

Investigations of Visual Pigment Changes in Fishes

By

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B.Sc., University of Victoria, 1998
M.Sc., Memorial University of Newfoundland/Universidade
Federal de Santa Catarina, 2001

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Abstract

Understanding why organisms possess particular combinations of visual pigments (VPs) is central to visual ecology. Species that adjust spectral sensitivity by changing retinal VPs provide a powerful tool to investigate this question. Changing chromophores (A_1 or A_2) and altering opsin expression are mechanisms used to adjust photoreceptor maximum absorbance (λ_{\max}). My research explored the function of such dynamic systems in two teleosts, coho salmon and zebrafish. I investigated temporal VP changes in relation to life history and environmental change. In coho, I found a correlation between chromophore shifts and seasonal variations in environmental variables for freshwater and marine life history stages (seasonal hypothesis). These findings provide an alternative explanation to the migration/metamorphosis hypothesis. The latter, suggests that shifts from A_2 to A_1 at metamorphosis, preceding seaward migration, are preemptive to changes in photic environment. Using exogenous thyroid hormone (TH), which plays a role in coho metamorphosis, I demonstrated that under various rearing conditions and times of year, TH consistently shifted VPs towards A_2 dominance. This increased A_2 is opposite to that occurring at metamorphosis, further supporting the seasonal hypothesis. While TH induced changes in rod λ_{\max} were consistent with a change in A_1/A_2 ratio, λ_{\max} variations in middle wavelength- and long wavelength-sensitive (MWS and LWS) cones were greater than predicted by a shift in A_1/A_2 ratio alone. I

proposed a change in opsin expression to explain MWS and LWS cone λ_{\max} variations. In support of this hypothesis, a novel RH2 opsin subtype (expressed in MWS cones) was isolated and sequenced. This second RH2 possessed an E to Q substitution at the position analogous to 122 in bovine RH1, which imposes a hypsochromic shift in λ_{\max} . Further investigation found that changes in coho MWS cone λ_{\max} were correlated with ontogeny and the frequency of MWS cones with λ_{\max} below 500 nm was reduced in marine compared to freshwater stages. The combination of changes in A_1/A_2 ratio and opsin expression provides coho with a dynamic spectral tuning mechanism. In zebrafish, I demonstrated the presence of an A_1/A_2 VP pair, which shifted to A_2 dominance with exogenous TH treatment, but not with temperature.

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List of abbreviations

A ₁	vitamin A ₁ -based chromophore = retinal = retinene ₁ = retinaldehyde = C ₁₉ H ₂₇ -CHO
A ₂	vitamin A ₂ -based chromophore = 3,4-didehydroretinal = retinene ₂ = 3,4-didehydroretinaldehyde = C ₁₉ H ₂₅ -CHO
A _{max}	absorbance maxima = the height of the main (α-band) in mO.D. as measured from baseline
CCD	charge-coupled device
E _a	Activation energy is the minimum energy required to take the molecule over the energy barrier from inactive to active conformation
ESP	extraction spectrophotometry procedure
HBW	half-band width
HPLC	high performance liquid chromatography
mO.D.	optical density x 10 ⁻³
MSP	microspectrophotometry or microspectrophotometer depending on the context
MWS, LWS, SWS, UVS	middle, long, short and ultraviolet wavelength sensitive respectively, referring to the four cone classes
MWS/LWS	middle/long wavelength-sensitive opsin class
nm	nanometers = 10 ⁻⁹ meters
OS	outer segment
PCR	polymerase chain reaction
RACE	rapid Amplification of cDNA Ends
RH1	rod opsin class (once called rhodopsin, but this name is inappropriate in this case as a rod could be expressing the RH1 but combining it with the A ₂ chromophore and thus by the original nomenclature this would have been a porphyropsin)
RH2	a middle wavelength opsin with an amino acid sequence with a high degree of homology with the RH1. In fishes the RH2 opsin is generally expressed in the MWS cones (Hunt <i>et al.</i> , 2001b)
ROS	rod outer segment
RPE	retinal pigment epithelium
SWS1	short wavelength-sensitive type 1 or ultraviolet opsin class
SWS2	short wavelength-sensitive type 2 or blue opsin class

T ₃	triiodothyronine
T ₄	tetraiodothyronine also commonly called thyroxine
TH	thyroid hormone
TSH	thyroid stimulating hormone
UTR	untranslated region
UV	ultraviolet = electromagnetic radiation that spans from approximately 10 – 380 nm, for the purposes of vision this range can be narrowed to about 300 - 380 nm
VP	visual pigment
α-band	alpha-band is the main absorbance band of vertebrate VPs and is the most long wavelength (~340 -640 nm) peak of the four absorbance peaks the position of which is referred to as the λ _{max} . The other peaks are the; β-band, (~380 – 410 nm) which is the absorbance of the cis band of the chromophore bound to the opsin, γ-band, (~278 nm) which is due to the aromatic amino acids (tryptophan and tyrosine), and the δ-band, (~231 nm) which is due to the organic bonds of the molecules.
λ _{max}	lambda max = spectral position of the main peak (α-band) of the absorbance spectrum of a visual pigment

Chapter 1 : General introduction

Vision and visual ecology

Vision is the ability to sense light, a process that begins in the photoreceptive cells. Photoreception in the simplest sense can be defined as the process of capturing light and converting it into a neural signal. While being succinct, these definitions fail to appreciate the complexity and diversity of the visual systems of many animals, which may confer the ability to decipher complex signals encoded in: wavelength (colour), frequency (spatial and temporal pattern), intensity, and polarization angle of light. The study of how these signals are created and detected and how they affect animal behaviour is visual ecology.

The process of vision begins with the capture of quanta of electromagnetic radiation (photons) by membrane bound receptors that are found in the photoreceptor cells of light sensing organs. The receptor molecules, known as visual pigments (VPs), are derived from a structure of an ancient origin shared with plants and bacteria which consists of a seven transmembrane protein (opsin) combined with a light capturing chromophore (based on vitamin A) (Nilsson, 1996). The variations in these two components, the chromophore and opsin, and how these variations affect the absorption of light by the photoreceptor cells are the foci of this thesis.

The absorption spectrum of a VP has a characteristic shape, with four distinct peaks, and while we have no comprehensive physical model to describe its precise shape, the attributes that cause each absorbance peak are known. The first peak (δ -band) occurs at 231 nm and represents the absorbance by organic bonds in the VP molecule. The second peak (γ -band) at 278 nm is the result of the absorbance of

aromatic amino acids (tryptophan, phenylalanine and tyrosine). The third peak (β -band) is due to the absorbance of the cis-band of the chromophore, which is covalently bound to the opsin molecule by a Schiff's base linkage. This peak may vary from approximately 300 nm to over 400 nm, depending on variations in both chromophore and opsin (Govardovskii *et al.*, 2000). The fourth peak, which is of particular interest to visual ecologists, is the α -band and the position of its maximum absorbance is referred to as the λ_{\max} . Absorption within the α -band is due to the visual pigment as a whole and varies with the chromophore-opsin combination. The absorption of light in the α -band results in a Z to E isomerization of the chromophore. This photoisomerization is the initial step in the phototransduction cascade that leads to the sensory perception of light.

Variations in either opsin or chromophore can alter the energy required to isomerize the chromophore, known as the energy of activation (E_a), thus altering λ_{\max} . Changes in both opsin and chromophore permit the λ_{\max} of vertebrate VPs to vary from as low as 350 nm to as high as 640 nm. The limits to the range of electromagnetic radiation that are suitable for vision are set by the energy per photon. At wavelengths below 300 nm, photons are high energy and capable of breaking organic bonds, while above 850 nm, photons have too little energy to isomerize the chromophore and are therefore not useful for vision.

One of the central themes in visual ecology has been to explain why different organisms possess VPs with different λ_{\max} values. The most common approach to address this question has been to compare the λ_{\max} values of VPs from organisms inhabiting different spectral environments. Another approach has been to look for

changes in the VPs of a single species relative to movements between different spectral environments or relative to periodic changes in the spectral environment of a single habitat. My thesis takes the second approach; examining the timing of changes in VP λ_{\max} relative to life history strategy and environmental variability.

Initial Discovery of Visual Pigments

Evidence for the existence of photolabile VPs in vertebrates was first published over a hundred years ago. In 1876, Boll noted the reddish colour of the excised frog retina, which faded when exposed to light (from: Wald, 1953). He named the pigment after its colour, *Sehrot* (visual red). He thus commenced a system of nomenclature (to be discussed below) that has been evolving ever since. Shortly thereafter, Kuhne (1878) and Kuhne and Sewall (1880) described a second pigment in the eyes of freshwater fishes. They called this new VP *sehporpur* (visual purple), again based on the colour of the retina when removed from the eye (from: Crescitelli, 1958; Kusmic & Gualtieri, 2000; Wald, 1939). In 1896, Köttgen and Abelsdorf extracted VPs from the retinas of several vertebrate species, using solutions of bile, and determined the approximate λ_{\max} of each by comparing their absorbance before and after bleaching (difference spectra). They found that mammals, birds, and amphibians generally had VPs absorbing maximally around 500 nm, while freshwater fishes have VPs absorbing maximally around 540 nm (Crescitelli, 1958).

Rhodopsin and Porphyropsin

Much of the pioneering work on VPs was done by Nobel Laureate George Wald, whose nomenclature for VPs is still used today despite some minor changes in definition. Wald published a series of studies (Wald, 1933, 1935a, b, 1936a, b) in which he demonstrated that retinol (vitamin A) could be extracted from a light adapted or bleached retina but not a dark adapted one, and that the destruction of the retina in darkness (with chloroform) caused the release of retinene (later called retinaldehyde or retinal for short). These same procedures were used to show that the differences in VP absorbance between terrestrial and freshwater vertebrates were due to the presence of vitamin A₁ and vitamin A₂ derivatives respectively (Edisbury *et al.*, 1937; Gillam *et al.*, 1938; Wald, 1939). Wald (1953) proposed new names for the two VPs using the existing term “rhodopsin” from Kuhne for the vitamin A₁ (retinal), and proposing “porphyropsin” for the vitamin A₂-based (3,4-didehydroretinal).

Wald’s nomenclature, while suitable for his simple two-VP model, soon became inadequate as the number of known VPs increased. The early nomenclature of Wald, implied that there were only two rod VPs, rhodopsin and porphyropsin, which he argued had fixed λ_{\max} values positioned at approximately 500 and 522 nm respectively (Wald, 1939, 1960). However, Wald (1937) had also named a cone opsin; the iodopsin found in chicken cones. He later (Wald *et al.*, 1953) went on to demonstrate that iodopsin with a λ_{\max} at 565 nm could be recombined with 3,4-didehydroretinal to form what he called cyanopsin with a λ_{\max} at 625 nm. This was the first evidence that retinal and 3,4-didehydroretinal were interchangeable within the same opsin, and that the replacement of A₁ for A₂ caused a long wavelength shift

in the λ_{\max} . The measurement of this reconstituted cyanopsin with its λ_{\max} at 625 nm came before such pigments had actually been identified.

The nomenclature began to get more complicated when yet another term chrysopsins (due to their golden appearance) was proposed for the blue shifted rod VPs of some deep sea fish. Wald took issue with this new term because these new chrysopsins were rod opsins based on vitamin A₁ which fell into his definition of a rhodopsin (Wald *et al.*, 1957). Furthermore, several other reports (Dartnall, 1952a, b; Denton & Wyllie, 1955) were being made in which VPs were found to have λ_{\max} values that clearly did not fit Wald's simple definition of rhodopsin and porphyropsin. To address the increasing complexity, Dartnall (1952b) proposed a new systematic naming system in which only the λ_{\max} of the VP would be given. To differentiate between which chromophores were used in the VPs, Munz (1957) suggested adding a subscript 1 or 2 to indicate whether the VP was based on retinal or 3,4-didehydroretinal respectively. Crescitelli (1958) later added that the λ_{\max} and subscript should be followed by the name of the organism, in brackets, from which the VP was measured. This new system was not particularly popular. Dartnall (1962) and Wald (1960) both suggested that the terms rhodopsin and porphyropsin might continue to be used provided their meanings were altered to imply VPs extracted from rods and based on A₁ and A₂ respectively.

To date, no systematic nomenclature has been derived for the naming of VPs, and confusion persists due to current usage of the terms rhodopsin and porphyropsin and the discovery of complex mixtures of VPs within single photoreceptors. Some authors have used the terms rhodopsin and porphyropsin to describe the incorporation

of retinal and 3,4-didehydroretinal with cone opsins (Archer, 1999), which increases ambiguity. Furthermore, none of these naming systems are suitable for labeling the λ_{\max} of receptors in which there exists a mixture of both A_1 and A_2 , or more than one opsin, let alone mixtures of both chromophores and multiple opsins. Given that mixtures of both opsins and chromophores have been found to exist within a single photoreceptor, I believe it is unlikely that any such systematic nomenclature is even feasible. In my thesis I will not use the terms rhodopsin and porphyropsin but instead A_1 - and A_2 -based VPs, and when discussing the chromophores themselves they will be referred to as A_1 and A_2 . When appropriate, I will use the nomenclature system outlined above, where the λ_{\max} value is followed by a subscript indicating the dominant chromophore type.

Since their discovery, several important differences between A_1 - and A_2 -based VPs have been uncovered: (i) the λ_{\max} of an A_1 -based VP is short wavelength shifted relative to the same opsin combined with A_2 ; (ii) the width of the absorbance curve at 50% of its peak height (half-band width; HBW) is narrower for an A_1 -based VP than its A_2 counterpart; (iii) A_1 -based VPs are less sensitive to thermal isomerization than the same opsin combined with A_2 ; (iv) the molar extinction of A_2 -based VPs are approximately 75 percent of that of A_1 -based VPs. The shifts in λ_{\max} and HBW associated with exchanging A_1 for A_2 in the same opsin are wavelength dependent, and while we have empirically based models describing these relationships there is no comprehensive physical theory to explain the phenomenon itself (Govardovskii *et al.*, 2000; Harosi, 1994). However, we can attribute the general shift in λ_{\max} and change in thermal isomerization rate to the change in E_a . The A_2 chromophore has an added

double bond in the β -ionone ring that increases the length of its conjugated π -electron system, lowering E_a (Fig. 1.1). Since energy is inversely proportional to wavelength, A_2 -based VPs can be photoisomerized by longer wavelength light than the same opsin combined with A_1 . Therefore, while the upper limit of λ_{\max} for A_1 -based VPs is around 580 nm (Blatz & Liebman, 1973), the upper limit for A_2 -based VPs is as high as 640 nm (Koskelainen *et al.*, 2000).

Two models to explain the timing of chromophore shifting

Until the 1960's, the predominant explanation for shifts in A_1/A_2 ratios was that they were correlated with movements between fresh water and the terrestrial or marine environments, a theory I call the migration/metamorphosis hypothesis. This hypothesis was an extension of the observations made on fresh water and terrestrial animals by Köttgen and Abelsdorf in 1896 (from: Crescitelli, 1958). Their findings were later reconfirmed by George Wald who demonstrated that the observed differences in maximum absorbance of terrestrial and fresh water vertebrate species were due to the presence of A_1 - and A_2 -based visual pigments. Based on his findings, Wald proposed a model that fresh water vertebrates used porphyropsin, terrestrial and marine vertebrates used rhodopsin, and metamorphosing or migrating vertebrates, such as amphibians and euryhaline fishes, used a combination of both, with the predominant VP being that which matched their spawning environment (*e.g.* porphyropsin for salmon that spawn in fresh water and rhodopsin for lamprey that spawn at sea; Wald 1939). Wald initially proposed that the presence of rhodopsin,

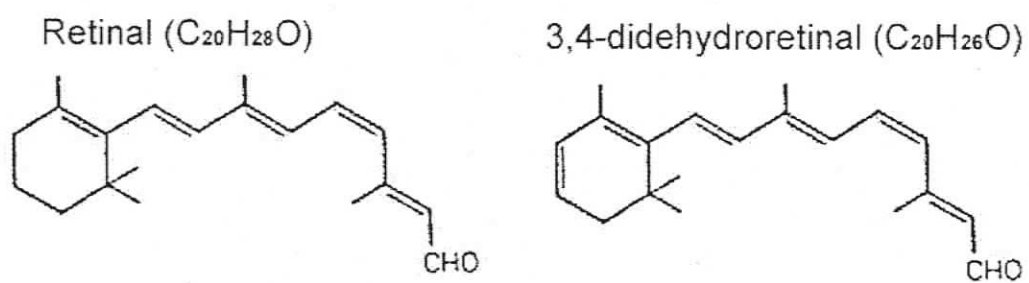


Figure 1.1 The structural and molecular formulas for retinal and 3,4-didehydroretinal; visual pigment chromophores that are based on vitamin A_1 and A_2 respectively.

porphyropsin, or a mixture thereof, was genetically predetermined and fixed (Wald, 1939). Eventually, he modified his hypothesis to acknowledge that rhodopsin/porphyropsin ratios were dynamic and could change within the lifetime of an organism in correlation with: (i) metamorphosis, (ii) migrations between fresh water and sea/land, or (iii) the changing seasons (Reuter *et al.*, 1971; Wald, 1973).

Wald's model that suggested shifts in rhodopsin/porphyropsin ratio were an adaptation to migration and metamorphic events was the most widely accepted and was a perfect match for the "sensitivity hypothesis" (Lythgoe, 1966), which suggested that the VPs of fishes tended to match the background photic environment. For example, deep-sea fishes have blue shifted VPs (Denton & Warren, 1956; Munz, 1957), coastal and freshwater fishes have green to yellow shifted VPs (Lythgoe & Partridge, 1991; Munz, 1958), and fishes in tannic stained water have red shifted VPs (Fuller *et al.*, 2003; McDonald & Hawryshyn, 1995). Accordingly, the proposed function of the A_1/A_2 shift was that it served as an adaptation that spectrally tunes the sensitivity of the organism to match the different photic environments encountered by migratory/metamorphosing species (Alexander *et al.*, 1994; Beatty, 1975a, 1984; Knowles & Dartnall, 1977; Kusmic & Gualtieri, 2000). I have named this the migration/metamorphosis hypothesis.

The migration/metamorphosis hypothesis was not adequate to explain variations in A_1/A_2 ratio that occur in some non-migratory species, and so a second hypothesis began to emerge, which I call the seasonal hypothesis. Dartnall *et al.* (1961) stumbled upon a seasonal pattern in the A_1/A_2 ratio in rudd (*Scardinius erythrophthalmus*, Linnaeus), a fish that increased its retinal proportion of A_2 in

winter and A_1 in summer. Seasonal patterns in A_1/A_2 ratio have since been observed in several other non-migratory freshwater teleosts (Allen, 1971; Allen *et al.*, 1982; Allen & McFarland, 1973; Bridges, 1964a, b), and a freshwater invertebrate (Suzuki *et al.*, 1985). Seasonal patterns in A_1/A_2 ratio have also been observed in metamorphosing/migrating species that were previously presumed to shift in correlation with their movements between habitats. Makino and Dodd (1996) demonstrated that bullfrogs (*Rana catesbeiana*, Shaw) continue to shift A_1/A_2 ratio even after they have metamorphosed. They showed that these shifts fit a seasonal pattern, despite previous evidence that the chromophore shift in this species was linked to the migration/metamorphic event (Crim, 1975; Ohtsu *et al.*, 1964; Wald, 1946; Wilt, 1959). The observations of seasonal patterns in A_1/A_2 ratio imply that either the spectral environment changes seasonally, for which consistent patterns have not been reported in the literature, or that an alternate functional explanation may exist. The nearly ubiquitous observations of increases in A_2 as temperature descends and increases in A_1 as temperature ascends among labile pigment pair species (reviewed in Beatty, 1984) has led to the proposal that the A_1/A_2 shift may play a role in modifying the thermal stability of the VP's (Ala-Laurila *et al.*, 2004b; Allen & McFarland, 1973; Williams & Milby, 1968).

In most migrating and metamorphosing species, the timing of movements are synchronized with season and it is therefore difficult to determine which came first, the correlation of chromophore shifting with migration/metamorphosis or with season. It is also possible that the two hypotheses are not in conflict, as chromophore shifting could serve more than one function. In addition to shifting spectral sensitivity

of migrating/metamorphosing species to match their new environment, the A_1 - A_2 shift could decrease thermal noise by shifting to a more thermally stable (A_1) chromophore in summer, thus improving/maintaining signal to noise ratio in the face of seasonally changing environmental variables (Allen & McFarland, 1973). This makes the line of distinction between these two hypotheses unclear, which is worsened by the fact that A_1 - A_2 shifting in several species has been attributed to both of these hypotheses on separate occasions, sometimes by the same researcher (*e.g.* coho salmon (*Oncorhynchus kisutch*, Walbaum) by Beatty, 1966).

