTGTTTGGGTTGGCTGCCAGTCTTCCCCCTCTGTTTGGCTGG
AGTAGATACATTCCAGAAGGTCTCCAGTGCTCCTGTGGACCAGACTGGTACACCACAAACAACAAATA
CAACAATGAGTCCTATGTGATGTTCCTCTTCTTCTTCTGCTTCGGAGTCCCATTCAGTGTCATTGTCTTCT
GCTATGCCCAGCTGCTCTTCATGATGAAGGCGGCTGCCGCGGCACAGGCAGACTCTGCCTCCACCCAG
AAGGCAGAGAAGGAGGTCACGAAGATGGTGGTGGTGATGGTGGTGGGCTTCCTAGTGTGCTGGATGCC
CTATGCCTCCTTTGCTGTCTGGGTTGTACAAAACCGCGGTGCACCCTTTGATCTCCGATTGGCCACCATA
CCTTCCTGCTTCTCCAAGGCCTCCACAGTCTGCAACCCTCTCATCTATGTCTTCATGAATAAGCTGTTCC
GTTCATGCATGATGAATTTGCTGGGATTAAAGTCCGGGGATGATGAGGAAGCATCATCAACATCCTCG
GTCACTCAAGTGTCTTCTGCTGGTTAA

Oncorhynchus kisutch RH1 Open Reading Frame GenBank Accession # AY214146
ATGAACGGCACAGAGGGACCAGATTTCTACGTCCCTATGTCCAATGCTACTGGCATTGTTAGGAACCCC
TATGAATACCCCCAGTACTACCTTGTCAGCCCAGCGGCGTACCCACTCATGGCTGCCTACATGTTCTTT
CTCATCCTCACCGGCTTCCCCATCAACTTCCTCACACTCTACGTCACCATCGAGCACAAAAAGCTGAGG
ACCGCCCTGAACTACATCCTGCTGAACCTGGCTGTGGCCGATCTCTTCATGGTAATCGGAGGCTTCACC
ACTACGATGTACACCTCCATGCATGGCTATTTCGTCTTTGGAAGAACGGGCTGCAACATCGAGGGATTC
TTTGCTACCCATGGTGGTGAGATTGCCCTGTGGTCCCTGGTTGTCCTGGCTATTGAGAGGTGGTTGGTC
GTCTGCAAACCTATTAGCAACTTCCGCTTCAGTGAGACCCATGCCATCATGGGCGTGGCCTTTACCTGG
GTCATGGCTGCTGCTTGCTCCGTGCCCCCTCTGCTTGGCTGGTCCCGCTATATCCCTGAAGGCATGCAGT
GCTCATGTGGAATTGACTACTACACACGCGCCCCTGACATCAACAATGAGTCCTTTGTCATCTACATGT
TCGTTGTCCACTTTATGATTCCCCTGTTCATCATCTCCTTCTGCTACGGCAACCTGCTCTGCGCTGTCAA
GGCAGCTGCCGCCGCCCAGCAGGAGTCTGAGACCACCCAGAGGGCTGAGAGGGAAGTGACCCGCATG
GTCATCATGATGGTCGTCTCCTTCCTAGTGTGCTGGGTGCCCTACGCCAGCGTGGCCTGGTATATCTTCT
GCAACCAGGGATCAGAGTTCGGCCCCGTCTTCATGACAATTCCGGCATTCTTTGCCAAGAGTTCGTCCC
TGTACAACCCTCTCATCTACGTGTTGATGAACAAGCAGTTCCGCAACTGCATGATCACCACCCTGTGCT
GTGGGAAGAACCCCTTCGAGGAGGAGGAGGGAGCCTCCACCACTGCCTCCAAGACCGAGGCCTCCTCC
GTGTCCTCCAGCTCCGTGGCTCCTGCATAA