Role of hormones on visual pigments chromophore composition

Hormones that play a role in metamorphosis have been shown to have effects on A_1/A_2 ratios in species that possess labile pigment pair systems. The application of exogenous prolactin and thyroid hormones (both tetraiodothyronine and triiodothyronine) have consistently caused increases in the proportion of A_2 -based VPs in teleosts (reviewed in Beatty, 1984). The precise role of these hormones on the A_1/A_2 ratio is not known but it is thought that they cause an increase in the activity of the presumed 3,4-dehydrogenase, which is proposed to convert retinal to 3,4-didehydroretinal (Dartnall, 1964) within the eye (Naito & Wilt, 1962). The effects of exogenous thyroid hormones are not due to use of pharmacological dosages as was demonstrated by the increase in A_2 in salmonids treated with thyroid stimulating hormone (Beatty, 1972). Prolactin and thyroid hormones (THs) are known to be involved in the metamorphic transitions that accompany changes in habitat in many labile pigment pair species. In salmonids, THs are linked to many of the physiological

and behavioural transitions that occur at smoltification (metamorphosis prior to seaward migration), including: silvering, changes in body shape, increased saltwater tolerance, rheotaxis and loss of UVS cones (Allison *et al.*, 2003; Folmar & Dickhoff, 1980; Grau *et al.*, 1982; Higgs *et al.*, 1982; Hoar, 1988; McBride *et al.*, 1982; Specker *et al.*, 2000; Staley & Ewing, 1992). Interestingly, in salmonids, the effect of exogenous TH on the A_1/A_2 ratio is opposite to the shift that occurs at smoltification. While salmonids decrease relative amounts of A_2 at the time of year when they go to sea, the application of TH, which stimulates smoltification, causes an increase in relative amounts of A_2 in all labile pigment pair species studied (Alexander, 1998; Allen, 1977; Allen & Munz, 1983; Allison *et al.*, 2004; Beatty, 1969a, 1972; Cristy, 1974; McFarland & Allen, 1977; Munz & Swanson, 1965; Tsin & Beatty, 1978).

Quantifying visual pigments

One of the most fundamental advances in the field of VP research was the recognition that the shape of the absorbance spectra of different VPs was uniform when plotted on an appropriate scale (Dartnall, 1953). The importance of this discovery was that it permitted characterization of the spectral absorbance of any VP with a single parameter, λ_{\max} . Dartnall's (1953) nomogram was based on curve fitting to the best available data at the time, obtained by extraction spectrophotometry techniques (ESP) on frog (*Rana spp.*) retinas. The principle of this first model was that the shape of the absorbance curve of all VPs was invariant when plotted on a frequency scale. The power of the template, as Dartnall (1953) pointed out, was that it allowed comparisons between spectral absorbance of the VPs alone and the spectral

sensitivity of the organism, as well as providing a means to estimate λ_{\max} from poor quality absorbance data.

Unfortunately, the universality of Dartnall's (1953) nomogram was soon brought into question. It was found that VPs with λ_{\max} values at longer wavelengths tended to have absorbance spectra that were slightly narrower (Marks, 1965), and those at shorter wavelengths were slightly wider, than Dartnall's nomogram (Ebrey & Honig, 1977). It was also discovered that VPs based on A_2 required a separate template altogether (Bridges, 1967; Munz & Schwanzara, 1967). These findings led to the proposal of new templates.

Following the introduction of Dartnall's (1953) nomogram, many attempts were made to find a universal template that described the shape of all VP absorbance curves. Various models were used, such as: (i) plotting the absorbance curves on different scales (Barlow, 1982); (ii) fitting the absorbance to multiple additive curves (Dawis, 1981; Ebrey & Honig, 1977; Harosi, 1976; Metzler & Harris, 1978; Mooij & van den Berg, 1983); and (iii) defining mathematical equations to predict the change in width with λ_{\max} (Govardovskii *et al.*, 2000; Harosi, 1994; Lamb, 1995; MacNichol, 1986; Mansfield, 1985; Palacios *et al.*, 1996; Partridge & De Grip, 1991; Stavenga *et al.*, 1993). However, one important fact remains; the shape of the absorbance curve has not yet been explained by any model of the underlying physical parameters of the VP itself. That is to say, we do not know why absorbance curves have the shape they do. For this reason, all templates have been based on empirical curve fitting. Govardovskii *et al.* (2000) have recently revisited the issue of a universal template. They provided an updated version of the template that is based on the shape of the A_1

based rod VP absorbance curve collected by Partridge and De Grip (1991) described by an equation that incorporates Lamb's (1995) formula and incorporates a modified version of the transformation provided by Mansfield (1985) and MacNichol (1986). As always, there are exceptions to every rule, and Govardovskii *et al.* (2000) provide a list of deviations from the universality of their template. What remains to be determined is whether these exceptions are due to the different measurement techniques that were being employed to collect the data, or if they reflect biologically relevant variations. Some evidence has been provided in recent studies (Carleton & Kocher, 2001; Glosmann & Ahnelt, 2002; Lukats *et al.*, 2002; Lyubarsky *et al.*, 1999; Makino & Dodd, 1996; Parry & Bowmaker, 2002; Röhlich *et al.*, 1994), and by my research, to suggest that at least some deviations may be expected if more than one VP opsin is expressed within single photoreceptors.

Models that predict the shift in λ_{\max} due to a change in chromophore

The effect of substituting A_2 for A_1 in the same opsin is a long wavelength shift in λ_{\max} . The magnitude of this shift in λ_{\max} ($\Delta\lambda_{\max} = A_2 \lambda_{\max} - A_1 \lambda_{\max}$) is wavelength dependent, such that $\Delta\lambda_{\max}$ is greater for VPs with λ_{\max} values at longer wavelengths (Bridges, 1965; Dartnall & Lythgoe, 1965; Tsin *et al.*, 1981). However, there is evidence that the relationship reaches an asymptote around 420 nm, below which $\Delta\lambda_{\max}$ begins to increase again (Harosi, 1994). As with the shape of the absorbance curve, there is no comprehensive physical model to describe the change in λ_{\max} when the same opsin is combined with A_1 and A_2 . Therefore, predictive models have been based entirely on empirical data sets. Our understanding of the relationship

between $\Delta\lambda_{\max}$ and λ_{\max} has therefore changed over time as the quantity and quality of measurements has improved.

Early models were only accurate for a narrow range in wavelength focused around the λ_{\max} of rod VP's (470 - 540 nm), from which nearly all of the recordings had been made. Therefore, extrapolations beyond these data sets were unreliable. The first two models to be published (Bridges, 1965; Dartnall & Lythgoe, 1965) proposed a linear relationship between $\Delta\lambda_{\max}$ and the λ_{\max} of the A₁-based VP (See Table 3.2 and figure 3.8). Both models predicted that as the λ_{\max} of the A₁-based VP increased so would $\Delta\lambda_{\max}$. If extrapolated below approximately 470 nm, these models predicted that the A₂-based VP λ_{\max} would be lower than that of the A₁-based VP (*i.e.* $\Delta\lambda_{\max}$ would be negative).

Later models took advantage of the technique of microspectrophotometry (MSP), introduced to the study of visual pigments by Hanoako and Fujimoto (1957), to expand the range of accuracy by measuring the λ_{\max} shifts in cones. The first of these post MSP models (Tsin *et al.*, 1981) included measurements of short wavelength sensitive (SWS) or blue cones, and predicted that $\Delta\lambda_{\max}$ would not be negative unless the A₁-based VP λ_{\max} was below approximately 400 nm. The three most recent models (Harosi, 1994; Parry & Bowmaker, 2000; Whitmore & Bowmaker, 1989) predicted non-linear relationships in which $\Delta\lambda_{\max}$ never reaches zero (see figure 3.8).

There is considerable variation even amongst the three most recent models, particularly in the long and short wavelength regions. I propose three possible reasons for the discord among the most recent models. Firstly, all three models used MSP

records collected from species that the authors presume to have 100 percent A₁- and A₂-based VPs in their photoreceptors or from VPs reconstituted with the chromophore of choice (Parry & Bowmaker, 2000). The assumption that in fact the photoreceptors actually possess 100 percent of either A₁ or A₂ is tenuous as there is considerable evidence to suggest that there is significant variation in A₁/A₂ ratio among individuals (Bowmaker *et al.*, 1975; Bridges, 1964b) and within individuals (temporal and spatial variability) (Bowmaker *et al.*, 1988; Bridges, 1982; Muntz & Northmore, 1971; Partridge *et al.*, 1989; Reuter *et al.*, 1971). In the study by Parry and Bowmaker (2000), although the retinas were bleached and photoreceptors supplied with specific chromophores for regeneration, it is possible that some of native chromophore remained and was reincorporated into the VPs. This possibility is more likely in cones that may use a different VP regeneration pathway, which does not require the retinal pigmented epithelium (RPE) (Mata *et al.*, 2002).

Secondly, more than just the chromophore ratio may have been changing. Most of the data used to create these models came from fishes, and recently both mammals and fishes have been shown to occasionally express more than one opsin within individual photoreceptor classes (Archer & Lythgoe, 1990; Carleton & Kocher, 2001; Cheng & Novales Flamarique, 2004; Hope *et al.*, 1998; Loew *et al.*, 2002; Lukats *et al.*, 2002; Parry & Bowmaker, 2002; Raymond *et al.*, 1996). If more than one opsin were present in varying ratios, the change in $\Delta\lambda_{\max}$ might not show a consistent pattern.

Finally, the shift in λ_{\max} due to chromophore substitution may not be explicable by any one single model. That is to say, the relationship between $\Delta\lambda_{\max}$ and

the λ_{\max} of the A₁-based VP may not be predictable by a single model. Recent evidence (Ala-Laurila *et al.*, 2004b) has shown that the E_a of different VPs does not vary with λ_{\max} according to the Stile-Lewis-Barlow (SLB) relation ($E_a = \text{constant} \times (1/\lambda_{\max})$). Ala-Laurila *et al.* (2004) proposed that this variation from the SLB relationship is the result of an enormously large degree of freedom in E_a that exists due to the numerous amino acid modifications that are possible in opsin molecules. If this is the case, then it is also possible that $\Delta\lambda_{\max}$ may not vary consistently among different opsins.

The opsins

The opsin is a seven transmembrane spanning G-protein-coupled receptor composed of approximately 350 amino acids (Nathans & Hogness, 1983). The seven hydrophobic transmembrane segments are believed to be α -helices, and together they form a palisade structure with an intramembranous chromophore-binding pocket that opens to the extracellular side of the cell membrane (Bowmaker, 1991; Hargrave & McDowell, 1992). Variations in amino acid sequence, particularly in the vicinity of the chromophore-binding pocket, can result in changes in the VP λ_{\max} . Vertebrate retinal opsins have been classified into five classes based on the general spectral location of their λ_{\max} and their amino acid sequence: (i) SWS1 (short wavelength-sensitive type 1, λ_{\max} 360-430 nm), (ii) SWS2 (short wavelength-sensitive type 2, λ_{\max} 440-460 nm), (iii) RH2 (rod opsin like, λ_{\max} 470-510 nm), (iv) MWS/LWS (long/middle wavelength-sensitive, λ_{\max} 510-560 nm), (v) RH1 (rod opsin, λ_{\max} 470-510 nm), (Bowmaker, 1991; Okano *et al.*, 1992; Yokoyama & Yokoyama, 1990;

Yokoyama, 2000). Many fishes express representatives of all five opsin classes and some express more than one copy of each (called subtypes, Bowmaker, 1995; Chinen *et al.*, 2005b; Dann *et al.*, 2004; Yokoyama, 2000). Thus far, opsin subtype expression has only been recorded in fishes and primates (Takechi & Kawamura, 2005), however, this is likely the result of sampling effort rather than being of phylogenetic importance.

Changes in opsin expression

Until recently, the existing paradigm was that each photoreceptor class contained only one opsin. With the availability of biomolecular techniques, such as immunohistochemistry and *in situ* hybridization, it has been found that more than one opsin may be expressed in a single photoreceptor. So far, this phenomenon has been most commonly found in fishes (Archer & Lythgoe, 1990; Hope *et al.*, 1998; Raymond *et al.*, 1996; Wood & Partridge, 1993; Zhang *et al.*, 2000) and mammals (Glosmann & Ahnelt, 2002; Lukats *et al.*, 2002; Lyubarsky *et al.*, 1999; Parry & Bowmaker, 2002; Röhlich *et al.*, 1994; Szel *et al.*, 1994), although there is evidence for coexpression in a species of salamander (Makino & Dodd, 1996) and gecko (Loew *et al.*, 1996). In some cases, the coexpression of more than one opsin in a receptor class may be the stable state (Lukats *et al.*, 2002; Parry & Bowmaker, 2002), however, it may also represent a transition state between the expression of two different opsins in a single receptor class (Cheng & Novales Flamarique, 2004; Hope *et al.*, 1998; Szel *et al.*, 1994; Wood & Partridge, 1993).

Changes in opsin expression within a photoreceptor class have the potential to alter the λ_{\max} of the receptor and, until recently, were considered fairly rare. The first evidence for a change in opsin expression was found in the rods of eels (*Anguilla spp.*) (Beatty, 1975b; Carlisle & Denton, 1959), but it was not until much later that it was demonstrated that this change in opsin occurred within individual receptors, *i.e.*, not by turnover of photoreceptors (Wood & Partridge, 1993; Wood *et al.*, 1992). Temporal (ontogenetic) and spatial (across the retina) changes in opsin expression have recently been reported among several families of fishes (Carleton & Kocher, 2001; Fuller *et al.*, 2004; Loew *et al.*, 2002; Loew & Sillman, 1993; Minamoto & Shimizu, 2005; Shand, 1993; Shand *et al.*, 2002; Shand *et al.*, 1988; Takechi & Kawamura, 2005; White *et al.*, 2004) and other vertebrates (Glosmann & Ahnelt, 2002; Lukats *et al.*, 2002; Lyubarsky *et al.*, 1999; Röhlich *et al.*, 1994). Work in this area is presently at the forefront of VP research, and to date little is known about the timing of changes in opsin expression or its functional significance.

Thesis objectives

The objective of my Ph.D. research was to investigate the timing of shifts in VP λ_{\max} in relation to life history transitions and environmental changes. I addressed different aspects of this question in four separate experiments, with the objectives of each listed below:

1. To determine if the timing of the shift from A_2 to A_1 at the time of smoltification in coho salmon is correlated with metamorphosis and the

ensuing seaward migration (migration/metamorphosis hypothesis) or with seasonal changes in environmental variables (seasonal hypothesis).

2. To explore the role of thyroid hormone (TH) on A_1/A_2 ratio in rods and cones of coho salmon under different rearing conditions and at different times of year as an extension of objective 1.
3. To examine the ontogenetic timing of changes in MWS and LWS cone λ_{\max} values relative to changes in A_1/A_2 ratio in rods of coho salmon.
4. To determine whether zebrafish possess an A_1/A_2 labile pigment pair system.

Outline

The first data chapter in this thesis (Chapter 2) investigates the timing of chromophore shifts in coho salmon. I attempted to differentiate between the seasonal and the migration/metamorphosis hypotheses, which have been used to explain the timing of chromophore shifting in salmonids and other vertebrates. Chapter 3 looks at the possibility that thyroid hormones may have differential effects on the A_1 - A_2 interchange system in coho under different rearing conditions and at different times of year. The observed shift in photoreceptor λ_{\max} is evidence for a shift in opsin expression in MWS and LWS cone classes. For the MWS cones, further support for a change in opsin expression came from the finding of a novel RH2 opsin sequence in coho salmon. In Chapter 4, the possibility that the two RH2 opsin subtypes are differentially expressed during various life history stages of coho salmon was investigated by comparing the λ_{\max} values of rods to those of MWS and LWS cones

for fish from different age classes. In the fifth and final data chapter, I describe an experiment that provided the first conclusive evidence for the existence of a paired pigment system in zebrafish (*Danio rerio*, Hamilton). A general discussion of my findings and areas for future research follows in Chapter 6.

Chapter 2 : Seasonal cycle in vitamin A₁/A₂-based visual pigment composition during the life history of coho salmon (*Oncorhynchus kisutch*)

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Introduction

Vertebrate VPs are composed of two components: a protein (opsin) and a chromophore (aldehyde of vitamin A). Spectral absorption properties of a VP are mainly determined by the amino acid sequence of the opsin protein and the type of chromophore bound to the opsin. Variations in amino acid sequence account for different classes of vertebrate opsins (*e.g.* ultraviolet, short, middle and long wavelength sensitive opsins, and rod opsins) and thus the λ_{\max} of the VP. However, some vertebrates, referred to as “paired pigment” or “labile pigment pair” species (Allen & Munz, 1983; Dartnall & Lythgoe, 1965), are capable of shifting between VPs based on vitamin A₁ (retinal) and vitamin A₂ (3,4-dehydroretinal).

Shifting from A₁- to A₂-based chromophores in the same opsin has the effect of shifting the λ_{\max} of the VP to longer wavelengths, broadening the HBW, decreasing the molar extinction coefficient, and increasing the frequency of thermal isomerization (Bridges, 1967; Brown *et al.*, 1963; Donner *et al.*, 1990; Wald & Brown, 1953). Because rods and cones both draw their VP chromophores from the same source, the RPE, shifts in A₁/A₂ ratio in rod photoreceptors are typically mirrored in neighboring cones (Liebman & Entine, 1968; Loew & Dartnall, 1976; Whitmore & Bowmaker, 1989). There are some exceptions *e.g.* deep sea fishes with neighbouring rods possessing A₁- or A₂-based VPs (Bowmaker *et al.*, 1988) and the use of Müller cells for VP regeneration in cones but not rods in the cone dominated retinas of ground-squirrels and chickens (Mata *et al.*, 2002).

Substituting A_2 for A_1 in the same opsin affects both λ_{\max} and thermal stability of VPs, but neither of these effects alone provides an explanation for the functional significance of the labile pigment pair system. Loew and Dartnall (1976) and Munz and McFarland (1977) proposed that a shift in A_1/A_2 ratio in rods may simply be incidental to a shift in A_1/A_2 ratio in cones. The functional outcome of the A_1/A_2 shift would be reflected in the photopic spectral sensitivity that has adaptive value to the fish. An alternate hypothesis is that the shift in A_1/A_2 content may optimize the signal to noise ratio for which temperature may be a limiting factor (Ala-Laurila *et al.*, 2004b; Barlow, 1956; Lewis, 1955; Stiles, 1948). Despite over sixty years of research on A_1/A_2 VP shifting, the proximate and ultimate causes of labile pigment pair systems remain unclear (Allen, 1971; Beatty, 1984; Bowmaker, 1991; Bridges, 1972; Crescitelli, 1991; Dartnall, 1962; Douglas, 2001; Knowles & Dartnall, 1977; McFarland & Allen, 1977; Munz & McFarland, 1977; Tsin & Beatty, 1979; Wald, 1939).

Observations of the timing of A_1/A_2 shifts in fishes and amphibians have led to two main hypotheses that address the ecological significance of labile pigment pair systems. First, shifts in A_1/A_2 ratio may be an adaptation that spectrally tunes the visual system to changes in the photic environment as a species moves from one habitat to another. Such movements are often associated with migration or metamorphic transitions. This hypothesis is supported by the observation that euryhaline fishes, which migrate between marine and fresh water, appear to shift between A_1 - and A_2 -based VPs at the time of migration (Carlisle & Denton, 1959; Crescitelli, 1956; Wald, 1957). Similarly, amphibians that metamorphose from

aquatic to terrestrial forms shift from A_2 to A_1 around the time of metamorphosis (Wald, 1946). Both of these observations generally agree with the fact that most marine and terrestrial vertebrates have A_1 -based VPs while most freshwater species use predominantly A_2 -based VPs (Wald, 1939). However, it is also notable that these early studies did not consider the possibility of seasonal shifts in A_1/A_2 ratio. Therefore, their observed correlations of A_1/A_2 ratio with migration/metamorphic events may have been confounded by the seasonal nature of these migration and metamorphic transitions. Table 2.1 illustrates species classified as shifting chromophore ratio with: (i) migration/metamorphosis, (ii) seasons, (iii) both migration/metamorphosis and season.

Second, shifts in A_1/A_2 ratio have been correlated with seasonal changes in environmental variables, such as temperature (Allen & McFarland, 1973; Suzuki *et al.*, 1985; Tsin & Beatty, 1977) and light quality/quantity (day length, intensity and wavelength) (Allen, 1971). Seasonal transitions in A_1/A_2 VP ratios were first observed in rudd (*Scardinius erythrophthalmus*, Linnaeus) by Dartnall *et al.* (1961) and have since been observed in many labile pigment pair species (reviewed in Beatty, 1984). Support exists for both the seasonal (Allen *et al.*, 1982; Beatty, 1969b; Bridges, 1964a; Suzuki *et al.*, 1985) and the migration/metamorphosis (Crescitelli, 1956, 1958; Kennedy, 1957; Wood *et al.*, 1992) hypotheses across different species (Table 2.1), and even within a single species, for example, coho salmon (Beatty, 1966). However, few studies have used a single species to investigate the evolutionary aspects of a paired pigment system by comparing evidence for and against both hypotheses outlined above.