Oncorhynchus kisutch RH2 Open Reading Frame GenBank Accession # AY214147
ATGCAGAACGGCACAGAAGGAAGCAACTTCTACATTCCCATGTCCAACCGGACTGGGCTTGTAAGGAG
TCCTTTTCTATACCAACAATACTACTTGGCGCCTCCATGGCAATACTATTGTCTTGCTGTCTATACGTTC
TTCCTGATCTGCTTTGGATTCCCCATCAACGGTCTGACCCTGTATGTCACAGCCACGAACAAGAAGCTC
CGGCAACCCCTTAACTTCATCCTGGTCAACTTGGCTGCAGCTGGAATGATCATGGTCCTCTTTGGATTC
ACCATCACCATCACATCTGCTGTCAATGGTTACTTCATCTGGGGACCATTGGGCTGTGCCATTGAGGGC
TTCATGGCTACACTTGGAGGTGAGGTTGCCCTGTGGTCTCTGGTAGTGCTGGCTGTCGAGAGATACATT
GTGGTCTGCAAGCCCATGGGCAGCTTCACGTTCACTTCCACCCATGCTGGTGCTGGTGTTGCATTCACC
TGGATAGCTGCTATGGCTTGTGCTGCTCCCCCACTGCTTGGCTGGTCCAGGTACATCCCAGAGGGCATG
CAATGCTCATGTGGACCTGACTACTACACCTTGGCCGAAGGCTTCAACAATGAATCATATGTGATCTAC
ATGTTCAGCTGCCACTTCATCATCCCAGTCTGTTTGATCGCCTACACTTATGGAAGTCTTGTCCTCACAG
TCAAGGCGGCTGCAGCTTCCCAGCAGGACTCAGCATCTACCCAGAAAGCTGAGAAGGAAGTGACACGT
ATGTGCATCCTGATGGTTTGTGGTTTCATGGTCGCCTGGACCCCCTATGCCACCCTCGCTGCCTATATCT
TCTTCAACAAAGGAATTGCCTTCAGTGCCCAGTCCATGGCTATACCTGCCTTCTTCTCCAAGAGCTCAG
CCTTGTTCAACCCCATTATCTATGTGTTGATGAACAAACAGTTCCGTGGCTGCATGCTGGCCGCTGTAG
GGATGAAAGCAGAAGAAGGTGAAACTTCTGTGTCAACAAGCAAGACAGAAGTGTCTTCTGCGGGCCCT
GCATAG

Oncorhynchus kisutch LWS Open Reading Frame GenBank Accession # AY214145
ATGGCAGAAAGCTGGGGAAGTGCTGCTTATGCAGCCAGGCGACAAAACCAAGATACAACGAGAGAAT
CTTCCTTTACCTTCACCAACAGCAATAACACCAAAGATCCCTTTGAGGGCCCCAACTACCACATTGCTC
CAAGATGGGTGTACAATGTTTCAACACTTTGGATGATCATTGTGGTCATCCTCTCAGTCTTCACCAATG
GCCTGGTACTGGTGGCCACTGCAAAATTCGAGAAGCTCCAACACCCTCTGAACTGGATCTTGGTCAACC
TTGCTATTGCTGACATTGGAGAAACACTTTTGGCAAGCACCATCAGCGTTTGCAACCAGTTTTTTGGCT
ACTTCATTCTGGGACATCCAATGTGTGTATTTGAGGGATACACTGTCTCCACTTGCGGAATTGCTGCTCT
GTGGTCCCTGGCTGTCATCTCTTGGGAGAGATGGGTGGTGGTGTGCAAGCCCTTTGGAAGTGTCAAGTT
TGATGCCAAATGGGCCATGGGAGGCATTATCTTCTCCTGGGTCTGGGCTGCTTTCTGGTGTGCCCCCCC
CATCTTTGGCTGGAGCAGGTACTGGCCTCACGGCCTGAAGACTTCCTGCGGACCTGATGTGTTCGGAGG
CAATGAGGATCCTGGAGTCAAGTCCTACATGATTACTCTCATGATTACATGCTGCTTCTTCCCCCTGTTC
GTGATCATCTTCTGCTACATTTTCGTGTGGCTAGCCATTCGTGCTGTTGCTCAACAGCAAAAAGACTCT
GAGTCAACACGGAAGGCCGAGAAAGAAGTGTCCAGGATGGTTGTTGTCATGATTATAGCTTACTGTGT
ATGCTGGGGACCTTATACCTGCTTTGCCTGCTTTGCTGCGGCTAACCCTGGGTATGCTTTCCACCCTCTG
GCAGCCGCAATTCCTGCCTACTTTGCCAAGAGCGCCACCATCTACAATCCAGTTATATATGTCTTCATG
AACAAACAGTTCCGTACTTGCATCATGCAGCTCTTTGGAAAGGCAGAAGATGATGGCACTGAAGTGTC
TACATCAAAAACAGAGGTTTCCTCTGTGGCACCTGCATAA

Oncorhynchus kisutch SWS1 Open Reading Frame GenBank Accession # AY214148
ATGGGAAAGAACTTCCATCTATACGAGAACATCTCTAAGGTCAGCCCATTTGAGGGGCCACAGTATCA
CCTGGCCCCAATATGGGCTTTCTACCTACAGACTGCTTTCATGGGCTTTGTGTTCTTTGCTGGAACACCC
CTAAACTTGATCATTCTCGTGGTGACAGTGAAGTACAAGAAGCTGAGACAACCGCTGAACTACATCCT
GGTCAATGTCTCGTTAGCAGGTTTTATCTTTGTGACTTTCTCTGTGAGTCAAGTGTTCGTTTCTAGTGCG
GGAGGATACTACTTCCTGGGTTACACCTTGTGTGCAATGGAAGCTTGCATGGGTTCAATAGCAGGGTTG
GTGTCAGCTTGGTCTCTGGCTGTCCTTGCCTTTGAGAGATACGTGGTCATCTGCAAACCCTTCGGCACCT
TCAAATTTGACAACAACCAGGCTCTGGCGGCTGTTGGCTTCACCTGGGTCATGGGGATCGGATGTGCCA
CCCCACCGTTCTTCGGCTGGAGCAGGTATATCCCTGAGGGTCTGGGCTGCTCCTGTGGACCTGACTGGT
ACACAAACAACGAGGAGTACCACTGTGCCAGCTACACCAAATTCCTTATTGTTACCTGTTTCCTCATGC
CCATGTCCATCATCTTCTTCTCCTACTCCCAGCTGCTGGGAGCACTACGGGCTGTGGCAGCCGCGCAGG
CTGAGTCAGCTTCCACCCAGAAGGCAGAGAAGGAAGTATCCAGGATGGTGATTGTGATGGTGTGCTCC
TTCATTCTGTGTTACGGCCCCTACGCCCTGGCAGGCTTGTACTTTGCCTACACAACTAGCGAGAACAAA
GACTACCGCCTGGTGACCATCCCTGCTTTCTTCTCTAAAAGCTCCTGTGTATACAATCCTCTTATTTACG
CCTTCATGAACAAACAGTTTAACGCCTGCATCATGGAGACTGTGTTCGGAAAGCAGATTGAAGAGACT
TCAGTTTCAGCCTCCAAGACTGAGGTCTCCACAGCATAA

Oncorhynchus kisutch SWS2 Open Reading Frame GenBank Accession # AY214149
ATGAACACAATGAGGTCAAATGCTCGCCCCGTGGAGCTCCAGGAGGGATTCTACATCCCTATCGCGCT
GGATACCATCAACATCACTTCACTCAGCCCCTTCCTGGTTCCTCAGGACCACCTGGCGGGCAGTGCTAT
CTTCTATGGCATGTCATTTTTCATGTTCTTCCTATTTGTTGCCGGCACTGCTATCAACGTCCTTACCATCG
TGTGTACTATCCAGTTCAAGAAGCTGCGATCCCATCTCAACTACATTCTGGTGAACTTGGCAATAGCTA
ACCTGCTGGTGTCCATGTTTGGATCCTCCACCGCCAGCTTGTCCTTTGCCTACAGATACTTTATCATGGG
ATCGGTTGCATGCCAGATTGAGGGATTTACAGCTACTCTTGGCGGTATGGTGAGCTTATGGTCTCTCTC
AGTAGTGGCGTTTGACAGATGGTTGGTTATTTGTAAACCAGTCGGTAACTTTCAATTCAAGAGCACACA
TGCAATAATCGGCTGTGCAATCACTTGGGTGTTTAGGTTGGCTGCCAGTCTTCCCCCTCTGTTTGGCTGG
AGTAGATACATTCCAGAAGGTCTCCAGTGCTCCTGTGGGCCAGACTGGTACACCACAAACAACAAATA
CAACAATGAGTCCTATGTGATGTTCCTCTTCTTCTTCTGCTTCGGAGTCCCATTCAGTGTCATTGTCTTCT
GCTATGCCCAGCTGCTCTTCATGATGAAGGCGGCTGCCGCGGCACAGGCAGACTCTGCCTCCACCCAG
AAGGCAGAGAAGGAGGTCACCAAGATGGTGGTGGTGATGGTGGTGGGCTTCCTAGTGTGCTGGATGCC
CTATGCCTCCTTTGCTGTCTGGGTTGTACAAAACCGCGGTGCACCCTTTGATCTCCGATTGGCCACCATA
CCTTCCTGCTTCTCCAAGGCCTCCACAGTCTACAACCCTCTCATCTATGTCTTCATGAATAAGCTGTTCC
GCTCATGCATGATGAATTTGCTGGGATTAAAGTCCGGGGATGATGAGGAAGCATCATCAACATCCTCA
GTCACTCAAGTGTCTTCTGCTGGTTAA