Table 2.1 Labile visual pigment pair species exhibiting changes in A_1/A_2 ratio correlated with seasons and/or a migration/metamorphic events or both.

Species	Common name	Author
<i>Species with a shift correlated to a migration or metamorphic event</i>		
<i>Anguilla anguilla</i>	European eel	(Wood et al., 1992) [†]
<i>A. rostrata</i>	American eel	(Beatty, 1975b)
<i>Petromyzon marinus</i>	Sea lamprey	(Crescitelli, 1956; Steven, 1950; Wald, 1957) [‡]
<i>Negaprion brevirostris</i>	Lemon shark	(Cohen et al., 1990)
<i>Rana pipiens</i>	Northern leopard frog	(Kennedy, 1957)
<i>Hyla regilla</i>	Pacific tree frog	(Crescitelli, 1958)
<i>Species with a shift correlated with the seasons</i>		
<i>Scardinius erythrophthalmus</i>	Rudd	(Dartnall et al., 1961)
<i>Notemigonus crysoleucas boscii</i>	Golden shiner	(Bridges, 1964b)
<i>N. crysoleucas aureatus</i>	Golden shiner	(Allen and McFarland, 1973)
<i>Richardsonius balteatus balteatus</i>	Redside shiner	(Allen, 1971)
<i>Luxilus (previously Notropis) cornutus</i>	Eastern common shiner	(Allen et al., 1982)
<i>Belonesox belisarius</i>	Topminnow	(Bridges, 1964a)
<i>Lota lota</i>	Burbot	(Beatty, 1969b)
<i>Procambarus clarki</i>	Red swamp crayfish	(Suzuki et al., 1985)
<i>Salmo trutta</i>	Brown trout	(Muntz and Mouat, 1984)
<i>Species that have been classified as both seasonal and migration/metamorphic</i>		
<i>Rana catesbeiana</i>	Bullfrog	(Makino et al., 1983; Wald, 1946)
<i>R. temporaria</i>	European common frog	(Muntz and Reuter, 1966*)
<i>Oncorhynchus clarki</i>	Cutthroat trout	(Allen et al., 1973)
<i>O. tshawytscha</i>	Chinook	(Beatty, 1966)
<i>O. nerka</i>	Sockeye	(Beatty, 1966)
<i>O. kisutch</i>	Coho	(Alexander et al., 1994*; Beatty, 1966) (present study)

*These studies did not recognize the seasonal pattern but provided sampling dates from which a pattern was discernable.

[†]This species does not show the pattern of increased A_2 in winter and decrease in summer.

[‡]Each of these studies found a predominance of A_1 or A_2 at different life history stages leading Bridges (1972) and Beatty (1984) to presume a shift with migration.

Coho salmon were chosen for this study because their range is restricted to temperate, seasonally variable regions and they possess a migratory life history strategy, which enables a comparison between the seasonal versus migration/metamorphosis hypotheses. On the west coast of North America, coho salmon hatch in late winter-early spring in cold freshwater streams and rivers. Young coho generally spend over a full year in fresh water as parr before undergoing a metamorphic event called smoltification, which prepares them for migration to the marine ecosystem. Once in the marine environment, coho usually spend 18 or more months at sea before maturing and returning to their natal streams to spawn. Since coho commonly spend at least a full year in both fresh and salt water, it is possible to follow the pattern of change in A_1/A_2 ratio in both habitats through all four seasons.

Previous studies examining A_1/A_2 shifting in coho salmon differ in their conclusions. Beatty (1966) arrived at the conclusion that chromophore shifting in coho during their fresh water residency was correlated with seasons, and not seaward migration, as implied by Crescitelli (1958) and Wald (1957). However, Beatty (1966), also maintained support for the migration/metamorphosis hypothesis and used it to explain the shift from vitamin A_1 to A_2 as spawning salmon returned from the ocean to fresh water. Interestingly, the shift observed in spawning coho from A_1 to A_2 in the fall, matched the trend of increasing A_2 recorded in coho parr at the same time of year. In contrast, a recent study by Alexander *et al.* (1994) supported only the migration/metamorphosis hypothesis and reported a correlation between the timing of the shift from A_2 to A_1 and the ability to regulate blood sodium levels at the time of smoltification. It was proposed that the shift in A_1/A_2 ratio could be used as an

indicator of smoltification in coho salmon. The functional explanation for the A_1/A_2 shift provided by Alexander *et al.* (1994) was that spectral sensitivity of coho salmon changed to match the presumed differences in spectral environment of the fresh and salt water environments.

In the present study, I tracked the variation in λ_{\max} as a correlate of A_1/A_2 ratio in rod photoreceptors of coho salmon at three life history stages throughout the year to differentiate between the migration/metamorphosis and seasonal hypotheses. In accordance with previous studies, I found that the degree of spectral shift in rod λ_{\max} in coho salmon matched that predicted for a change in A_1/A_2 ratio. I also compared hatchery-reared fish to those captured from the wild to determine whether hatcheries resembled the natural environment with respect to the A_1/A_2 ratio. These results provide new evidence to support the notion that seasonal variables such as temperature and photoperiod play a dominant role in determining A_1/A_2 VP ratios in this paired-pigment species.

Materials and Methods

Animals and care

Coho salmon alevin, parr and smolts were collected from Robertson Creek hatchery on Vancouver Island, B.C. (49.322°N 124.936°W) and Kispiox River hatchery in central B.C. (55.435°N 127.647°W). Target Marine salmon farms, Sechelt, B.C. (49.479°N 123.782°W) provided ocean-going juveniles. Wild coho parr, smolts and ocean going juveniles were captured in the field from populations

associated with both hatcheries. Fish were shipped to the University of Victoria and arrived within 24 hours, with the exception of wild samples collected from the open ocean. Eyes from fish captured early in the trip were enucleated and kept on ice. Once onsite, fish were held in holding tanks at 11 ± 1 °C for no more than 48 hours.

Rearing conditions at the two hatcheries differed, as did natural photoperiod and ambient temperatures (6° latitudinal difference between hatcheries). Coho at Robertson Creek were reared in outdoor earthen ponds from post hatch until smoltification at which point they were released into the Somass River to make their seaward migration. Temperature of outdoor ponds varied from a minimum of 3.4 °C in winter to a maximum of 16.5 °C in summer. Ponds were exposed to natural lighting conditions, which included canopy shade in the morning, direct sunlight at mid-day and patchy light in the afternoon. The Kispiox River hatchery reared their coho in indoor tanks with patchy natural light from windows and skylights with supplemental light from overhead fluorescent lighting. Tanks were covered with screen mesh to eliminate escapement. Temperature in indoor tanks was maintained at 7 ± 1 °C throughout the year, while wild fish collected from a nearby creek resided in waters that were subject to surface freezing from late December to late April and only rose above 10 °C during July and August.

Microspectrophotometry

Fish were dark adapted for at least one hour prior to sacrifice by an overdose of 300 mg l⁻¹ MS-222 (tricaine methanesulphonate, Crescent Research Chemicals, Pheonix, AZ, USA) followed by cervical transection. The body mass and standard

length of the fish varied widely depending on where and when they were sampled (alevin, juvenile and adult; marine or fresh water). The right eye was enucleated and hemisected along an anterior-posterior axis. A piece of retina 1 - 2 mm² was cut out of the dorsal-most section of the dorsal hemisphere of the retina. The dorsal retina was chosen because the dissection technique was repeatable with clear landmarks for orientation. A standardized location was required to decrease between fish variation, since dorsal-ventral variations in A₁/A₂ ratio were observed in coho, despite a previous report by Munz and Beatty (1965) that found no interhemispheric variation using the VP extraction technique. I found a significant difference ($t_{12} = -3.59$, $p = 0.04$, $r^2 = 0.854$, paired t-test) in the mean λ_{\max} of rods sampled from the dorsal versus ventral hemisphere in the same eye using MSP. The mean λ_{\max} of rods from the dorsal retina was lower than those from the ventral retina, consistent with findings in other salmonids and other paired-pigment fishes (Bridges, 1982; Denton *et al.*, 1971; Muntz & Northmore, 1971). The retinal sample was teased apart on a glass cover slip and a drop of Minimal Essential Media (MEM; Sigma, St. Louis, MO, USA; pH adjusted to 7.4 - 7.6) was applied to the sample. A second cover slip was placed over the sample and sealed with paraffin. All procedures were performed under deep red illumination (>650 nm) or using a dissecting scope equipped with infrared LED (800 nm) illumination and monitored with a charge-coupled device (CCD) camera. Care and treatment of fish was in accordance with the University of Victoria Animal Care Committee, under the auspices of the Canadian Council for Animal Care.

The CCD-MSP instrument used in this study has been described in detail in Hawryshyn *et al.* (2001). Here I provide a brief description. A short duration (500

msec) flash of full spectrum (300 - 800 nm; 150 W xenon arc light source – intensity regulated, Oriel), unpolarized light was projected as a small beam ($\sim 2 \times 3 \mu\text{m}$) through the rod outer segment (ROS). The transmitted light passed through a spectrograph (300 nm blazed grating, Acton Research Co, MA) and onto a Peltier cooled ($-45 \text{ }^\circ\text{C}$), back-illuminated, CCD-detector (NTE/CCD-1340/400-EMB Princeton Instruments, Roper Scientific, Inc., Trenton, NJ). ROS absorbance ($\log_{10}(I_R/I_M)$) was calculated by comparing transmitted intensity through the ROS (I_M) to the transmitted intensity through an area clear of debris and in media adjacent to the ROS referred to as the reference (I_R).

The retinal sample was examined under infrared illumination (Schott RG850 filter, Ealing Optics) and monitored by an infrared camera (Canadian Photonics Laboratory). The search image and infrared filtered beam (Schott RG850 filter) were displayed on a computer monitor. The position of the ROS relative to the measurement beam was controlled by a motorized stage (Marhauser-Wetzlar GmbH & Co., Germany). The path of the motorized stage was plotted on-screen to prevent repeated measurements of ROSs.

A preliminary analysis suggested that there was considerable variation in percent A_2 between individual ROSs within a fish (mean standard deviation = 22.7 percent, $n = 851$ fish), which was greater than the variation between means for each fish (mean standard deviation = 10.1 percent, based on 79 samples of ~ 10 -fish). In order to maximize my sampling effort and minimize the number of fish sacrificed I made MSP recordings of at least 20 ROSs, from each of 10 fish on each date, from each geographic sampling location. During the course of these experiments, I

collected recordings from 851 coho. Approximately 17,000 MSP records met the criteria for inclusion in the database. Criteria for acceptance of absorbance spectra were: (i) presence of a baseline on the long wavelength arm (Harosi & MacNichol, 1974); (ii) peak absorbance near the expected wavelength (~500-530 nm for coho rod photoreceptors); (iii) minimal absorbance by photoproduct and; (iv) signal to noise ratio of the α absorption band greater than 5 to 1. These criteria rejected approximately ten percent of MSP recordings. Determinations of λ_{\max} , and percent A_2 from absorbance records were performed offline subsequent to initial sampling.

A custom designed analysis program was used to determine λ_{\max} from ROS's and the A_1/A_2 ratio from absorbance records using existing templates (Govardovskii *et al.*, 2000; Munz & Beatty, 1965). Raw absorbance curves, which consisted of 1340 points recorded between 300 and 750 nm, were linearly detrended if necessary (Harosi, 1987). The raw data was smoothed using a nine-point adjacent averaging function, and the smoothed curve was normalized to zero at the baseline on the long wavelength arm and to one at the center of the α -peak. Normalized curves were analyzed with a nonlinear least-squares routine, to the upper 20 percent of the weighted A_1/A_2 averaged Govardovskii *et al.* (2000) template (based on the center of the α -peak ± 40 nm). A second estimate of λ_{\max} was attained using a minimum variance fit to the Munz and Beatty (1965) template, which fits the curve from λ_{\max} to a point at 20 percent of the maximum on the long wavelength arm (Fig. 2.1). The average of these two estimates was used for the calculation of percent A_2 as described below.

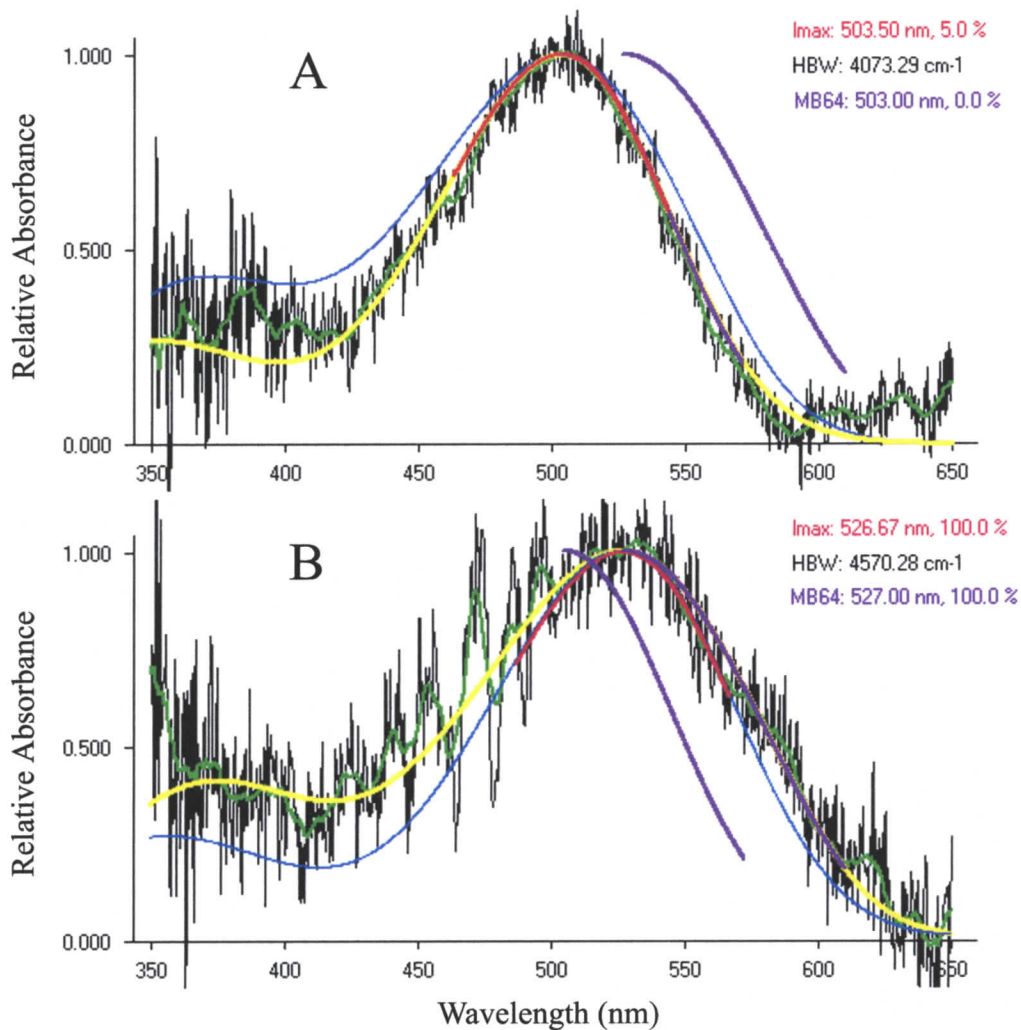


Figure 2.1. Normalized spectral absorbance curves from individual rod photoreceptors from coho salmon (*Oncorhynchus kisutch*, Walbaum). Selected examples of (A) 100 percent A₁-based VP and (B) 100 percent A₂-based VP determined based on λ_{max} values. Both spectra are the result of single transverse MSP measurements through the outer segment of individual rod photoreceptors. Black lines are the 1340 raw data points collected between 300 and 750 nm. Dark green lines are nine-point adjacent averaging smoothed lines. Red lines are the nonlinear least-squares fit of the upper 20 percent of the weighted A₁/A₂ averaged Govardovskii *et al.* (2000) template. Purple lines are the 503 and 527 nm Munz and Beatty (1965) template curves which are fit from λ_{max} to a point at 20 percent of the maximum on the long wavelength arm. Thin blue lines are the 100 percent A₂ (A) and 100 percent A₁ (B) template, predicted if the VP had these chromophore ratios respectively. Thick yellow lines are the best-fit template based on the calculated λ_{max} . Values in the upper right hand corner are estimates based on template fitting, λ_{max} = Govardovski *et al.* (2000) fit, and MB64 = Munz and Beatty (1965) fit, HBW = half-band width of the curve at 50 percent of the peak height measured on a scale of cm⁻¹.

Data analysis

Coho salmon rod VPs vary in λ_{\max} from 503 to 527 nm depending on the ratio of A₁- and A₂-based chromophores (Munz & Beatty, 1965). Munz and Beatty (1965) created a template for calculating percent A₂ in retinal extracts. The template was based on the range of λ_{\max} values observed in coho and by incorporating the known difference in molar extinction coefficients of A₁- and A₂-based VPs (Bridges, 1967; Wald & Brown, 1953). From their template, which provided percent A₂ at 10 percent intervals, the following third order polynomial expression was derived to calculate percent A₂ from the observed λ_{\max} (nm),

$$\text{Percent A}_2 = -363082.74 + 2073.4743 \times \lambda_{\max} + -3.9522863 \times \lambda_{\max}^2 + 0.0025151612 \times \lambda_{\max}^3. \quad (1)$$

Percent A₂ values for all ROS absorbance curves from each fish were used to calculate mean percent A₂ values for individual fish. For each location or age group, monthly mean percent A₂ values were calculated based on mean percent A₂ from all fish sampled for that location or age group in the specified month. Annual variations in mean percent A₂ for specific geographic locations or age classes were fit, by the method of least squares, to cosine curves with a period of one year. This was accomplished by varying the mean, amplitude (of oscillation) and lag time (number of months the cosine curve was advanced or retarded to maximize the fit) (Legendre & Dutilleul, 1992). Akaike's information criterion (AIC) approach for model selection was used to compare the fit of mean monthly percent A₂ data to various models.

$$\text{AIC} = \text{deviance} + 2 \times \text{number of parameters} \quad (2)$$

This technique of model selection penalizes models that use a greater number of parameters, and provides a relative weight value (Akaike's weight = w_i ; where larger values indicate greater support for the model) that can be used as a criterion for model selection (Wagenmakers & Farrell, 2004). I compared the trend in mean monthly percent A_2 to a linear model, a least squares cosine curve, and to temperature and day length values, where appropriate. Periodic samples collected from the wild were considered too infrequent to justifiably fit to cosine curves. Instead, mean monthly percent A_2 values were compared to fish from the associated hatchery, sampled on the same date, using a two-way ANOVA followed by independent sample t-tests for comparisons on each sampling date ($p \leq 0.05$). Collection of MSP records from 10-20 fish per sample date required that MSP measurements be made throughout the day, with recordings made between 7 am and 10 pm. To determine if time of day may have had an effect on A_1/A_2 VP composition I performed five replicates of a 24 hour sampling series. Every three hours for 24 hours consecutively, I dark adapted one fish for three hours and made MSP recordings from 50 rod photoreceptors. I found no significant effect of time of day on either mean λ_{\max} ($F_8 = 1.166$, $p = 0.348$; Fig. 2.2) or peak absorbance (measured in mOD; $F_8 = 1.465$, $p = 0.241$; Fig. 2.3).