Salmo Salar RH2 Open Reading Frame GenBank Accession # AY214132
ATGCAGAACGGCACAGAAGGAAGCAACTTCTACATTCCCATGTCCAACCGGACTGGGCTTGTAAGGAG
TCCTTTTCTATACCAACAGTACTACTTGGCACCTCCATGGCAATACCATGGTCTTGCTGTCTACATGTTC
TTCCTGATCTGCTTTGGATTCCCCATCAACGGGCTGACCTTGTATGTCACAGCCACAAACAAGAAGCTT
CGGCAACCCCTTAACTTCATCCTGGTCAACTTGGCTGCAGCTGGAATGATAATGGTCCTCTTTGGATTC
ACCATCACCATCACATCTGCTGTCAATGGCTACTTCATCTGGGGACCATTGGGCTGTGCTATTGAGGGC
TTCATGGCTACACTTGGAGGTGAGGTTGCCCTGTGGTCTCTGGTAGTGCTGGCTGTCGAGAGATACATT
GTGGTCTGCAAGCCCATGGGAAGCTTCACGTTCACTTCCACCCATGCTGGTGCTGGTGTTGCATTCACC
TGGATAGCTGCTATGACTTGTGCTGCTCCCCCACTGCTTGGATGGTCCAGGTACATCCCAGAGGGCATG
CAGTGCTCATGTGGACCTGACTACTACACCTTGGCCGAAGGCTTCAACAATGAATCATATGTGATTTAC
ATGTTCAGCTGCCACTTCATCATCCCAGTCTGTTTGATCGCCTTCACTTATGGAAGTCTTGTCCTCACAG
TCAAGGCGGCTGCAGCTTCCCAGCAGGACTCAGCATCTACCCAGAAAGCTGAGAAGGAAGTCACTCGT
ATGTGCGTCCTGATGGTTTGTGGTTTCATGATCGCCTGGACCCCCTATGCCACCCTCGCTGCCTACATCT
TTTTCAACAAAGGAATTGCCTTCAGTGCCCAGTCCATGGCTATACCTGCCTTCTTCTCCAAGAGTTCAG
CCTTGTTCAACCCCATTATCTATGTGTTGATGAACAAACAGTTCCGTGGCTGCATGCTGGCCACTGTAG
GGATGAAAGCAGAAGAAGATGAGACTTCTGTGTCAACAAGCAAGACAGAAGTGTCTTCTGTGGGCCCT
GCATAG

Salmo Salar LWS Open Reading Frame GenBank Accession # AY214131
ATGGCAGAAAGGTGGGGAAGTGCTGCTTATGCAGCCAGGCGACAAAACCAAGATACAACGAGAGAAT
CTTCCTTCACCTTCACCAACAGCAATAACACCAAAGATCCTTTTGAGGGCCCCAACTACCACATTGCTC

CAAGATGGGTGTACAATCTCTCAACACTTTGGATGGTCATAGTGGTCATCCTCTCAGTCTTCACCAATG
GCCTGGTACTGGTGGCCACTGCAAAATTCAAGAAGCTCCAACACCCTCTCAACTGGATCTTGGTCAACC
TTGCTATTGCTGACATTGGAGAAACACTTTTGGCAAGCACCATCAGCGTTTGCAACCAGTTTTTTGGCT
ACTTCATTCTGGGACATCCAATGTGTGTCTTTGAGGGATACACTGTTTCTGTGTGTGGTATTGCTGCTCT
GTGGTCCCTGGCTGTTATCTCTTGGGAGAGATGGGTGGTGGTGTGCAAACCTTTTGGAAGTGTCAAGTT
TGATGCCAAATGGGCCATGGGAGGCATTATCTTCTCCTGGGTCTGGGCTGCTTTCTGGTGTGCCCCCCC
CATCTTTGGCTGGAGCAGGTACTGGCCTCATGGCCTGAAGACTTCCTGCGGACCTGATGTGTTCGGAGG
CAATGAGGATCCTGGAGTCAAGTCCTACATGATTACTCTCATGATTACATGCTGCTTCTTCCCCCTGTTC
GTGATCATCTTCTGCTACATTTTCGTGTGGCTAGCCATTCGTGCTGTTGCTGCGCAGCAGAAAGACTCT
GAGTCAACACAGAAGGCCGAGAAGGAAGTGTCCAGGATGGTTGTTGTCATGATTATAGCTTACTGTGT
ATGCTGGGGACCTTATACCTGCTTTGCCTGCTTTGCTGCGGCTAACCCTGGGTATGCTTTCCACCCTCTG
GCTGCTGCAATTCCTGCCTACTTTGCCAAGAGCGCCACCATCTACAATCCAGTTATATATGTCTTCATG
AACAGACAGTTCCGTACTTGCATCATGCAGCTCTTTGGAAAGGCAGAAGATGATGGCACTGAAGTGTC
TACATCAAAAACCGAGGTTTCCTCTGTGGCACCTGCATAA

Salmo Salar SWS1 Open Reading Frame GenBank Accession #AY214133
ATGGGAAAGGACTTCCATCTGTACGAGAACATCTCTAAGATCAGCCCATTTGAGGGGCCACAGTATCA
CCTGGCCTCAATGTGGGCTTTCTACCTACAGGCTGCTTTCATGGGCTTTGTGTTCTTTGCTGGAACACCC
CTAAACTTTATAATTCTCGTGGTGACAGTGAAGTACAAGAAACTGAGACAACCGTTGAACTTCATCCTG
GTCAACATCTCGTTAGCAGGTTTCATCTTTGTGACGTTTTCTGTGAGTCAAGTGTTTGTTTCTAGCGCGA
GAGGATACTACTTCCTGGGTTACACCTTGTGTGCAATGGAAGCTTGCATGGGTTCAATAGCAGGGTTGG
TGTCAGCTTGGTCCCTGGCTGTCCTTGCCTTTGAGAGATACGTGGTCATCTGCAAACCCTTCGGCACCTT
CAAATTTGACAACAACCAGGCTCTGGCGGCTGTTGCCTTCACCTGGGTCATGGGGATCGGATGTGCCAC
CCCACCGTTCTTCGGCTGGAGCAGGTATATCCCTGAGGGTCTGGGTTGCTCCTGTGGACCTGACTGGTA
CACAAACAACGAGGAGTACCACTGTGCCAGCTACACCAAATTCCTTATTGTTACCTGTTTCCTCATGCC
CATGTCCATCATCTTCTTCTCCTACTCCCAGCTGCTGGGAGCACTACGGGCTGTGGCAGCTGCCCAGGC
TGAGTCAGCTTCCACCCAGAAGGCAGAGAAGGAAGTATCCAGGATGGTGATTGTGATGGTGTGCTCCT
TCATTCTGTGTTACGGCCCCTACGCCCTGGCAGGCTTGTACTTTGCCTATACAACAAGCGAGAACAAAG
ACTACCGCCTGGTGACCATCCCTGCTTTCTTCTCTAAAAGCTCCTGTGTATACAATCCTCTTATTTATGC
CTTCATGAACAAACAGTTTAACGCCTGCATCATGGAGACTGTGTTCGGAAAGCAGATTGAGGAGACTT
CAGTTTCAGCCTCCAAGACTGAGGTCTCCACAGCATAA

Salmo Salar SWS2 Open Reading Frame GenBank Accession # AY214134
ATGAACACAATGAGGTCGAATGCTCGCCCCGTGGAGCTCCAGGAGGGATTCTACATCCCTATCGCGCT
GGATACCAACAACATCACTTCACTCAGCCCCTTCCTGGTTCCTCAGGACCACCTGGCGGGCAGTGCTGT
CTTCTATGGCATGTCATTTTTCATGTTCTTCCTATTTGTTGCCGGCACTGCTATCAACGTCCTTACCATCG
TGTGTACTATCCAGTTCAAGAAGCTGCGATCCCATCTCAACTACATTCTGGTGAACTTGGCAATAGCTA
ACCTGCTGGTGTCCATGTTTGGATCCTCCACCGCCTGCTTGTACTTTGGCAACAGATACTTTATCATGGG
ATCGGTTGCATGCCAGATTGAGGGATTTACAGCTACTCTTGGCGGTATGGTGAGCTTATGGTCTCTCTC
AGTAGTGGCGTTTGAAAGATGGTTGGTTATTTGTAAGCCAGTCGGTAACTTTCAATTCAAGAGCACACA
TGCAATACTTGGCTGTGCAATCACTTGGGTGTTTGGGTTGGCTGCCAGTCTTCCCCCTCTGTTTGGCTGG
AGTAGATACATTCCAGAAGGTCTCCAGTGCTCCTGTGGACCAGACTGGTACACCACAAACAACAAATA
CAACAATGAGTCCTATGTCATGTTCCTCTTCTTCTTCTGCTTCGGCGTCCCATTCAGTGTCATTGTTTTCT
GCTATGCCCAGCTGCTCTTCATGATGAAGGCGGCTGCCGCGGCACAGGCAGACTCTGCCTCCACCCAG
AAGGCAGAGAAGGAGGTCACCAAGATGGTGGTGGTGATGGTGGTGGGCTTCCTAGTGTGCTGGATGCC
CTACGCCTCCTTTGCTGTCTGGGTTGTACAAAACCGCGGTGCACCCTTTGATCTCCGATTGGCCTCCATA
CCTTCCTGCTTCTCCAAGGCCTCCACAGTCTACAACCCTCTCATCTATGTCTTCATGAATAAGCTGTTCC
GCTCATGCATGATGAATTTGCTGGGATTGAAGTCCGGAGATGATGAGGAAGCATCATCAACATCCTCA
GTCACTCAAGTGTCTTCTGCTGGTTAA