Results

General observations across coho populations

Absorbance spectra of rod photoreceptors were measured from retinal tissue in the dorsal hemisphere of coho salmon eyes. Fish were collected from two

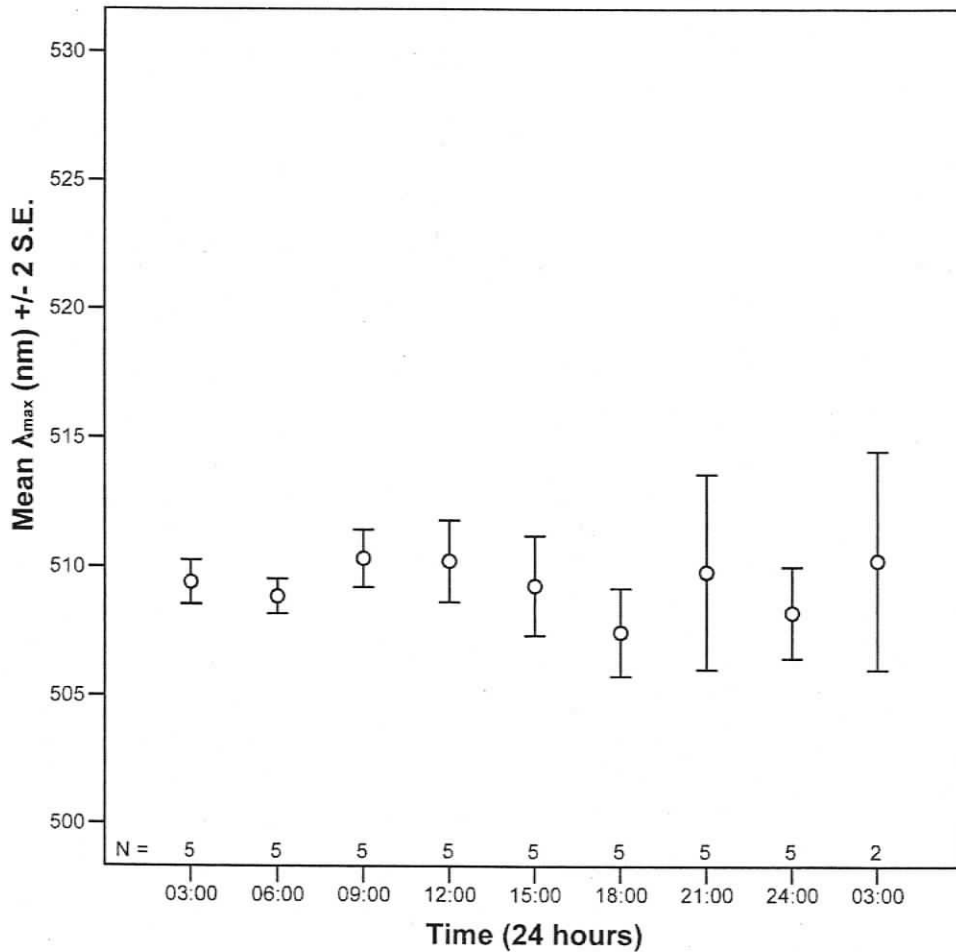


Figure 2.2. The mean rod λ_{\max} (nm) \pm 2 S.E. for coho salmon (*Oncorhynchus kisutch*, Walbaum) over 24 hours. Each point represents the mean of five replicates of a 24 hour sampling protocol, in which one or two (on the last replicate) fish were sampled every three hours. λ_{\max} was measured using MSP on individual rod outer segments. Note the final time period (03:00) was only repeated twice. The mean of each fish was based on the mean of 50 rods sampled from the dorsal retina. Replicates of the 24 hours sampling were performed on coho parr from Robertson Creek Hatchery in the summer of 2004 on June 17/18, 23/24, July 22/23, and August 23/24 (this replicate included two fish every three hours).

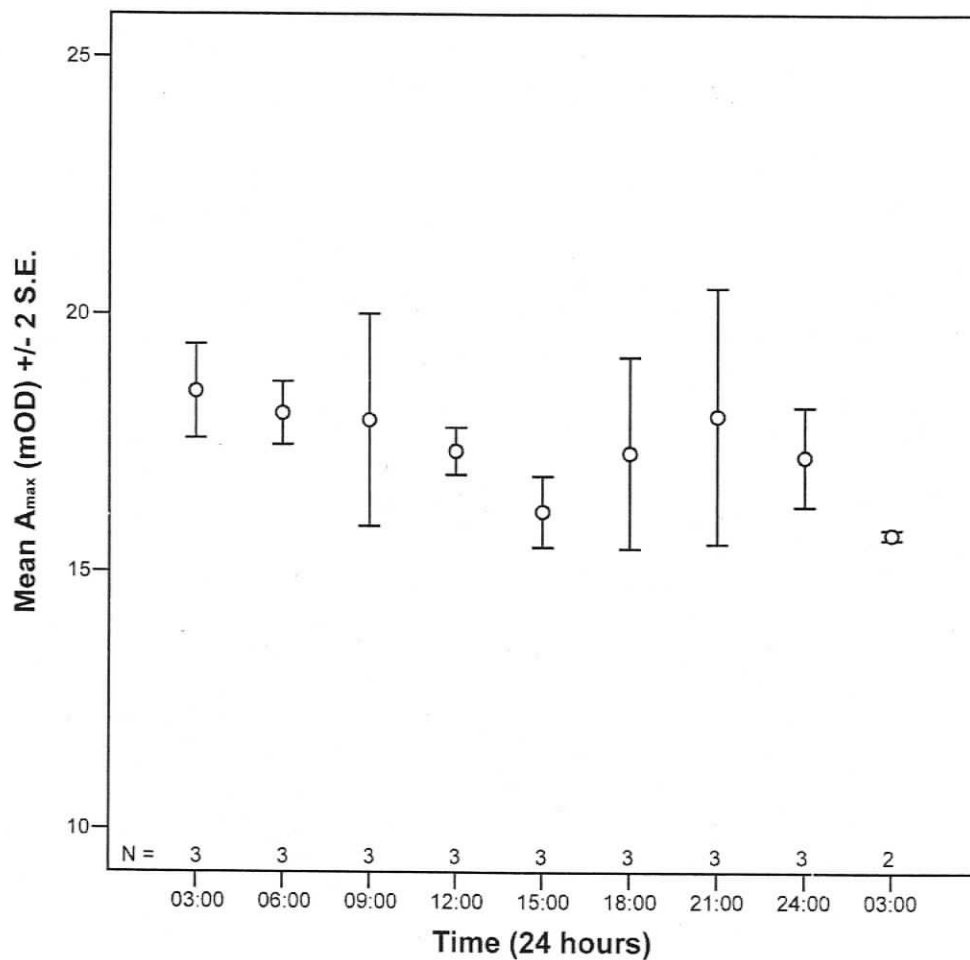


Figure 2.3. The mean rod A_{max} (mOD) \pm 2 S.E. for coho salmon (*Oncorhynchus kisutch*, Walbaum) over 24 hours. Each point represents the mean of three replicates of a 24 hour sampling protocol, in which one or two (on the last replicate) fish were sampled every three hours. A_{max} was measured using MSP on individual rod outer segments. A_{max} was corrected for rod angle and diameter. Note the final time period (03:00) was only repeated twice. The mean of each fish was based on the mean of 50 rods sampled from the dorsal retina. Replicates of the 24 hours sampling were performed on coho parr from Robertson Creek Hatchery in the summer of 2004 on July 22/23, and August 23/24 (this replicate included two fish every three hours).

hatcheries and from wild populations associated with both hatcheries, as well as from the open ocean and a marine aquaculture facility. Over a two-year period 851 coho were sampled. Twenty rod absorbance spectra were measured from each fish.

Although sampling took place over two years, no one population was sampled for more than 12.5 months. Therefore, data in the following figures have been plotted as a single 12-month cycle permitting all populations and age classes to be compared directly at any given time during the year.

Mean rod λ_{\max} per fish ranged from 502 to 527 nm, which is in accordance with previous findings for coho salmon and reflects the predicted shift from retinal to 3,4-didehydroretinal dominance within a single rod opsin (Alexander *et al.*, 1994; Beatty, 1966; Munz & Beatty, 1965). Monthly variation in λ_{\max} , and percent A_2 for all fish from all locations and age classes (except wild population from near the Kispiox River hatchery) were plotted in Figure 2.4. The cyclical pattern in mean monthly λ_{\max} and percent A_2 in Figure 2.4 was best fitted by a cosine curve (method of least squares; Table 2.2) with a period of one year. This cyclical pattern had a peak in late winter (February – March) with a mean of 509.23 nm (32.4 percent A_2) and amplitude of 3.07 nm above and below the mean. The well-defined relationship between λ_{\max} and percent A_2 -based VP composition for rod photoreceptors in this species means that there is little difference between expressing my results in either form. Therefore, only percent A_2 values are provided for the remainder of the results section. AIC weights support the hypothesis that mean monthly percent A_2 values for coho salmon follow a seasonal/cyclical pattern (Table 2.2).

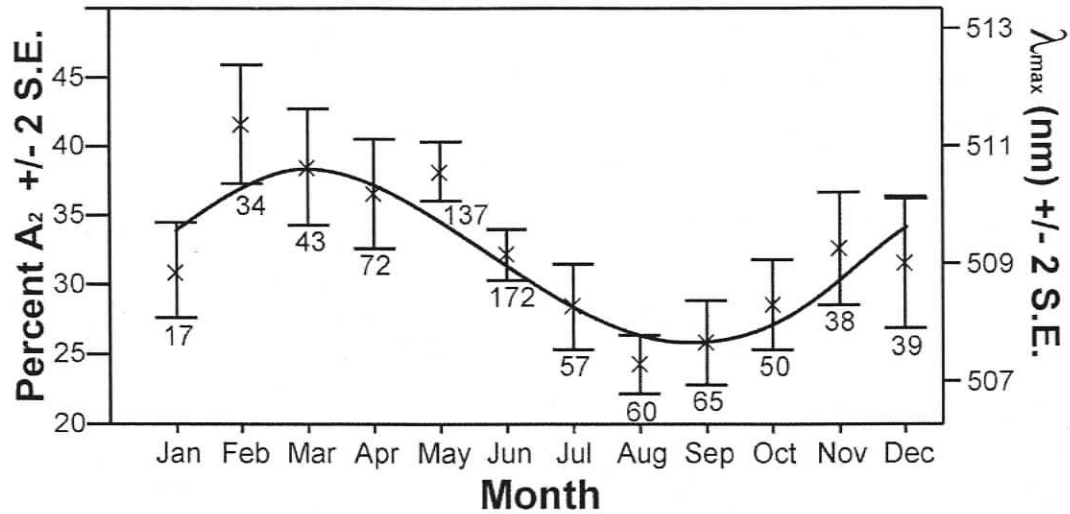


Figure 2.4. The annual cycle in A_1/A_2 ratio for coho salmon (*Oncorhynchus kisutch*, Walbaum) at all life history stages collected from Kispiox River hatchery and Robertson Creek hatchery (as well as wild individuals from the associated river). Each point is the mean λ_{\max} (scale on right side), and percent A_2 (scale on left side) for all individual fish (sample size (n) below each error bar) collected in a given month over a two-year period, from all age classes at each location. Cyclical variation is best fit with a least squares cosine model. Error bars equal two standard errors of the mean. MSP measurements were taken from an average of 18 rod photoreceptors per fish, sampled from the dorsal retina. Percent A_2 values for each rod were calculated using the λ_{\max} obtained from MSP and the Munz and Beatty's (1965) template derived for coho salmon rods. This figure represents MSP measurements of 14 159 rods, from 784 coho.

Table 2.2 Model comparisons using Akaike's information criterion (AIC); ΔAIC = the difference between the AIC score for that model and the lowest AIC score, W_i = AIC weight value.

Location	Model	ΔAIC	W_i
All sites (without Kispiox wild)	Linear	84.158062	$5.312623e^{-19}$
	Least squares cosine	-	≈ 1.000000
Robertson Creek	Linear	68.544709	$1.305285e^{-15}$
	Least squares cosine	-	≈ 1.000000
	Temperature	48.581375	$2.822854e^{-11}$
Kispiox River	Linear	60.464896	$7.416767e^{-14}$
	Least squares cosine	-	≈ 1.000000

ΔAIC = the difference between the AIC score for that model and the lowest AIC score, W_i = AIC weight value

Wild versus hatchery coho

Wild populations associated with each hatchery were periodically sampled to determine if hatcheries mirrored the natural environment with respect to variables that might affect the A_1/A_2 VP ratio. Mean percent A_2 -based VP composition of coho from Robertson Creek hatchery did not differ ($F_1 = 1.413$, $p = 0.240$; 2-way ANOVA) from that of the wild population in the Somass River (release river - Robertson Creek Hatchery fish). There was however, a significant difference among coho that were sampled on different dates ($F_2 = 17.545$, $p < 0.001$; 2-way ANOVA) (Table 2.3), in accordance with the observed seasonal cycle.

Conversely, mean percent A_2 -based VP composition of coho reared at the Kispiox River hatchery differed ($F_1 = 255.650$, $p < 0.001$; 2-way ANOVA) from that of wild fish caught nearby in Clifford Creek. On three separate occasions, in the months of May and June, wild fish were sampled from a near-by tributary of the Kispiox River and mean percent A_2 values were compared to those of hatchery fish. In each case, wild fish had higher mean percent A_2 values than hatchery-reared coho (Table 2.3). Similarly, wild fish collected in August from the mouth of the Skeena River 300 km from the Kispiox hatchery, had higher percent A_2 values than fish taken from the hatchery at the same time (Table 2.3). Differences between sampling dates were significant ($F_3 = 12.956$, $p < 0.001$; 2-way ANOVA), following the pattern of seasonal change in percent A_2 . The differences in A_1/A_2 ratio between wild and hatchery fish at the Kispiox River hatchery appear to be correlated with differences in water temperature. Temperatures measured in Clifford Creek rose above 10 °C for only a two-month period in late summer and were close to 0 °C for five months from

Table 2.3 Comparison of the percent A_2 between hatchery and wild coho from the same location on the same day.

Location	Date	Hatchery	Wild
		Mean percent A_2 ± 1 S.E.M.	Mean percent A_2 ± 1 S.E.M.
Robertson Creek	May 20	47.8 \pm 2.8 (9)	47.4 \pm 5.2 (10)
	May 24	43.4 \pm 2.6 (8)	35.2 \pm 3.8 (8)
	Aug 28	23.9 \pm 2.5 (10)	24.4 \pm 2.9 (10)
Kispiox River	May 18	27.0 \pm 2.6 (19)	75.0 \pm 2.9 (8) ^a
	May 29	30.8 \pm 2.8 (20)	69.4 \pm 4.6 (10) ^a
	Jun 12	30.0 \pm 1.9 (16)	75.3 \pm 3.5 (10) ^a
	Aug 20	20.4 \pm 2.8 (10)	47.8 \pm 3.9 (20) ^a

^a Means are different at $\alpha = 0.05$

late December until late April, and hatchery water temperature was kept constant at 7 ± 1 °C.

Differences between hatcheries

Coho from Robertson Creek hatchery were reared in outdoor raceways under natural temperature and light conditions (see next section), whereas coho from Kispiox River hatchery were reared indoors under constant temperature (7 ± 1 °C) with natural light supplemented with room lights. When compared, both populations exhibited a seasonal cycle in A_1/A_2 ratio, with winter peaks and summer troughs in percent A_2 but the amplitude of oscillation at the Kispiox River hatchery was dampened relative to the trend at Robertson Creek hatchery (Fig. 2.5). Robertson Creek hatchery data fit a cosine function; with a period of one year, a mean of 36.6 percent A_2 , amplitude of 8.1 percent, and a peak in the month of February and a minimum in August (Fig. 2.5). When various models (linear, cosine, temperature and day length) were compared using the AIC approach, the least squares cosine curve provided the best fit to the data (Table 2.2). Note that day length is not included in the analysis because it follows a cosine function and provided an equivalent fit to the least squares cosine curve. Kispiox River hatchery data also fit a cosine function; with a period of one year, a mean of 27.3 percent A_2 , an amplitude of 6.8 percent, and a peak in mean percent A_2 in March – April and minimum in September – October. Because temperature did not vary at this hatchery mean monthly percent A_2 values were compared only to linear and cosine models (Table 2.2).

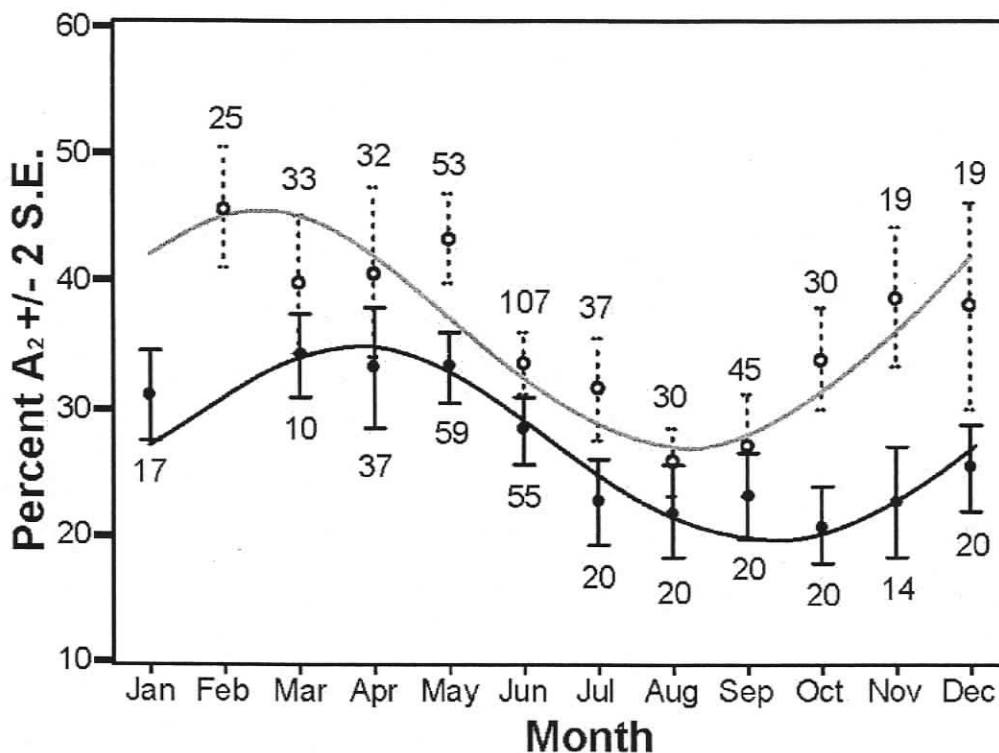


Figure 2.5. Differences in monthly mean percent A_2 content for coho salmon (*Oncorhynchus kisutch*, Walbaum) from two hatcheries with different rearing techniques. Each point is the mean monthly percent A_2 for all fish sampled (sample size (n) above and below the error bars) from the Robertson Creek hatchery (grey open circles) and Kispiox River hatchery (black closed circles). Robertson Creek hatchery fish are kept outdoors under natural conditions of photoperiod and temperature, while the Kispiox River hatchery fish were reared indoors under natural light from windows and a constant temperature of 7 ± 1 °C. Error bars equal two standard errors of the mean. Values for the mean percent A_2 for each fish are based on percent A_2 from an average of 18 rod photoreceptors per fish, sampled from the dorsal retina. Percent A_2 values for each rod were calculated using the λ_{max} obtained from MSP and the Munz and Beatty's (1965) template derived for coho salmon rods. Mean monthly percent A_2 was best fit by a least squares cosine model.

Environmental variables

Monthly mean percent A_2 values were more closely correlated in time to temperature than day length at Robertson Creek hatchery (Fig. 2.6A, B). For fish collected from Robertson Creek hatchery, temperature and percent A_2 were negatively correlated (Fig. 2.6A). Percent A_2 peaked in late February one month after temperature reached its annual low. Cross correlation between percent A_2 and inverse temperature was highest ($r^2 = 0.821$) when the cosine function was phase lagged by one month relative to temperature (Fig. 2.6A). Cross correlation between percent A_2 and day length was highest ($r^2 = 0.855$) when there was a two-month phase lag between percent A_2 and inverse day length (Fig. 2.6B). For fish collected from Kispiox Hatchery, a cross correlation with temperature was not possible since this hatchery maintained a constant rearing temperature year round. However, a cross correlation between percent A_2 and inverse day length peaked ($r^2 = 0.977$) when the curve fit to percent A_2 data was advanced by three months relative to day length (Fig. 2.6C). The variation in percent A_2 found in ocean going coho also showed an inverse relationship to ocean water temperatures (see Fig. 2.7 in following section).