Oncorhynchus mykiss *rtp12.5* Full length cDNA GenBank Accession # AY255832
TGATGAAAGAAAGATGGCGTCTCCAAATGGAGGTGATGATTTTGAATCATCTCTGCTGAGTTTTGAGA
AGCTGGATAGAGCATCTCCTGACCTATGGCCAGAGCAATTGCCAGGAGTCGCAGATTTTGCTGCATCAT
GCAAAAATCCAATTACCAATTCCCCACCCAAATGGATGGCTGAACTGGAGAGTGAGGACATTGAAATG
TTAAAAAAAGCTGGGGAGCCTGACCACAGCCAACCTGATGGAGAAGGTCAAAGGGCTACAGAACTTGG
CCTACCAGCTGGGCCTCGAGGAGTCCAGGGAGATGACCAGGGGAAAGTTCCTGAACATCCTGGAGAG

ACCCAAAAAGTGAAGACCCAGGATGTGTCTGCACAGAGAGTAAAACAACACCCAGACGACTGACTTG
AGACAGAAGAGGAATCAGACCTATGCCTTTATGTATACACTCATACTACTACTACCACCACCATCAAGT
CCCTGGATTTGAATATTGACACTAAATTCATTCACTGCGGAGTTCCCCTCGAGTCAAAGGGCAGCAACA
CTTTTCACCCCTTGACCTGTACATTTATTTTGTCCTCAAAGGCAGTTCCATTTACTTTTATAATGATTTGA
AATGGTGAATTACTTTCCTGTTTAGCTAGCTTGCAGGACTGTTTTGAAATGTGTATATTCTGTATTTATT
ATTCTTCTATTTGGAGCATTCTAAAAAAAAAAGTGCAATTTGTCAAAGAAAATTGTGTAATTCTTCAAA
AAAATTAATAAATTGCTCCCTCTTTTAGGCTATAACATGTTTATTAATTGTCCTTGTAATTGGTAATTAC
CCTCAGTGTGCATCTATGGATACAATTTAATCTTTCAATGGTTTAGGATACAATTAAATTCACTTCAGA
AACTGGAAAAAAAAAAAA

Oncorhynchus mykiss *sep15* Full length cDNA GenBank Accession # AY255833
CGCGGATCCAGACGCTGCGTTTGCTGGCTTTGATGAAAAATCGCCAAAATGTCGGGGGAGGTGTATAT
TTTGTGGCTTCTTTCTCTCATACAAACGCTGTCGGCCTATGGAGCCGACCTGTCCTCAGAGGCATGCAG
GGAGCTGGGCTTCTCCAGCTGCTGGGGGTGCAGCTCCTGTGACCTGCTGGGGGAGTTCAGCCTGAGCTC
GATCCAGCCTGTCTGCAAACAGTGCTGCCAGCAGGAGGTCCATATGGAGTCCAGGAAGCTCTACCCTG
GGGCCATCCTGGAAGTGTGTGGATGAAAATTGGGGAGGTTCCCTCAAGTCCAAGCTTTTGTCAGGTCTG
ACAAGCCGAAGATGTTCAAGGGTCTTCAAATTAAGTATGTGAGAGGCGCAGACCCTATACTAAAGCTG
CTGGACGATAACGGGAACATTGCTGAAGAACCCAGCATCCTCAAGTGGAACACGGATAGTGTGGAAG
AATTCCTGAGCGAGAAGTTGGAAGTATATAATACCGATTTGTAGTACTCATCACCATTTTCTTTTGTGC
ACTGTTTTTGGATTTTATTGTTTAAAGGTACTGCACTATTGTATTATTTCACTTCCCCCGTAGTACCTGA
AAATCATGAACTTCGTCCGTAGATTTTGTGAACTGACATTATGTGCCAGGATGTTAAACGGCTGTTTTA
TAGATTTGTAAAGTACCTGAACTTACCTGCAAAATACCTGGCATTAACGTCAAAAATATTGGAATGAA
GTAGCCAAAGAAAGACTAAAGATGTACACTGCTTGTTACCTTTTTTGTTCTCCAATCTGTCAGTTGTCTC
TGTGGTTTAAAAAAAAATATAACTTGTGGGATCTTTGGGTGGGGTCCAAGTCGTTCGCTTCATGCAGAT
GTTCCGGGGAGGAACACATTAATGAAGGCCTCACAGCAAACCACATTTTGGGATGTGAGACCAGATGT
CCCTGTGTGTCACTCTGGGCATCTTGCAGTGGAGAGAGACTGGATTCCCCAGTGTGTGATTAAGAATTG
GGGAGGGAAAAACACTGCAATGAATAAAATGTCCCCATGTTAAAAAAAAAA