Life history stage

I found a seasonal cycle in A_1/A_2 ratio in coho salmon at all life history stages sampled. Coho parr (0+ age class), which remain in fresh water for over a year, followed the same cyclical pattern in their A_1/A_2 ratio as smolts (1+ age class) that were preparing to undergo smoltification and migrate to the marine environment. At

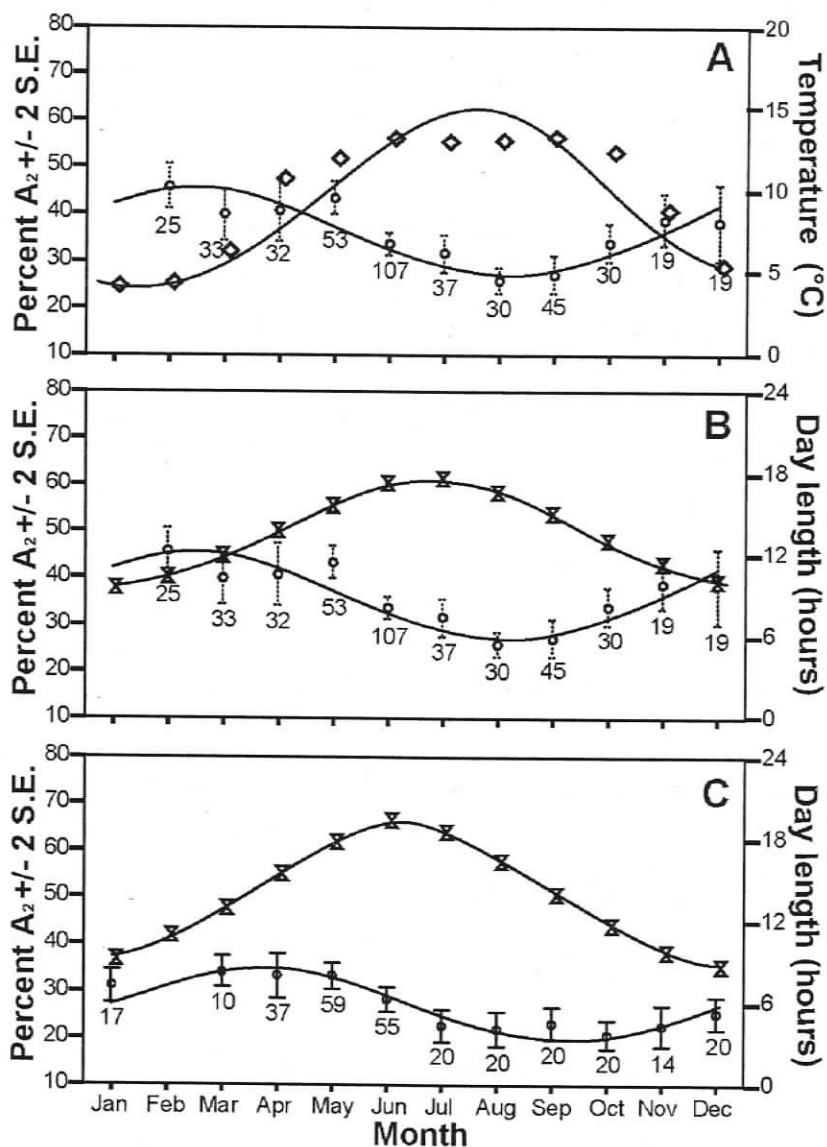


Figure 2.6. Comparison of mean monthly percent A_2 in coho salmon (*Oncorhynchus kisutch*, Walbaum) with day length and temperature. (A) Seasonal variation in mean monthly temperature (diamonds) for outdoor ponds at Robertson Creek hatchery was best fit by a least squares cosine model, and shows a negative correlation with mean monthly percent A_2 (open circles) with a one month offset of the cosine curves. (B) The strength of negative correlation between the cosine curves fit to mean monthly percent A_2 (open circles) and day length (double triangles) at Robertson Creek hatchery was at a maximum when the curves were offset by two months. (C) Temperature was constant at the Kispiox River hatchery; however, the strength of negative correlation between the least squares cosine curves fit to mean monthly percent A_2 (open circles) and day length (double triangles) was at a maximum when they were offset by three months. Error bars are equal two standard errors of the mean and the number of fish sampled per month is labeled below the error bars.

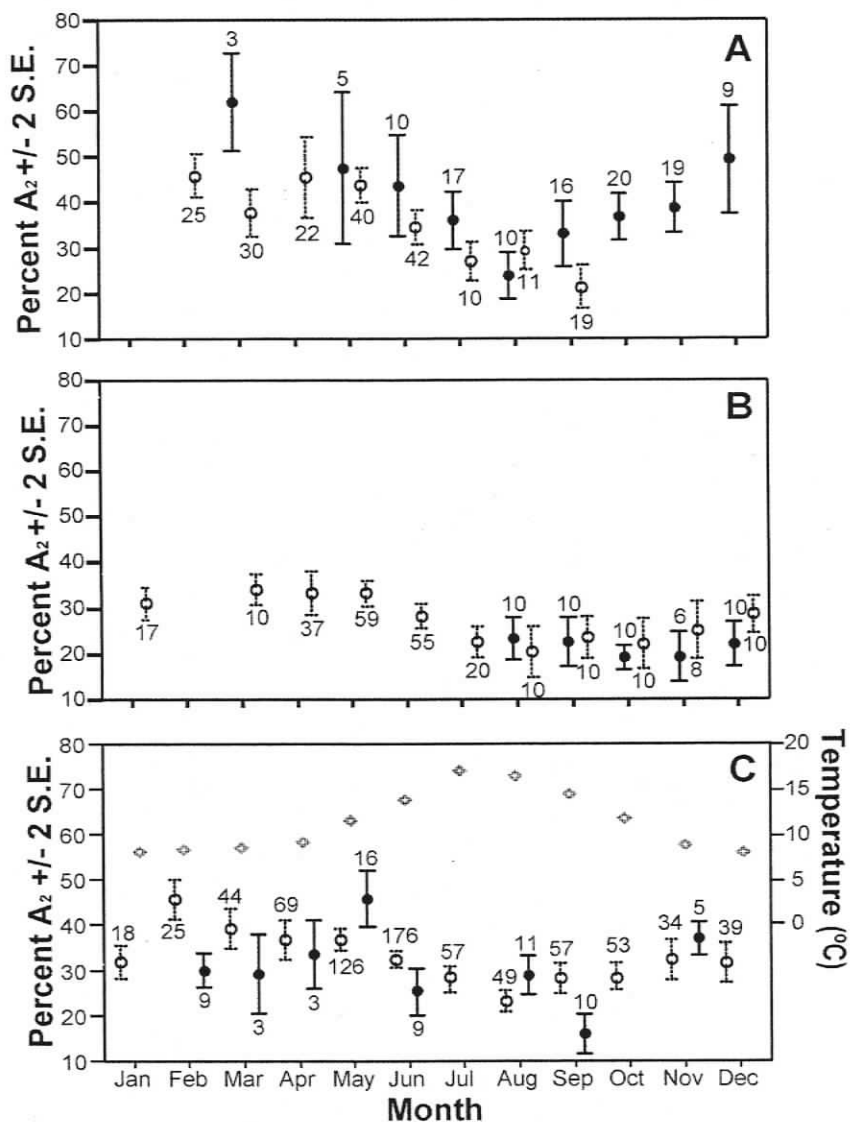


Figure 2.7. Comparison of mean percent A_2 between age classes, and between fresh water and ocean going coho (*Oncorhynchus kisutch*, Walbaum). (A) Both parr (closed circles) and smolts (open circles) at the Robertson Creek hatchery exhibit a seasonal cycle in percent A_2 with a peak in February - March and minima in July - August - September. (B) Parr (closed circles) at the Kispiox River hatchery were not sampled for the entire year; however, mean percent A_2 in the summer and fall does not differ from smolts (open circles) from the same hatchery. (C) Shows a seasonal cycle in mean percent A_2 for ocean going coho (closed circles) that followed the same pattern as coho parr and smolts in fresh water (open circles) with increased percent A_2 in winter and decreased A_2 in summer. There was an inverse relationship between temperature (open grey diamonds) and percent A_2 for ocean going coho (closed circles) as was observed at the Robertson Creek hatchery (Fig. 2.5A). Ocean water temperatures were recorded at the Target Marine farm site. Error bars are equal two standard errors of the mean and number of fish sampled per month is labeled above or below the error bars.

Robertson Creek hatchery, both parr and smolts showed a decrease in percent A_2 in summer and an increase in winter (Fig. 2.7A). Although, parr were only sampled for five months during the summer from the Kispiox River hatchery, the low percent A_2 values matched that observed in post-smolts. Post-smolts represented a small group of fish that were not released from the hatchery at the time of smoltification (May – June) and instead were held back in fresh water an additional eight months so that measurements could be made in both groups concurrently. These observations indicate that shifts from vitamin A_2 to A_1 VP dominance were not restricted to salmon undergoing smoltification (Fig. 2.7B). Collections of juvenile coho from the open ocean were made eight times throughout the year and the data appear to corroborate the hatchery data set showing increased percent A_2 in winter and decreased A_2 in summer following the same inverse relationship with temperature (Fig. 2.7C) observed during the freshwater phase (Fig. 2.6A). Increased variance in mean percent A_2 for ocean samples was due to the practical problem of obtaining fish (see n-values in Fig. 2.7C).

Discussion

General observations for coho

I found a seasonal pattern in the A_1/A_2 ratio in coho salmon at all life history stages that were measured. My approach of making long-term collections from several populations removed confounds encountered by previous studies. For example, if the sampling of smolts and/or adults was limited to the time of migration,

instead of at regular intervals throughout the year, earlier studies may have inadvertently missed the seasonal correlations with both photoperiod and temperature. Furthermore, sampling from only one population, particularly a hatchery population has implications for the interpretation of the results regarding the species as a whole. I found that percent A_2 in rod photoreceptors of all age classes of coho salmon reached a maximum in winter and a minimum in summer. This seasonal variation in A_1/A_2 VP composition occurred: (i) in smolts (1+ age class) that were preparing to migrate to sea; (ii) in parr (0+ age class), those fish that remain in fresh water for another year; and (iii) in ocean going juveniles (2+ age class) that would not be returning to fresh water to spawn for another one to two years. This is the first description of seasonal variations in A_1/A_2 ratio reported in ocean going (2+) coho. My results corroborate and extend the previous findings of Beatty (1966) made on parr and sexually mature coho salmon.

Overall, the seasonal variation in percent A_2 was not large (24 – 41 percent) and was comparable to the small amount of variation in rod λ_{\max} observed in another recent MSP study on coho salmon (Novales Flamarique, 2005). In both studies, the amplitude of shifts in A_2 was less than has been reported previously in coho salmon based on studies using ESP (18 – 58 percent, Beatty 1966; 16 – 78 percent, Alexander *et al.* 1994). In the present study, the reduced variance is likely due to the standardized sampling location in the dorsal retina and the large sample size. The dorsal retina was significantly (see Materials and methods section) lower in percent A_2 than the ventral retina in coho, as observed in other fish species (reviewed in Bowmaker 1991). It is important to note that the site specific retinal sampling

employed in this study did not alter the interpretation of the results, which is focused on the timing of the change in A_1/A_2 ratio and not the absolute values.

Environmental variables

My observation of an annual cycle in A_1/A_2 ratio is not unique among fishes. Several other labile pigment pair species have been found to alter their A_1/A_2 ratio seasonally and many have been shown to shift in response to changes in temperature and photoperiod (reviewed in Beatty, 1984; Bridges, 1972). In this study, the seasonal pattern in A_1/A_2 ratio was more closely correlated in time with variations in temperature than day length. The same trend has been observed in other temperate fish species. Both Dartnall *et al.* (1961), studying rudd (*Scardinius erythrophthalmus*, L.) in Britain, and Allen *et al.* (1973), studying cutthroat trout (*Oncorhynchus clarki*, Richardson) in Oregon state, recorded decreases in percent A_2 in late summer/fall (July – November) and increases in late winter/spring (January – May). The decreases in A_2 content occurred approximately three months after the longest day and only one to two months after water temperatures reached their summer maximum. The same seasonal shift was also recently observed in both freshwater and marine populations of Japanese dace (*Tribolodon hakonensis*, Günther) by Ueno *et al.* (2005). Japanese dace reached their lowest proportion of A_2 in summer (August) and the highest in winter (January). Ueno *et al.* (2005) also found a closer temporal correlation between the proportion of A_2 and water temperature than day length, and as observed here the amplitude of shift in the marine population was reduced compared to that of the fresh water group.

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1957). Because the rate of thermal noise is about four orders of magnitude higher in cones than rods (Rieke & Baylor, 2000), the effect of temperature may pose limits on photopic vision using A_2 -based VPs. The seasonal shift in A_1/A_2 ratio may reflect a trade-off between increasing spectral breadth of sensitivity by using A_2 -based VPs and maximizing signal to noise ratio by shifting to A_1 when temperatures rise.

A cautionary note regarding life history plasticity

Variations in life history strategies of coho salmon make it difficult to differentiate between seasonal and migration/metamorphosis hypotheses for the A_1/A_2 interchange system. Coho salmon migrate from fresh water to sea in spring and summer coincident with the shift from A_2 - to A_1 -based VPs. The return migration to fresh water occurs in late summer, fall, and winter, at the same time that coho shift back from A_1 to A_2 (reviewed in Groot & Margolis, 1991). The traditional view of the shift from A_2 (often referred to as the freshwater pigment) to A_1 (terrestrial and marine pigment) and back to A_2 at spawning, fit well with Wald's (1960) migration/metamorphosis hypothesis based on his observations in lamprey (*Petromyzon marinus*, L) and bullfrog (*Rana catesbeiana*, Shaw). However, coho parr and ocean going adults both normally spend at least a full year in freshwater and marine environments, respectively. During their extended residency in these habitats I have shown that coho continue to exhibit cyclical shifts in the A_1/A_2 ratio. This evidence strengthens support for the contention that VP chromophore shifting is most likely correlated with seasonal patterns in environmental variables.

A potential confound for the interpretation of VP shifting is that coho salmon populations are known to vary the amount of time spent in both freshwater and marine environments. Some populations of coho salmon may migrate to sea in their first year (0 winters spent in freshwater) or may spend over four years in fresh water (Drucker, 1972; Pritchard, 1940). Likewise, during their marine residency coho may return to spawn after just a few months at sea (jacks) or they may wait more than a full year (Bilton *et al.*, 1984). Therefore, an alternate hypothesis to explain the seasonal pattern I observed here is that the annual cycle in A_1/A_2 ratio, correlated to temperature and day length, could act to prepare fish of all age classes for a potential migration each year if conditions (physiological and environmental) were appropriate. Thus individuals within a population could shift their life history strategy. This plasticity could be interpreted as adaptive in the context of environmental change. My data do not explicitly differentiate between the two hypotheses (seasonal vs. migration/metamorphosis) in light of this potential plasticity. However, the potential to vary life history strategy would be dependent on differential timing of hormonal action on metamorphosis and VP shifting at the parr to smolt transformation (Baggerman, 1960; Beatty, 1972).

Hormonal action on visual pigment shifting

Exogenous thyroid hormones, which have been linked to the metamorphosis/migration event in salmonids, cause a shift to increased A_2 which is opposite to the direction of shift that occurs naturally at smoltification. Thyroid hormones are associated with several of the physiological changes that occur at

smoltification *e.g.* silvering, changes in body shape, increased saltwater tolerance, rheotaxis and loss of UVS cones, and all of these changes can be induced with exogenous TH or TSH (Allison *et al.*, 2003; Folmar & Dickhoff, 1980; Grau *et al.*, 1982; Higgs *et al.*, 1982; Hoar, 1988; McBride *et al.*, 1982; Specker *et al.*, 2000; Staley & Ewing, 1992). However, the effect of exogenous TH on the A_1/A_2 ratio is opposite to the shift that occurs at smoltification. Many studies have applied exogenous TH or TSH to fishes. The results have consistently shown increases in the relative amount of A_2 -based VPs in photoreceptors, not decreases as might be expected if TH were affecting chromophore ratio in conjunction with smoltification (Alexander, 1998; Allen, 1977; Allen & Munz, 1983; Allison *et al.*, 2004; Beatty, 1969a, 1972; Cristy, 1974; McFarland & Allen, 1977; Munz & Swanson, 1965; Tsin & Beatty, 1978). Only one exception has been reported, in which a small but significant (~6 percent over one month, $p < 0.05$) decrease in the ratio of A_2 was found when cold (5 °C) water acclimated (A_2 dominated) coho were treated with TH (Alexander *et al.*, 1998). The same study reports an approximate 50 percent increase in A_2 in one month when warm (15 °C) water acclimated fish were treated with TH. As this is the only study found to show a decrease in percent A_2 in photoreceptors in fish treated with an exogenous application of TH, the question of temperature effects on the direction of shift warrants further investigation. While TH has been closely tied to smoltification/metamorphosis in most respects, there is some doubt as to its role in the A_2 to A_1 shift at the time of smoltification and migration to sea in coho salmon.

By comparison, bullfrogs go through a single metamorphic event and were reported to shift from A_2 - to A_1 -based VPs as they metamorphose from aquatic tadpoles to semi-terrestrial adults (Crim, 1975; Ohtsu *et al.*, 1964; Wald, 1946; Wilt, 1959). However, studies examining the A_1/A_2 ratio over longer periods of time found that adult bullfrogs shifted seasonally, from A_2 dominance in winter to A_1 dominance in summer (Makino *et al.*, 1983; Reuter *et al.*, 1971). Furthermore, Bridges (1970) was able to alter the A_1/A_2 ratio of bullfrog tadpoles during metamorphosis, using a paradigm of constant light or darkness, with no effect on development, evidence against a link between metamorphosis and chromophore shifting. Further support for the hypothesis that possession of a labile pigment pair is related to seasonal changes, comes from a broad survey of freshwater fishes by Schwanzara (1967), where A_1/A_2 pigment pair systems were less prevalent (one third as common) in fishes from the tropics than fishes from temperate latitudes, where the magnitude of seasonal variability is increased.

Conclusions

In summary, the balance of evidence supports the hypothesis that the labile pigment pair system in coho and possibly most salmonids represents an adaptation to seasonal fluctuations in environmental variables. My evidence shows that coho salmon do not shift their VPs strictly to accommodate migrations between fresh and salt water (Hoar, 1976; Wald, 1960). I therefore suggest that the A_1/A_2 ratio is not a suitable indicator for the parr-smolt transition in salmonids as was recently proposed by Alexander *et al.* (1994). While my discussion has addressed aspects of the possible

ultimate causes of labile pigment pair systems, further research is required to understand the proximal mechanisms of vitamin A₁ - A₂ chromophore shifting.

Despite considerable evidence in support of seasonal variation in A₁/A₂ VP shifting across many taxa, seasonal fluctuations in environmental variables do not account for all of the variation in A₁/A₂ ratios in all species. There are undoubtedly multiple factors involved, which might explain the many exceptions, such as: (i) the presence of A₂ in deep sea fishes (Bowmaker *et al.*, 1988); (ii) the fact that anadromous sturgeons do not shift from their A₂ dominance upon migration to sea (Sillman *et al.*, 1999); and (iii) that dorsal ventral differences in A₁/A₂ ratio exist across the eyes of bullfrogs (Reuter *et al.*, 1971) and fishes (Bridges, 1982; Denton *et al.*, 1971; Muntz & Northmore, 1971).

Chapter 3 : Effects of exogenous thyroid hormones on visual pigment composition in coho salmon (*Oncorhynchus kisutch*)

This chapter is based on a collaboration with the following authors and is in preparation for submission to the Journal of Experimental Biology as cited below:

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Introduction

In many vertebrates, the combination of VPs possessed by an individual is not static and fixed, as once thought, but dynamic and flexible. Changes in VP composition can alter spectral sensitivity, which has direct consequences on the efficiency of target detection. Knowledge of what influences the timing of changes in VP composition within vertebrate photoreceptors has implications for our understanding of the adaptive significance of the VPs to the organism. The evidence collected in Chapter 2, for coho salmon, pointed towards a correlation between the timing of shifts in A_1/A_2 ratio and seasonal changes in environmental variables such as temperature and day length. However, the temporal coincidence of the seaward migration with the spring increase in day length and temperature (Alexander *et al.*, 1994; Beatty, 1966; Temple *et al.*, 2006) made it difficult to distinguish among the seasonal and migration/metamorphosis hypotheses. A possible solution to this dilemma would be to find a means of disentangling the two factors.

Seaward migration in salmonids is preceded by a distinct transition event called smoltification. This transition includes modifications in both physiology and behaviour, which prepare the freshwater parr for life at sea as smolts. Thyroid hormones (TH) are known to play a role in smoltification. TH levels increase in parr in the weeks that precede seaward migration, rising in late February – March and peaking in April - May (Dickhoff *et al.*, 1982; Hoar, 1988).

Experiments that have used TH injections, TH treated food, or TH immersion, have stimulated many of the measurable effects of smoltification

including; silvering, (Robertson, 1949), a decrease in condition factor, increased hypo-osmoregulatory abilities (Folmar & Dickhoff, 1980; Higgs *et al.*, 1982), a change in rheotaxis (Specker *et al.*, 2000), changes in salinity preference (Baggerman, 1960), and loss of UVS cones (Allison *et al.*, 2003; Browman & Hawryshyn, 1994). Interestingly, it has been repeatedly shown that exogenous TH causes increases in the relative proportion of A₂ in the retina of salmonids and other teleosts. This TH induced shift is opposite in direction to that occurring at the time of smoltification in spring, which is a decrease in retinal A₂ (Allen, 1977; Allen & Munz, 1983; Allison *et al.*, 2004; Beatty, 1969a, 1972; Cristy, 1974; McFarland & Allen, 1977; Munz & Swanson, 1965; Tsin & Beatty, 1978). Administration of exogenous thyroid stimulating hormone was found to be equally effective at inducing an increase in A₂ (Beatty, 1972), thus demonstrating that the reverse response (*i.e.* increase in A₂ in response to TH and not a decrease as occurs at smolting) was not the effect of pharmacological doses of TH. One criticism of experiments using exogenous TH is that they have not accounted for the underlying physiological state of the organism at the time of hormone treatment, specifically in relation to known lunar and seasonal variations in the endocrine system (Hoar, 1988; Larsen *et al.*, 2001), and temporal expression patterns of TH receptors (Power *et al.*, 2001).