Oncorhynchus mykiss RH1 proximal promoter GenBank Accession # AY305662
GTTTGTAGGATGTTTGGGAGGAAATGACATTGAAATGCATGAGTAATCCTATCTCCCGCACACACACA
CACACACACACATAAACGTGCAAACATGCATGCACACGCACAATCTAAAAATCTTAGTCACACAATAA
GCATCAGCTATAATACTGCAACATAAGCACAAGTACTCAATGAATGACCTGTGAATTACATGTAACTTT
ATGTTGCTACACTTAATGTTCTATTAAAATAATATTTTGCATTATATTGATTTAAAATCTATAAATATTG
CTATACCTAATATTAATACAATTATGTAATATGTTCAGGATGAGGACGTTACACGTCTCAGACCATTAT
CTATAAAAACATCCGTCAATCACCATATAGTATTTCCTGGTGGTGCAGCTATTCTACTTTGTGCCTATGT
ATTACCACAGCTTAACGTGTTTCTTATCGCAACTTATAATAAACCATCTGTTCTCCCACAGCACTCCGTC
TCTTCACTCTTTTGTGCTTGGAGTATGAAGCTTCAAACATGTCTCTAGAATATATTGAAATATATCTTCA
AAAGAGAAACTGTAAATTTAACAGTGTTTAAAAGTTGTATGCAAATGACTAACTCAGTTCTGAGTATAT
GGATGAGGCCTAGGTGTGTAACAGCCAATGCAGCGTTTCAGATTAAGCCATCATCTCCTGATAGGTAT
GAGCAGGTGTAACCTTAATAACTTGATGAGTGGCCAGTTATGTTTGCAGTGGTGGCTGGATTATATATT
AACAGTGGCCTAAATCACATTGTGCTGACTGATTGGGAAAGTGATCATCTAACACCTTAAGATGTGCCT
ATAAAATCAGGAGTCTGACCTCCTGGGTGCATTTGTAGAAAGGACCCTTTCCATCTCTCTTTCTGTACA
GCCGGAACACCATCTGAAGAAGGGCTGATCGCCACCGCAAGACCGCAACC

Oncorhynchus mykiss RH2 proximal promoter GenBank Accession # AY305661
ACTATAGGGCACGCGTGGTCGACGGCCCGGGCTCGTCTGGATTTTTTCTAATATTTATTAGGCCTACAT
CTTGGCGTAGTAGAAACATTTTTGAAAATGCAAGCCAATTTCCTGTAATTTCTAGATGGAGCTGAGAGA
AAATGTTGCCATTTTAAAGCTAATTTCCTGCATTTATATGCATTTTGCCATGGCTAATCCTGTGTTCTTTT
GCTCAAACATAACAACAAAATCAAAACTGATAAATTCATTGTTTTGGGAATTTTCATGTCTCTCTGACT
GTCTAGTTTTTATTTTGGCGATTGCTAGTTCTCAAAGATGATATGATCAAAAGAACAATATTTTTACTAC
ATACTCAACGTCTGGTTTTAGTCATTTGTACACTGTTCAAACATACGTGTCCTACGTGTCCTCTTACACA
GTAACTGGGTGCAAGCAGAAGCTATAGACTACCCATCCATACTTAGTTGCATTATCCTACAGAGGTATA
GTAATAAAAAACAAACAATAATAAAACATGTTTTACATTAAAAACAAATGTTTAAACAAGGTCAGTGT
GATAATCTTTATTCCCTGATGAGTTAGACAAAATCCACAGTGATTACGTGTTATAATCCCTTCCCTAAT
ATCTGAGGTTAAGATCAATTAACGAATTAGTTCGTAACAACCCCGCCTAAGCTAATTCAGCCAATTTTA
GCCAATGACATAGGGCCCCAAAAGGGTATAAAACTCCCTGCCAGGACACCCTAGCATAGGCAAAAGG

TTCACAACCAGATCAGATCTCATCTTGCCTTTCTCTTGCATTCATTGGACATCCACTGAGTAAAGATGC
AGAACGGCACAGAAGGAAGCAA

Oncorhynchus mykiss LWS proximal promoter GenBank Accession # AY305663
AGACCAAACTCACATGACACACCCGTTATGAATTGTCTGGTAAAGCGGATAGGATAGAGATTATCTCA
GCAAATCAGGCAAAAGGCCGAAGTGATCCACTTTCATCATTAAGTTGTTTTGTATTTGCATGAGGAAAA
GATAGAACGTAGAATGTAGTAAATTAAATCATTGCTCTGATTATCCCCTCAAAGAATTCTTCCCTGTTT
ACTTTGTTATAGTAAAAACATTCATATTGTCAAAATGTGTTTTCTTTATTTCAGAAGTAATAAGAACAA
TTGTTAAGTCAACTATTTTCTCTTTTAAGCATGAAGAGGATAAGGAATTTCACCTGACTGCTGAGTAAC
ATGACCCCAAAAGCACTTCTCATCCAAGAAGGATTAACCTTAACTTCAAACCTTGGCTTCAGTTCGACT
GGCCGGCATAACTCAAGTTCTTTTTGGTGTGTGTGTGTATAAGTCTTAGATCTTAATCTCGATGACGAC
ATGGCTGGTATAAAACCCCACAGATTGGGGTACACCATCTTGTTGGTTGCTAACACCACAACAGGTGA
TCAGCAAGACAAGACAACAGAAGCCAACCACAAGGACAGAGAAAAAGCGTCCAA

Oncorhynchus mykiss SWS1 proximal promoter GenBank Accession # AY305659
ATAGGGCACGCGTGGTCGACGGCCCGGGCTGGTCAATCAATTAATTCATTGGTTGATGGATTAGCACTT
AACAATAATGAATCGTTAAAGTGGAGTTTCCAAACATTGTTGATGTGATTGCCACTGCCCAGTCAGCTT
GTTCTTTTTACCCCTGTTCACCACATCCCCATCCCCCTCCCCTTAGGTTACGTTCGTGGAAGGTTAATGT
CTATCAAAGATTAGTCCCTCAGAGATTAATGGGAACTGGATAAGGCAGTTACCACCTCATTAGAACAG
CCTACACAGCTTAGCTTGATTCAGTGATATACATTACAAACTCCAGAAGTATGGCAATGCTTTCTATCT
TAACACTGTCCGCACCAACAAAAACAACGTGTCTCTGTTATAAATTCAATTATTCTTCTAAATTGTATTC
TTTCACACTGGCTCTTTAAGATGGATTACTGCGCCCAACAAAGTAAGGTCTCTACTTACGGGTCATGGT
CATGGGGGCAGCTTAATCCTAAAGTTTATTCTTCCACCTACAAGATCAGGAAGATCCACAGGAGAAGC
TTGTTCCTGACATAATGTAGAGGTTTATAAGTGGTTTGTGGTCATATAAAAGGGAGCCTTTCAGCGTCA
CAGTGCACATCTAAGGGACGGACGGGTAGGGGTACTGAGTCAGAGAGGAGCCAAGATGGGAAAGAAC
TTCCATCTGTACGAGAACATCTCTAAGGTCAGCCCATTTGAGGGGCCACAGTATCACCTGGCCCCAATA
TGGGCTTTCTACCTACAGGCTGCTTTCATGGGCTTTGTGTTCTTTG

Oncorhynchus mykiss SWS2 proximal promoter GenBank Accession # AY305660
AGGTTTCAAACAGTCAGGACAGCTGTTGTAAGCTTATTAGATAAACACCAAAGACCTGAGCTATTTTTT
CATGTTACCCTATTTCAATTGTTTGTGCGATTCAGTCCTCTGTTTCTGAAGAAAGTGTTGATGGCACGTC
AAAAGCATTTATGGATAAGTCCCTAAAGACCAAGACATCCAGCACAAAGCCTGAGTTAAACCAGCAGA
GCGCAGATTACAAAAGGCTGAAATGCGTACCATTAGCCGGCTTAACAGCATTGGAAAGAAGATTAGCT
GGCTGCAGTAACACTGAACTGCTATCTATGTAAATGGGATCATCCTTATCATCCAGGTTTTTCTGCACG
GAAAGAAGATTACGTTCAGGGACTGTGTCTTATATACATGTGCAGGTGTGTGTGGTTGTATGAGAGGA
CTTGTGTGTTGAGGAAATTACTTGACATCTTTCATTTGAGGGTGGGGCACTGTGATATCACTCTTAAGG
CCTTATAAAAGTCCACTTAGTTCCCCAACAGGACATGCGATCAACCTTCAAGCTCCTGAGGTAGACACC
AGGCAATTAACGCATTTTGAATTACTTATTCTCATTTTAATGGAATAGATCTTTGTAGTTATAAGACCTT
GGTAGTGGGGAAACCTTGGTAGTGGGGATT