In a literature survey, I was able to find only two studies that took season into consideration when manipulating TH levels with respect to A₁/A₂ ratio and the results of both were equivocal. Cristy (1974) found that rainbow trout treated with TH showed a greater increase in molar percent A₂ in summer than winter, however, dosage effects may have confounded these results. In a second study, Alexander *et al.*

(1998) investigated the effect of exogenous TH on coho that had been artificially brought to their winter and summer states (high and low A_2 respectively) by using light and temperature treatments. They found that coho that were already in a state of A_2 dominance underwent a small but significant decrease in the proportion of A_2 when fed TH treated food (Alexander *et al.*, 1998). Their result was used to support the hypothesis that the shift in A_1/A_2 ratio was linked to smoltification in coho salmon (Alexander *et al.*, 1994). However, while these authors suggested that a high A_2 content might be a prerequisite for a TH induced shift from $A_2 - A_1$, my findings from both wild and hatchery populations (Chapter 2) suggest that such a high A_2 content is rarely attained under natural conditions. As an alternate explanation for the reverse effect of TH, Alexander *et al.* (1998) also suggested the direction of the A_1/A_2 shift might be affected by temperature. This hypothesis was formally tested here.

In the present study, I sought to determine the effects of TH treatment on fish held under different rearing conditions or on fish treated at different times of year. Secondly, I examined whether rods and cones shifted chromophore ratios concomitantly in coho, as has been observed in other teleosts (Allison *et al.*, 2004; Loew & Dartnall, 1976; Parry & Bowmaker, 2000; Saszik & Bilotta, 1999; White *et al.*, 2004). I measured photoreceptor λ_{\max} values using MSP and found that coho salmon shifted to A_2 dominance when treated with exogenous TH under all rearing conditions and at all times of year tested. There was a simultaneous increase in the λ_{\max} values of both rods and cones. However, the extent of spectral shift in λ_{\max} observed in both MWS and LWS cones exceeded that predicted using any published conversion formula for predicting the shift in λ_{\max} due to a chromophore substitution

alone. To account for the increased shift in λ_{\max} observed in the MWS and LWS cones, I proposed that multiple opsin subtypes exist for these cone classes in coho salmon. In support of this hypothesis a second RH2 opsin subtype (expressed in the MWS cones) was isolated, cloned and sequenced.

Materials and Methods

Animal care and experimental design

Coho salmon were obtained from two local hatcheries (Robertson Creek, Department of Fisheries and Oceans, Canada hatchery; Port Alberni, British Columbia and Target Marine, commercial hatchery; Sechelt, British Columbia) and transported to the University of Victoria aquatics facility. I conducted five experiments in which coho were treated with exogenous TH while being kept under different rearing conditions at different times of year. Details of the rearing conditions, dates, fish age and morphometrics *etc.* are included in Table 3.1.

I used two different delivery systems for treating coho with exogenous TH; a food and a bath treatment. Both techniques have proven efficacy in stimulating smoltification like transitions in coho (Alexander, 1998; Alexander *et al.*, 1998; Munz & Beatty, 1965) and A_1/A_2 shifts in this and other species (Allison *et al.*, 2004; Beatty, 1969a, 1972). In the first two experiments, I used a large number of fish for weekly sampling. The large volume of water required to keep many fish meant that a TH food treatment was more practical and less wasteful. However, it was not possible to control the dosage of TH delivered to each fish because it was consumption

Table 3.1 Details of rearing conditions, treatments and dates of Experiments I-V.

Experiment	Dates	Light Source [†]	Water Temperature	Photoperiod	Treatment Type	Fish age (months)	Fish Length (cm)	Fish weight (g)
I	29.01.2002- 25.03.2002	6500°K Fluorescent	15 ± 1°C	12L:12D	T ₃ /T ₄ on food	12	9.2 ± 0.7	9.2 ± 1.6
II	21.06.2002- 05.08.2002	6500°K Fluorescent	15 ± 1°C	12L:12D	T ₃ /T ₄ on food	5	5.9 ± 0.6	2.2 ± 0.8
III	20.01.2005- 03.03.2005	Patchy Sunlight	4.5 ± 2°C	Natural	T ₄ in water	12	11.0 ± 0.7	12.3 ± 2.5
IV	04.02.2005- 30.04.2005	6500°K Compact Fluorescent	3 ± 2°C	7L:19D	T ₄ in water	12	8.6 ± 0.7	6.4 ± 1.6
V	25.04.2005- 06.06.2005	Patchy Sunlight	11 ± 1°C	Natural	T ₄ in water	3	5.4 ± 0.3	1.8 ± 0.4

[†]Spectral irradiance measures provided in Appendix A

dependent. High variation in A_1/A_2 ratio among the treated groups and incomplete transition to A_2 dominance led me to try a bath treatment for the remaining experiments in which fewer fish were used. Detailed descriptions of both treatments are provided below.

For Experiments I and II, 300 fish were separated into two 750 l cylindrical fiberglass tanks. Regular water replacement was necessary to accommodate high water quality and the oxygen demands of coho salmon (Tarazona & Munoz, 1995). A TH bath was therefore not economical and instead TH was applied to the food. The treatment group received a diet of commercial salmon pellets sprayed with alcohol containing dissolved L-thyroxine and 3,5,3'-triiodo-L-thyronine (Sigma, St. Louis, MO, USA). I used a mixture of 120 ppm by weight L-thyroxine (T_4) and 12 ppm 3,5,3'-triiodo-L-thyronine (T_3), a ratio that approximates plasma TH levels found in salmonids prior to smoltification (Ebbesson *et al.*, 2000; Plate, 2001). The T_3/T_4 diet was provided to the treatment group for five weeks in Experiment I and four weeks in Experiment II, after which the fish were put onto the control diet for the remaining two weeks. The control group was fed the same commercial salmon pellets sprayed only with alcohol, and both control and treatment fish were fed to satiation every other day. At weekly intervals 3-10 fish were sampled from both control and treatment groups (for details see n-values in results).

For Experiments III – V, TH treatment involved adding T_4 dissolved in 1.5 ml of 0.1 M NaOH to the tank water for a final concentration of $300 \mu\text{g l}^{-1} T_4$. The bath treatment was used in these later experiments to attempt to reduce some of the variation in A_1/A_2 ratio among the treated fish, which may have been due to a dose

response caused by differential food consumption. The choice to switch from a mixture of T₃/T₄ in food to only T₄ as a bath treatment was based on the success of previous experiments in our lab. While an investigation into the differences between these treatments may be fruitful, it was beyond the scope of this research. Fish were transferred to fresh TH treated water three times per week. Control fish received the vehicle only (1.5 ml of 0.1 M NaOH). Both control and treatment groups were fed to satiation every other day with commercial salmon pellets. Five to 10 fish (see results for sample sizes) were sampled from both control and treatment groups after 4 - 6 weeks of treatment. Care and treatment of fish was in accordance with the University of Victoria's Animal Care Committee, under the auspices of the Canadian Council for Animal Care.

Microspectrophotometry

Fish were dark adapted for at least one hour prior to sacrifice by an overdose of Eugenol 100 mg l⁻¹ (ICN Biomedicals Inc., Irvine, CA.), followed by cervical transection. The right eye was enucleated and hemisected along an anterior posterior axis. A piece of retina 1 - 2 mm² was cut out of the dorsal most section of the dorsal hemisphere. The retinal sample was teased apart on a glass cover slip and a drop of MEM (Sigma; pH adjusted to 7.4 - 7.6) was applied to the sample. A second cover slip was placed over the sample and sealed with paraffin. All procedures were performed under deep red illumination (>650 nm) or using a dissecting scope equipped with infrared LED (800 nm) illumination and monitored with a CCD-camera.

I used a CCD-MSP that has been described previously (Hawryshyn *et al.*, 2001), to measure spectral absorbance of individual photoreceptors. The CCD-MSP device delivered a short flash (0.05 - 0.5 seconds)* of full spectrum light (300-800 nm; 150 W xenon light source – intensity regulated, Oriol) to the photoreceptor outer segment. The beam size was approximately $2 \times 3 \mu\text{m}$ at the focal plane of the outer segment. The transmitted beam passed through a spectrometer (300 nm blazed grating, Acton Research Corporation) and onto a 1340×400 pixel, Peltier cooled (-45°C), back-illuminated CCD-detector (Princeton Instruments, Roper Scientific, Inc., Trenton, NJ, USA). Photoreceptor absorbance ($\log_{10}(1/T)$) was calculated by comparing the transmitted intensity through the photoreceptor (I_M) to the transmitted intensity through an area clear of debris adjacent to the photoreceptor (reference (I_R)) thus, $T = I_M/I_R$). If acceptable, the record was then stored for later analysis.

The retinal sample was examined under infrared illumination (Schott RG850 filter, Ealing Optics) and monitored by an infrared camera (Canadian Photonics Laboratory). The search image and infrared filtered beam (Schott RG850 filter) were displayed on a computer monitor. The position of the ROS relative to the measurement beam was controlled by a motorized stage (Marhauser-Wetzlar GmbH & Co., Germany). The path of the motorized stage was plotted on-screen to prevent repeated measurements of ROSs.

Criteria for acceptance of absorbance spectra were: (i) presence of a baseline on the long wavelength arm (Harosi & MacNichol, 1974); (ii) peak absorbance near the expected wavelength ($\sim 500 - 530 \text{ nm}$ for coho rod photoreceptors); (iii) minimal

* Duration was dependent on intensity and was set to deliver an optimum number of photons per exposure time = total counts see Appendix B

absorbance by photoproduct and; (iv) signal to noise ratio of the α absorption band greater than 5 to 1. These criteria rejected approximately ten percent of MSP recordings. Determinations of λ_{\max} , and percent A_2 from absorbance records were performed offline subsequent to initial sampling.

A custom designed analysis program was used to determine the λ_{\max} from absorbance records using existing templates. Each MSP record consisted of 1340 points between 300 and 750 nm. Each record was linear detrended if necessary (Harosi, 1987). A nine-point adjacent averaging function was used for line smoothing. The smoothed curve was normalized to zero at the baseline on the long wavelength arm and to one at the center of the α -peak. The fit of the normalized curve was compared with a nonlinear least-squares routine to the upper 20 percent of the weighted A_1/A_2 averaged Govardovskii *et al.* (2000) template (based on the center of the α -peak ± 40 nm).

For some rods, I also obtained a second estimate of λ_{\max} based on a template created by Munz and Beatty (1965) for rod pigments of coho salmon. The absorbance curves were compared (minimum variance fit) to the Munz and Beatty (1965) template, which extends from the λ_{\max} to a point at 20 percent of the maximum on the long wavelength arm. The Munz and Beatty (1965) template assumes that the λ_{\max} of coho rods varies from 503 – 527 (nm). Their model is in close agreement with Harosi's (1994), which predicts that an A_1 -based VP with a λ_{\max} of 503 nm would shift to 529 nm if A_1 was replaced with A_2 (see results). However, many of the rods measured had λ_{\max} values that exceeded 527 nm and therefore could not be fit to the

Munz and Beatty (1965) template. In such cases, I used the estimate obtained by the fit to the Govardovskii *et al.* (2000) template.

Gene Discovery

One coho parr was dark-adapted for one hour and then killed by immersion in 300 mg/L tricaine methanesulfonate (Crescent Research Chemical, Phoenix, AZ, USA) for a period of ten minutes. The right eye was enucleated and the neural retina was dissected free of pigmented epithelium under deep red light. Immediately after dissection, total RNA was isolated from the tissue using TRIzol reagent (Invitrogen Canada Inc., Burlington, Ontario, Canada) as per the manufacturer's recommended protocol. The retina was placed in a 1.5 ml microcentrifuge tube containing 700 μ l TRIzol and was homogenized using a disposable Kontes® Pellet Pestle® with cordless motor tissue grinder (Kimble Kontes, New Jersey, USA). Isolated RNA was resuspended in 50 μ l RNase-free water. RNA concentration was determined by measuring absorbance using spectrophotometry at a standard wavelength of 260 nm. Total cDNA was synthesized using 1 μ g of total RNA. Each RNA sample was annealed with 500 ng random hexamer oligonucleotide (Amersham Biosciences, Baie d'Urfe, Québec, Canada) and cDNA prepared using Superscript II RNase H⁻ reverse transcriptase (Invitrogen) as described by the manufacturer's protocol.

A degenerate forward primer (5'-GCTATTGAGAGGTACATNGT-3') was designed based on an alignment of consensus RH2 opsin open reading frame (ORF) sequences and was synthesized by Operon Biotechnologies, Inc. (Huntsville, AL, USA). A degenerate reverse primer designed by Johnson *et al.* (1993) was also used

(5'-RAANATNACNGGRTTTRAA-3'). All primer pairs used in this study were diluted and combined in an equimolar ratio to a final concentration of 10 μ M. Primers were used to amplify cDNA synthesized from 1 μ g parr retinal total RNA. The 20 μ l reaction contained 20 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 200 μ M dNTPs, 1 μ M of each primer, 2 μ l cDNA diluted 1:20 and 1.0 U Platinum Taq DNA polymerase (Invitrogen). The thermocycle program was 94°C for 9 minutes, followed by 40 cycles of 94°C for 30 seconds, 50°C for 1 minute and 72°C for 1.5 minutes, and a final extension at 72°C for 10 minutes. Amplicons were separated in a 1.5 percent agarose gel and visualized by ethidium bromide staining. The DNA band was excised from the gel and extracted by freeze-squeeze centrifugation (Smith, 1980). Extracted DNA was cloned into PCR2.1-TOPO vector using the TOPO TA Cloning Kit (Invitrogen). Plasmid DNA was purified using QIAprep Spin Miniprep Kit (Qiagen Inc., Mississauga, Ontario, Canada) and sequenced (Centre for Biomedical Research DNA Sequencing Facility, University of Victoria, Victoria, BC, Canada). A partial RH2 opsin sequence was obtained using the degenerate primers that differed from the previously reported RH2 sequence for coho (Dann *et al.*, 2004).

The full-length RH2B coding sequence was isolated using the BD SMART RACE cDNA Amplification Kit (BD Biosciences Clontech, Mississauga, Ontario, Canada) according to the manufacturer's protocol; with the exception that Platinum Taq (Invitrogen) was used in the PCR reactions. Primers used in the RACE reactions were synthesized by Operon Biotechnologies, Inc. and are as follows: 3'-RACE primer (5'-CTATGCCAGCTTTGCTGCCGTGATT-3'); 5'-RACE primer (5'-GGCAGCACAGGCCATTGCCATGAC-3'). The 5'-RACE reaction utilized the

following thermal profile: initial denaturation at 94°C for 9 minutes followed by 5 cycles at 94°C for 30 s, 72°C for 3 min followed by 5 cycles at 94°C for 30 s, 70°C for 30 s, 72°C for 3 min, followed by 35 cycles at 94°C for 30 s, 62°C for 30 s and 72°C for 3 min. The 3'-RACE reaction thermal profile, was 94°C for 9 minutes, followed by 35 cycles of 94° for 30 s 72°C for 90 s. Amplicons were gel-purified, cloned and sequenced as described above.

Sequences from 5' and 3' RACE were assembled and compared to sequences in GenBank using Blastn (www.ncbi.nlm.nih.gov/BLAST/). Sequence alignments were performed using ClustalW (www.ebi.ac.uk/clustalw/#; Chenna *et al.*, 2003).

Data analysis

Statistical analyses were performed using the mean λ_{\max} value obtained for each receptor class from individual fish (see Allison *et al.*, 2005; Jokela *et al.*, 2003). This approach is not typical of MSP studies, which typically report the mean for each class of receptor from all fish observed (*e.g.* Cummings and Partridge (2001), Harosi and Kleinschmidt (1993), Hawryshyn *et al.* (2001), Nawrocki *et al.* (1985), and Novales Flamarique (2005)). However, it is statistically correct to treat the fish as the sample unit, not the individual photoreceptors, since photoreceptors from the same fish lack independence (pseudoreplication). When comparisons were made between fish for a particular receptor class I used the mean λ_{\max} of all receptors collected from each fish (fish mean λ_{\max}). When comparison were made between control and treatment groups for a particular receptor class I used the mean λ_{\max} of all fish *i.e.* the mean of the fish mean λ_{\max} values for all fish in each group (group mean λ_{\max}).

In Experiment II, in which I recorded both rod and cone photoreceptors, I was unable to record from an equal number of cones from each fish. On occasion as few as one to as many as 18 MWS or LWS cones were recorded from a single fish. To account for the unequal contribution of receptors from each fish to the mean for a particular group or sampling date I performed a weighted analysis using the square root of n divided by the mean number of receptors measured for that cone type on that sampling date from that group of fish. This procedure decreases the relative contribution of fish with a lower number of records, and maintains the correct n -value (number of fish) for the analysis of variance between treatments.

One-way analysis of variance was used to detect differences in means among groups over time. Independent sample t -tests were used for comparisons between the control and treatment groups at specific time points ($\alpha = 0.05$).

Results

In all five experiments, application of exogenous TH resulted in typical parr-smolt like transitions. TH treated fish exhibited many visible physical characteristics associated with coho smolts; loss of parr marks, increased silvering, blue-green dorsal colouration, and a decreased condition index (body weight to length ratio) (Fig. 3.1).

Experiment I

Experiment I began at the end of January and terminated in March, a period when the relative proportion of vitamin A₂ in the retina is at a peak (Alexander, 1998; Beatty, 1966; Temple *et al.*, 2006). At day zero ($T = 0$), before dividing the fish



Figure 3.1. TH treated (upper) and control (lower) coho alevin. Both fish were from Target Marine hatchery, Sechelt, B.C., hatched in March of 2005 and were transferred to the University of Victoria aquatic facilities on April 13, 2005. The treated fish were kept in water ($11 \pm 1^\circ\text{C}$) to which T_4 dissolved in 0.1 M NaOH was added to a concentration of $300 \mu\text{g T}_4$ per litre of tank water for four to six weeks. Control fish were kept under identical conditions except that they received only the vehicle (0.1 M NaOH). These images were taken on the same day after approximately four weeks of treatment.

into the two groups, the group mean λ_{\max} (for definition see methods) for rods was 514.6 ± 3.2 nm ($n = 10$; Fig. 3.2). After five weeks the rod λ_{\max} of the treatment group had increased to 521.0 ± 4.8 nm ($n = 3$), which was significantly ($t_4 = -3.561$, $p = 0.024$) higher than the control group (510.40 ± 6.3 nm; $n = 3$). Two weeks after switching the treatment group to the control diet the group mean λ_{\max} had decreased to 509.3 ± 2.6 nm ($n = 10$), which was not significantly ($t_{18} = -0.574$, $p = 0.573$) different from the control group (508.6 ± 2.6 nm; $n = 10$). During the course of the experiment the group mean λ_{\max} of rods from the control group decreased, resulting in a significant ($f_6 = 4.218$, $p = 0.003$; one-way ANOVA) decrease in λ_{\max} over time (Fig. 3.2). These results demonstrate that TH was effective in causing an increase in rod λ_{\max} , consistent with an increase in the proportion of A_2 , in winter when background levels of A_2 in the retina are naturally higher.

Experiment II

The same pattern of increasing λ_{\max} with increased duration of TH treatment was observed in Experiment II, which was performed in July – August (Fig. 3.3). The initial group mean λ_{\max} for rods sampled from the dorsal retina was 511.9 ± 4.4 nm ($n = 10$). After six weeks the group mean λ_{\max} of the control fish had declined to 508.8 ± 2.0 nm ($n = 6$). After four weeks of TH treatment the group mean λ_{\max} of the treated fish increased to 515.2 ± 4.9 nm ($n = 8$), which was significantly ($t_{14} = -3.042$, $p = 0.009$) higher than the control group, which had a group mean λ_{\max} of 509.5 ± 1.8 nm ($n = 8$) at that time point. After switching the treatment group back to the control diet, the group mean λ_{\max} returned to the level (509.9 ± 2.6 nm) of the controls (508.8 ± 2.0 nm).

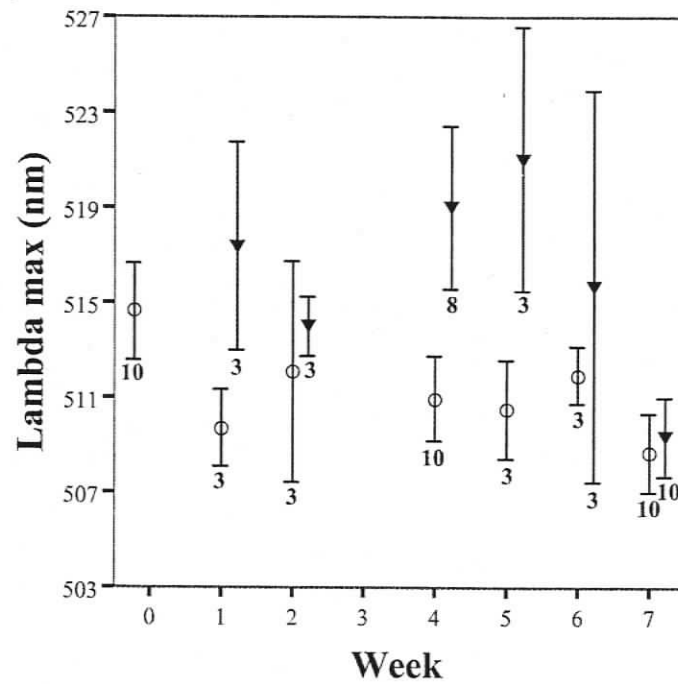


Figure 3.2. Group mean $\lambda_{max} \pm 2$ S.E. for the control group (circles) and TH treatment group (triangles) from Experiment I performed in February - March. Each point represents the mean rod λ_{max} of all coho salmon (*Oncorhynchus kisutch*, Walbaum) sampled at the specified time point (n-values below error bars equal number of fish). The mean λ_{max} for each fish was based on the mean λ_{max} of approximately 20 rods measured using MSP. Treated fish received commercial salmon pellets sprayed with 12 ppm T_3 and 120 ppm T_4 by weight dissolved in alcohol.

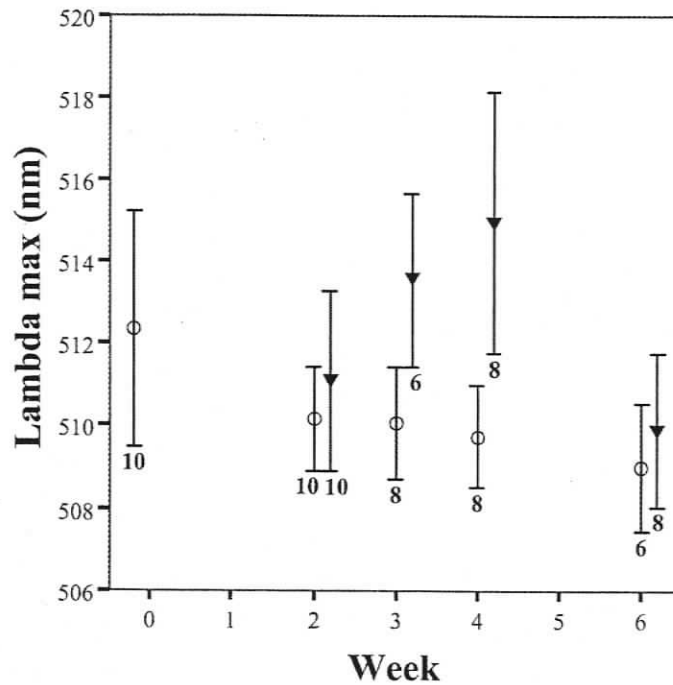


Figure 3.3. Group mean $\lambda_{\max} \pm 2$ S.E. for the control group (circles) and TH treatment group (triangles) from Experiment II performed in July - August. Each point represents the mean rod λ_{\max} of all coho (*Oncorhynchus kisutch*, Walbaum) measured at that time point (n-values below error bars equal number of fish measured). The mean λ_{\max} for each fish was based on the mean λ_{\max} of approximately 20 rods measured using MSP. Treated fish received commercial salmon pellets sprayed with 12 ppm T_3 and 120 ppm T_4 by weight dissolved in alcohol.

nm) and the two groups were no longer significantly different ($t_{12} = -0.846$, $p = 0.414$; Fig. 3.3).

In Experiment II, I recorded from both rods and cone photoreceptors. Coho salmon have five photoreceptor classes: ultraviolet- (UVS) short- (SWS), middle- (MWS) and long- (LWS) wavelength sensitive cones, which express SWS1, SWS2, RH2 and LWS opsin genes respectively, while rods express rod opsin RH1. The SWS1 and SWS2 opsins are expressed in single cones, while the RH2 and LWS opsins are expressed in the two outer segment members of double cones in *Oncorhynchus* spp. (Allison *et al.*, 2003). My sampling methodology resulted in an insufficient number of UVS and SWS cones being recorded for many individual fish thereby preventing statistical analysis for these two cone classes. Similar problems have been reported by other investigators using similar techniques, and it is clear that MSP records do not reflect relative numbers of each cone type (Shand, 1993; White *et al.*, 2004). Despite the difficulties of obtaining an adequate number of absorbance measurements from single cones a large number of MWS ($n = 275$) and LWS ($n = 282$) double cone spectra were recorded, permitting the use of individual fish as the sampling units, as was done for rods.

MWS cone group mean λ_{\max} for control fish started at 514.6 ± 2.8 nm ($n = 4$) and decreased to 510.4 ± 10.7 nm, ($n = 6$) over the six weeks of the experiment. For the treatment group, MWS cone λ_{\max} increased for the duration of the TH treatment reaching 522.0 ± 6.4 nm ($n = 8$) after four weeks, which was significantly higher ($t_{11} = -2.421$, $p = 0.031$) than the control group (512.2 ± 9.2 nm, $n = 7$). Two weeks after

stopping the TH treatment the group mean λ_{\max} of the MWS cones decreased to 514.0 ± 7.8 nm ($n = 7$), which was no longer significantly different ($t_{11} = -0.696$, $p = 0.501$) from the control group (510.4 ± 10.7 nm, $n = 6$).

For LWS cones the group mean λ_{\max} at $T = 0$ was 571.9 ± 11.9 nm ($n = 4$) and the control group decreased to 566.9 ± 6.7 nm ($n = 6$) over the six weeks of the experiment. After four weeks of TH treatment the group mean λ_{\max} of the LWS cones had increased slightly to 576.0 ± 11.0 nm ($n = 8$), which was not significantly different ($t_{12} = -0.984$, $p = 0.345$) from the control group (569.8 ± 12.3 nm, $n = 6$) at the same time point. By the end of the experiment, two weeks after removing the treatment, the group mean λ_{\max} of the LWS cones for the treated fish had decreased to 566.6 ± 8.9 nm ($n = 7$), which was not significantly different ($t_{11} = 0.057$, $p = 0.955$) from the control group (566.9 ± 6.7 nm, $n = 6$).

Experiment III-V; photoperiod, light intensity and temperature varied

In Experiments III - V, application of exogenous TH resulted in a significant ($p < 0.001$) increase in the group mean λ_{\max} of rods, MWS and LWS cones sampled from the dorsal retina (Fig. 3.4). Group mean λ_{\max} values for rods of treated fish in all three experiments were greater than 530 nm (Exp. III = 531.5 ± 1.4 nm, $n = 5$; Exp. IV = 532.5 ± 0.9 nm, $n = 7$; Exp. V = 533.0 ± 1.0 nm, $n = 5$). The group mean λ_{\max} for rods from the dorsal retina was significantly lower than from the ventral retina for fish in the control groups in Experiments III (dorsal = 513.0 ± 3.8 , ventral = 518.5 ± 4.4 , $t_{14} = -2.7$, $p = 0.018$) and IV (dorsal = 510.8 ± 1.9 , ventral = 517.5 ± 2.2 , $t_7 = -4.3$, $p = 0.008$). For Experiment V, the group mean λ_{\max} of rods in the ventral retina

was still greater than the dorsal retina but the difference was not significant at $\alpha = 0.05$ (dorsal = 509.6 ± 0.7 , ventral = 511.9 ± 2.7 , $t_{10} = -1.8$, $p = 0.110$). There was no significant dorsal/ventral difference in rod group mean λ_{\max} for TH treatment groups. Both dorsal and ventral retina were above 530 nm in all three experiments (Exp III, dorsal = 532.3 ± 1.1 ; ventral = 530.7 ± 2.8 , $t_{12} = 1.5$, $p = 0.148$; Exp IV, dorsal = 531.5 ± 1.4 , ventral = 533.0 ± 1.3 , $t_8 = -1.8$, $p = 0.117$; Exp V, dorsal = 533.0 ± 1.0 , ventral = 533.3 ± 1.6 , $t_8 = -0.3$, $p = 0.737$). When all rods measured from all fish in Experiments III – V were plotted in a single frequency histogram the range of λ_{\max} values extended from approximately 500 to 540 nm (Fig. 3.5).

The range of group mean λ_{\max} values for MWS cones (501.5 – 547.7 nm; Fig. 3.4) was greater than predicted for a shift from A_1 to A_2 in a single opsin (Table 3.2). The range of λ_{\max} values from all individual MWS cones from all fish from Experiments III – V extended from below 490 nm to above 550 nm (Fig. 3.6). The wide range of MWS cone λ_{\max} values was apparent even within individual fish (Fig. 3.7). The six published models (Table 3.2) that predict the difference in λ_{\max} when the same opsin is combined with either A_1 or A_2 chromophores predict a shift in λ_{\max} of between 16.6 nm (Parry and Bowmaker 2000) and 36.0 nm (Whitmore and Bowmaker 1989) for VPs with λ_{\max} values in the range of 495 - 512 nm (Fig. 3.8). I observed a shift in MWS cone λ_{\max} of between 46.2 - 60 nm (range of values based on group mean λ_{\max} and individual cone λ_{\max} respectively) that was greater than could be explained by any of these models (Table 3.2). This observation led me to the hypothesis that more than one RH2 opsin subtype was expressed in the MWS cones.

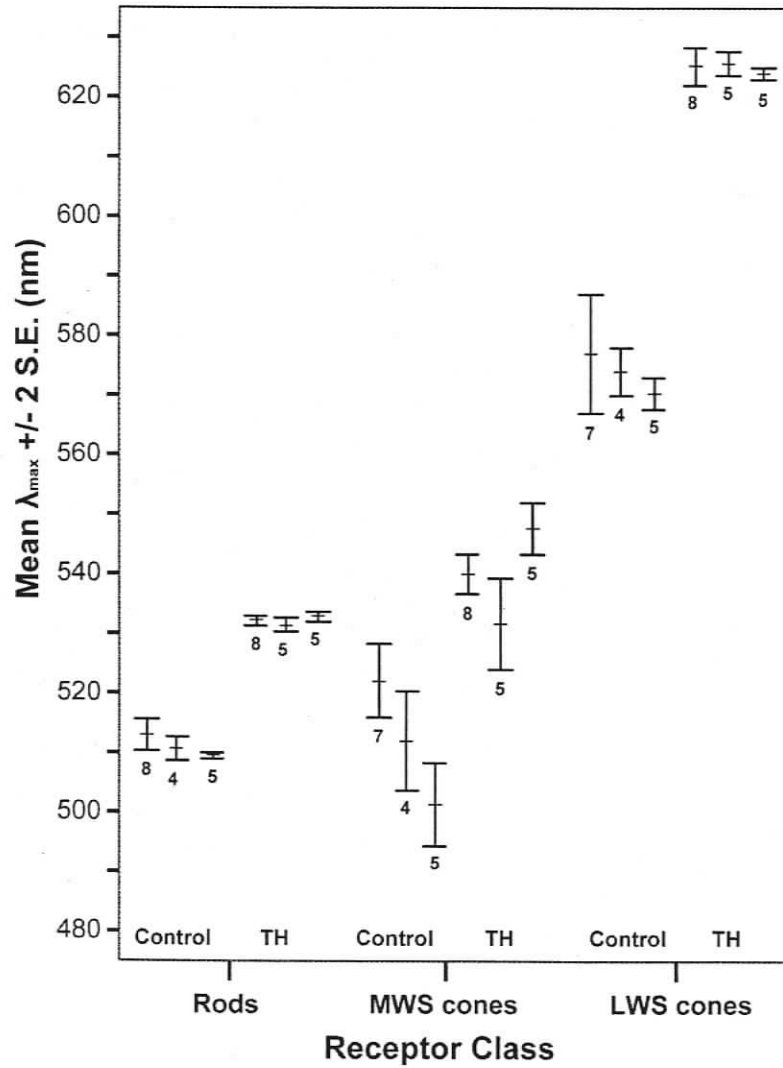


Figure 3.4. Group mean $\lambda_{max} \pm 2$ S.E. for the rods, MWS and LWS cones from control and treatment groups for Experiments III – V (plotted in numerical order from left to right). Absorbance spectra of individual photoreceptors from the dorsal retina of coho salmon (*Oncorhynchus kisutch*, Walbaum) were measured using MSP. The group mean λ_{max} is based on the mean of all fish sampled for that group, and the mean λ_{max} for each fish is the mean of approximately 20 rods, 10 MWS or 8 LWS cones on average. The control and TH treated group mean λ_{max} values for rods, MWS and LWS cones were significantly different from one another at p-value of < 0.001 in all three experiments.

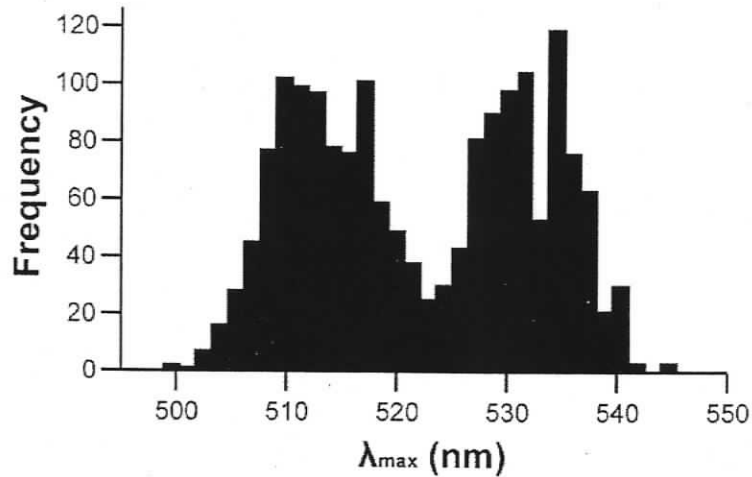


Figure 3.5. Frequency histogram of the λ_{\max} values of all rods recorded from both the dorsal and ventral retinal hemispheres of all coho salmon (*Oncorhynchus kisutch*, Walbaum) used in Experiments III - V.

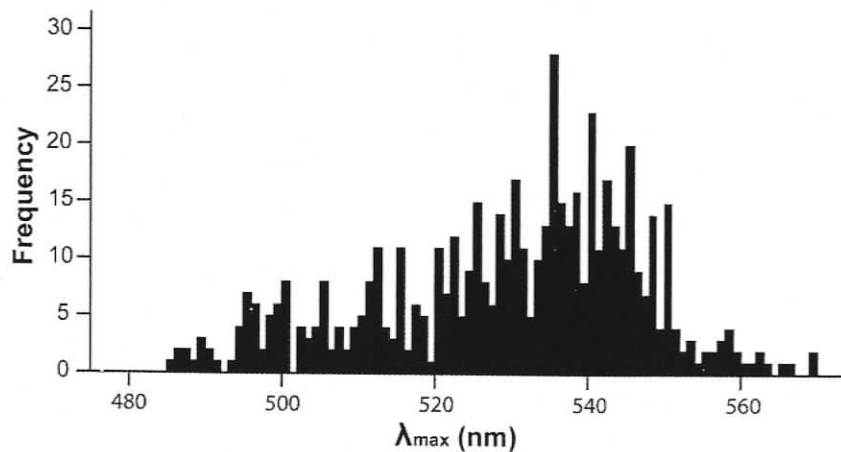


Figure 3.6. Frequency histogram of the λ_{\max} values of all MWS cones recorded from both the dorsal and ventral retinal hemispheres of all coho salmon (*Oncorhynchus kisutch*, Walbaum) used in Experiments III-V. The distribution of λ_{\max} values for the MWS cone class is greater than can be explained by the model of a single RH2 opsin combined with a range of A_1/A_2 values (see text).

Table 3.2 Models for calculating A₂ and A₁ λ_{max} values from known A₁ and A₂ λ_{max} values and the predicted values based on my MSP observations.

Source	Receptor class	Opsin class	A ₁ λ _{max} (nm)	Equation for A ₁ - A ₂ where λ = A ₁ λ _{max} in nm except in Harosi's (1994)	Calculated A ₂ λ _{max} (nm)	A ₂ λ _{max} (nm)	Inverse equation for A ₂ - A ₁ were λ = A ₂ λ _{max} in nm except in Harosi's (1994)	Calc. A ₁ λ _{max} (nm)
Bridges (1965)	Rod	RH1	503.0		527.8	534.0		506.8
	LWS	LWS	563.0		624.9	633.0		568.0
	MWS-1	RH2A	495.0		514.8	522.0 [§]		499.4
	MWS-2	RH2B	512.0 [†]	= 1.6187 × (λ ₁ - 286.42)	542.4	548.0	= (λ ₂ + 286.42) / 1.6187	515.5
Dartnall and Lythgoe (1965)	Rod	RH1	503.0		529.1	534.0		506.1
	LWS	LWS	563.0		623.6	633.0		569.0
	MWS-1	RH2A	495.0	= -263.1382 + 1.57505 × λ ₁	516.5	522.0 [§]	= (λ ₂ + 263.182) / 1.57505	498.5
	MWS-2	RH2B	512.0 [†]		543.3	548.0		515.0
Tsin, Liebman, Beatty and Drzymala (1981)	Rod	RH1	503.0		530.0	534.0		506.2
	LWS	LWS	563.0		605.0	633.0		585.40
	MWS-1	RH2A	495.0		520.0	522.0 [§]		496.6
	MWS-2	RH2B	512.0 [†]	= (λ ₁ - 79) / 0.8	541.3	548.0	= (λ ₂ × 1.8) + 79	517.4
Whitmore and Bowmaker (1989)	Rod	RH1	503.0		534.1 [√]	534.0		502.9
	LWS	LWS	563.0		626.5	633.0		566.8
	MWS-1	RH2A	495.0	= e ^{(LN(λ₁ / (52.5)(0.4) + 250))}	523.0 [√]	522.0 [§]	= (λ ₂ - 250) ^{0.4} × 52.5	494.3
	MWS-2	RH2B	512.0 [†]		548.0	548.0		512.7 [√]
Harosi (1994)*	Rod	RH1	503.0		528.9	534.0		506.4
	LWS	LWS	563.0		632.2	633.0		563.4 [√]
	MWS-1	RH2A	495.0	= λ ₁ - (27.91483 - 2.35989 × λ ₁ + 0.05054 × λ ₁ ²)*	517.2	522.0 [§]	= (3.35989 - √(3.35989*3.35989 - 4*0.05054*(λ ₂ + 27.91483))) / (2*0.05054)*	498.3
	MWS-2	RH2B	512.0 [†]		542.6	548.0		515.4
Parry and Bowmaker (2000)	Rod	RH1	503.0		521.9	534.0		512.2
	LWS	LWS	563.0		606.4	633.0		580.2
	MWS-1	RH2A	495.0	= e ^{(λ₁ / (400 + 5))}	511.6	522.0 [§]	= 400 × (ln(λ ₂) - 5)	503.0
	MWS-2	RH2B	512.0 [†]		533.8	548.0		522.5

*In Harosi's (1994) equation the λ_{max} values are in reciprocal λ_{max} so λ = 10000/λ_{max} in nm.

√Values marked in bold are calculated values that were chosen because the model predicted the greatest difference between the A₁ and A₂ states (see text for details).

†The λ_{max} value of the second MWS cone opsin RH2B when combined with A₁ is unknown, this value simply represents a possible value derived from back calculating from the observed values for the upper limits of the MWS cone λ_{max} values in figure 3.10 using Whitmore and Bowmaker's (1989) formula.

§The λ_{max} value of the second MWS cone opsin RH2A when combined with A₂ is unknown, this value simply represents a possible value derived from back calculating from the observed values for the lower limit of the MWS cone λ_{max} values in figure 3.10 using Whitmore and Bowmaker's (1989) formula.

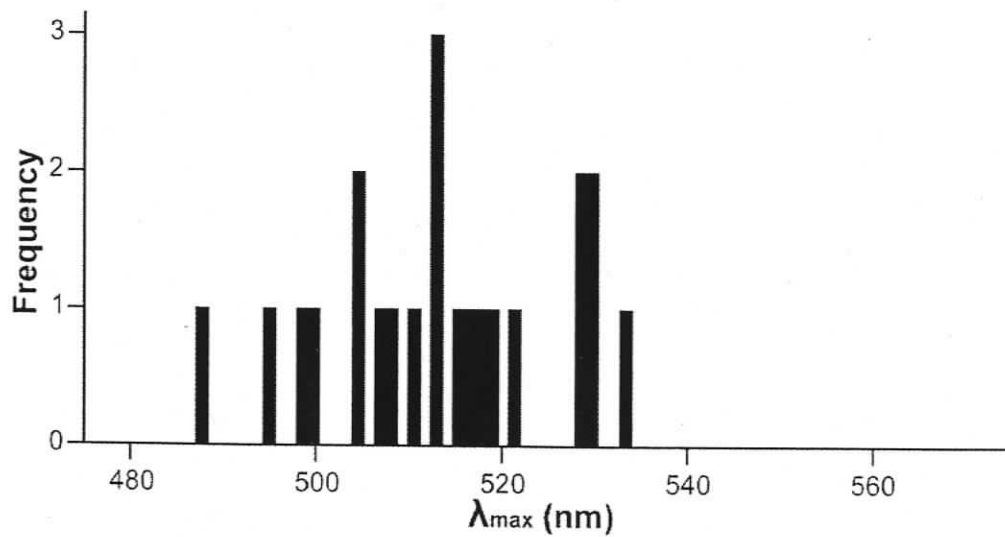


Figure 3.7. Frequency histogram for the λ_{max} values of all MWS cones recorded from an individual coho salmon (*Oncorhynchus kisutch*, Walbaum), from the control group in Experiment V. The distribution of λ_{max} values for the MWS cone class is greater than can be explained by the model of a single RH2 opsin combined with a range of A_1/A_2 values (see text).

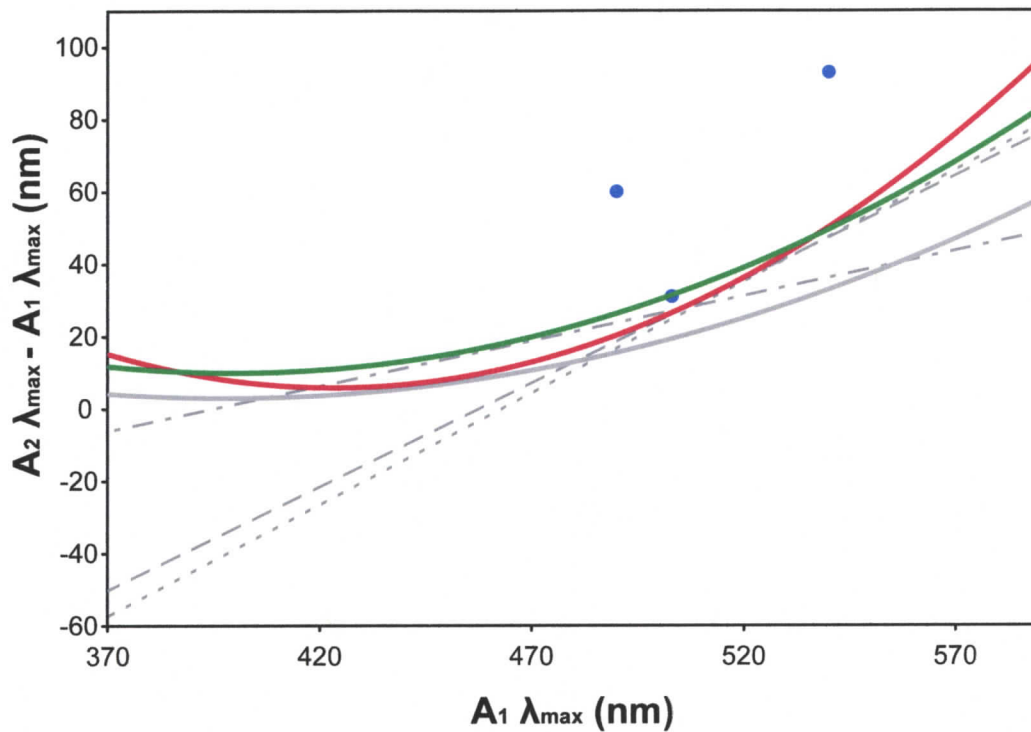


Figure 3.8. Six models that predict the expected shift in VP λ_{\max} when one opsin is combined with A_1 and A_2 chromophores, plotted as the spectral shift between the A_1/A_2 pairs as a function of the λ_{\max} of the A_1 member. These models are compared to the observed values for rods, and MWS and LWS cones in coho salmon (*Oncorhynchus kisutch*, Walbaum). Lines representing each model are: Bridges 1965 = grey dotted, Dartnall and Lythgoe (1965) = grey dashed, Tsin *et al* (1981) = grey dotted and dashed, Whitmore and Bowmaker (1989) = solid green, Harosi (1994) = solid red, and Parry and Bowmaker (2000) = solid grey. The observed range for rods, MWS and LWS cones based on the mean per fish are plotted as blue dots. The spectral shift observed in rods values fall within the predicted range of the models indicating that the observed variance in rod λ_{\max} can be explained by a change in chromophore ratio. The spectral shift of both the MWS and LWS cones lies outside the range predicted by all six models and therefore the variance in λ_{\max} of the MWS and LWS can not be explained by a shift in chromophore ratio alone.

Under the assumption that more than one RH2 opsin subtype was being expressed in a single individual, it was not appropriate to treat all MWS cone records as a single normally distributed population. Therefore, MWS cone λ_{\max} values are hereafter presented as frequency histograms representing all λ_{\max} values recorded from individual MWS cones from each group of fish.

Scatter plots of the λ_{\max} of one photoreceptor class against another may provide information about the A_1/A_2 ratio in different receptor classes. Previous reports have suggested that the A_1/A_2 ratio is determined by the retinal pigment epithelium (Bridges, 1972, 1973; Loew & Dartnall, 1976; Makino-Tasaka & Suzuki, 1984; Reuter *et al.*, 1971). Loew and Dartnall (1976) found a correlation between the λ_{\max} of neighbouring rods and cones that was nearly linear when the λ_{\max} of MWS and LWS cones were plotted against the λ_{\max} of nearby rods. I created a similar plot to determine if the same relationship held when the mean λ_{\max} of all MWS and LWS cones from individual fish were plotted against the mean λ_{\max} of all rods from the same fish (Fig. 3.9). Since MWS cones (and possibly LWS cones) appeared to express more than one opsin, it may not have been appropriate to treat each class of receptors from each fish as if originating from a single population. Furthermore, after teasing apart the retina, there was no way of knowing if the rods and cones I was recording from had been in close proximity in the intact retina. To circumvent these issues, I compared λ_{\max} values from both outer segments of individual double cones. If A_1/A_2 ratios are determined by RPE cells or other cells in proximity to the OS's (e.g. Müller cells, Mata *et al.*, 2002) then it would be expected that both double cone OS's should have similar, or at least certainly not highly dissimilar A_1/A_2 ratios.

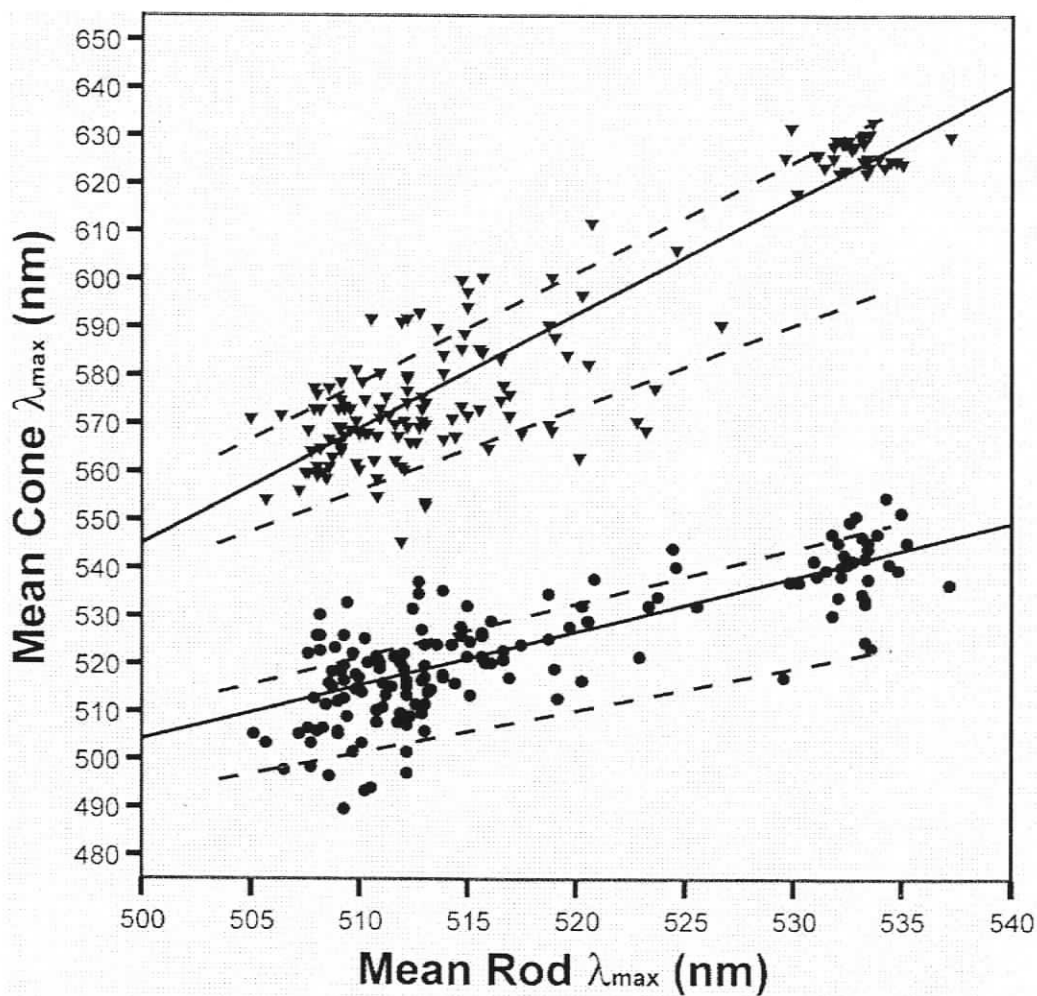


Figure 3.9. Mean MWS and LWS cone λ_{\max} per fish versus the mean rod λ_{\max} for coho salmon (*Oncorhynchus kisutch*, Walbaum) from Experiments I - V. Solid lines are the lines of best fit to the observed data, dashed lines represent possible scenarios of multiple RH2 and LWS opsins that might explain the variation in the observed λ_{\max} values. For the MWS cones most of the data points fall between the two dashed lines which demarcate the A_1 - A_2 range for opsins having λ_{\max} values of $495_1 - 523_2$ nm and $512_1 - 548_2$ nm. For LWS cones two possible opsins are predicted, having λ_{\max} values ranging from $545_1 - 600_2$ nm and $563_1 - 633_2$ nm.

The distribution of MWS versus LWS cone λ_{\max} values did not fit the pattern expected for a single opsin in each OS combined with a mixed ratio of A_1 or A_2 . I plotted λ_{\max} values for LWS versus MWS cone OS's for approximately 100 double cones from which both OS's had been recorded (Fig. 3.10). The expectation for such a plot, for a species with only one opsin in each of its double cone OS's and using only one chromophore type, would be a single tightly clumped distribution of λ_{\max} values. For a species with only one copy of each opsin but the ability to use both A_1 and A_2 , as was thought to be the case here, the prediction would be a distribution of points tightly grouped along a straight line that would extend diagonally away from the origin. The observed distribution did not meet either of these expectations. Instead the distribution appeared as if it were a diagonal straight line stretched out on the horizontal axis. I suggest that this distribution is indicative of the presence of more than one opsin subtype being expressed in the double cone OS's.

Using the distribution of values from the MWS versus LWS plot I was able to obtain estimates of the λ_{\max} values for each of the proposed opsin subtypes when combined with A_1 and A_2 . This was accomplished by defining the limits of the scattered distribution of λ_{\max} values from both OS members of individual double cones (Fig. 3.10) with lines that account for a measurement error of ± 3 nm on all sides, except the lower side (see below). From this I obtained an estimate of the A_1 and A_2 based extremes for the LWS (563 – 633 nm) and MWS (495 – 548 nm) cones. Inserting the upper and lower λ_{\max} values into equations from the six models in Table 3.2, I obtained estimates of the corresponding A_1 - or A_2 -based λ_{\max} values (Table 3.2). As a conservative measure, to reduce the probability of Type I error (rejecting

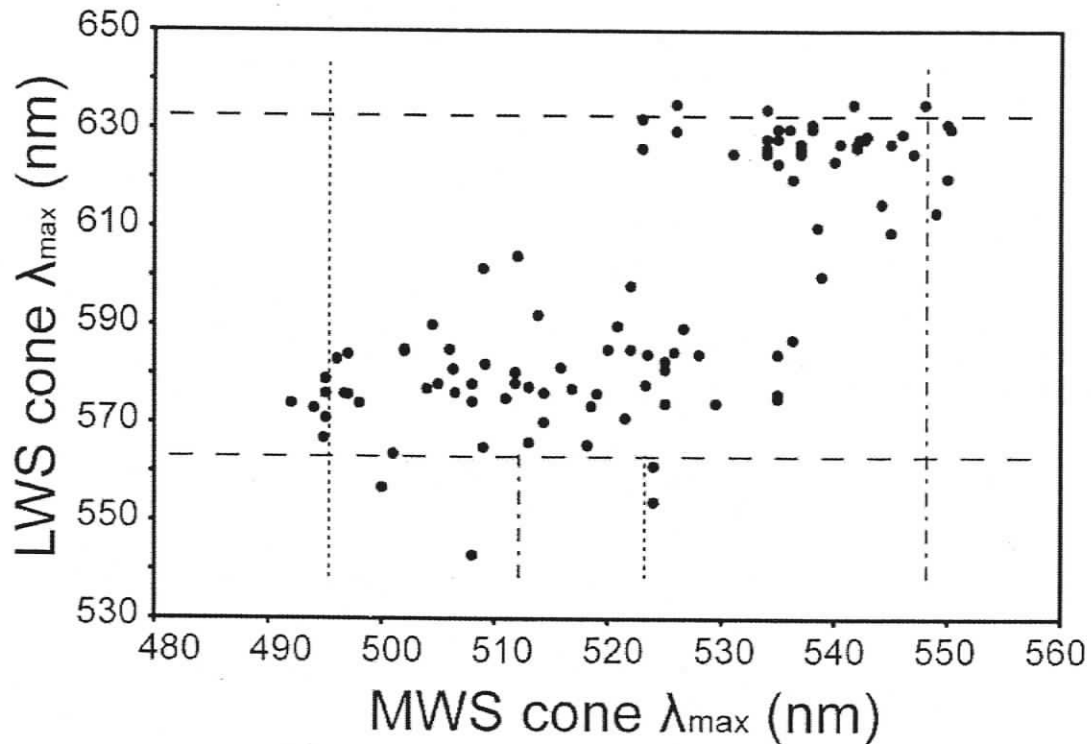


Figure 3.10. Scatter plot showing λ_{\max} values for MWS and LWS outer segments of individual double cones. The horizontal dashed lines demarcate the minimum and maximum λ_{\max} values predicted for a single LWS opsin with a λ_{\max} range of 563₁ - 633₂ nm using Harosi's (1994) model (for model selection criteria see text). Vertical dotted lines predict the range of λ_{\max} values for the MWS cones at the short wavelength range of the data set. Vertical dotted and dashed line predicts the range of λ_{\max} values based on the long wavelength range of the data set. All data points fit between these vertical and horizontal limits within the measurement error of the MSP device (± 3 nm), except those in the lower range of the LWS data set (see text). Using the lower value of 495₁ nm as the A₁ observed value I used Whitmore and Bowmaker's (1989) model to calculate that the same opsin would have a λ_{\max} of 523₂ nm if combined with an A₂ chromophore. Using the inverse of Whitmore and Bowmaker's (1989) model I estimated that the A₁ based VP that corresponded to the 548₂ nm MWS cone value at the long wavelength end of the range would have a λ_{\max} of 512₁ nm.

the null hypothesis, that there is only one MWS opsin subtype, when it is in fact true), I compared my observed data set to the model that predicted the greatest range for the given A₁-A₂ pigment pair. For the long wavelength end of the spectrum, Harosi's (1994) model predicts the greatest shift in λ_{\max} . The observed range of λ_{\max} values for most of the LWS cones (560 – 635 nm) could be explained by a single LWS opsin combining with A₁ and A₂ to give a range of 563₁ – 633₂ nm. For the middle wavelength region, Whitmore and Bowmaker's (1989) model predicts the greatest shift. However, the range of λ_{\max} values observed for the MWS cones was greater than could be explained by a single opsin combining with a mixed ratio of A₁ and A₂. The simplest model to account for the range was to assign the longest value as the A₂ state and the shortest values as the A₁ state. Using this approach the data set can be fit by a minimum of two opsins, one with the range 495₁ – 523₂ nm and the other 512₁ – 548₂ nm. Despite this added complexity, the general trend remained consistent with the hypothesis of a simultaneous shift in rods and cones. The λ_{\max} of the MWS and LWS cones increased when fish were treated with exogenous TH (Fig. 3.4).

Comparable to rods, the LWS cone λ_{\max} values were significantly increased by the application of exogenous TH in all three experiments. The difference between the control and treatment groups was significant ($p < 0.001$) for LWS cones sampled from the dorsal retina in all three experiments (Exps III – V; Fig. 3.4). The range of λ_{\max} values was generally consistent with the presence of only one LWS opsin that shifted from 563₁ – 633₂ nm. However, there were two cases where both OS members of double cones were measured and the λ_{\max} of the LWS OS was substantially below 563 nm (Fig. 3.9). There were also several LWS cones recorded in the control groups

of the three experiments that had λ_{\max} values below 563 nm, more frequently in the dorsal retina, which contains less A₂ (Fig. 3.9). These results suggest that more than one LWS opsin subtype may be present in the LWS cone class of coho salmon.

Two RH2 opsin subtypes

To account for the broader than expected range in MWS cone λ_{\max} , I hypothesized that coho may express a second RH2 opsin subtype. In order to test this hypothesis, retinal material was collected from an individual coho that had been observed to possess MWS cones with λ_{\max} values at both the low and high end of the distribution. From this sample, two RH2 opsins subtypes were isolated and cloned. One was the previously identified (Dann *et al.*, 2004) RH2, here renamed RH2A, and the second was a novel RH2 sequence that I have named RH2B (GenBank Accession No. DQ309027) in accordance with the nomenclature recently used for other fish opsin subtypes (Carleton & Kocher, 2001; Collin *et al.*, 2003).

Alignment of the RH2A and RH2B sequences (Fig. 3.11) revealed 48 amino acid differences (86.1 percent amino acid sequence identity; compared to 86.8 percent nucleotide identity see Appendix C). The possession of several amino acids that are conserved among vertebrate opsins suggested that the RH2B was likely a functional opsin. The predicted RH2B amino acid sequence possessed a lysine at position 296, which is known to form a Schiff's base linkage with the chromophore (Wang *et al.*, 1980). It also had a glutamate at position 113, which is considered to be the counterion of the protonated Schiff's base (Sakmar *et al.*, 1989; Zhukovsky & Oprian, 1989). The RH2B also had cysteine residues at positions 110 and 187, which

RH2A	MQNGTEG	SNF	YIPMSNRTGL	VRSPFE	YYPQY	YLAPPWQ	YYC	LAVYTFFLIC	50																																										
RH2B	MQNGTEG	NNF	YIPMSNRTGL	VRSPFL	YQQY	YLADPWQ	FYL	LAVYMFFLIC	50																																										
RH2A	FGFPINGLTL	YVTATNK	KL	R	QPLNFIL	VNL	AAAGMIM	VLF	GFTITITS	SAV	100																																								
RH2B	FGFPINGLTL	YVTATNK	KL	Q	QPLNFIL	ANL	AAAGMIM	VMF	GFTITITS	SAV	100																																								
RH2A	NGYFI	WG	P	L	G	CAIEG	F	MATL	GG	E	VAL	W	SLV	VLA	V	E	R	Y	I	V	CKPMGS	S	F	T	F	150																									
RH2B	NGYFV	F	G	P	M	G	CAIEG	F	MATL	GG	Q	VAL	W	SLV	VLA	I	E	R	Y	I	V	CKPMGS	S	F	T	F	150																								
RH2A	S	T	H	A	G	A	V	A	F	T	W	I	A	A	M	A	C	A	A	P	P	L	L	G	W	S	R	Y	I	P	E	G	M	Q	C	S	C	G	P	D	Y	Y	T	L	A	E	G	F	N	200	
RH2B	T	T	H	A	G	A	C	A	F	T	W	V	M	A	M	A	C	A	A	P	P	L	V	G	W	S	R	Y	I	P	E	G	M	Q	C	S	C	G	P	D	Y	Y	T	L	A	E	G	F	N	200	
RH2A	N	E	S	Y	V	I	Y	M	F	S	C	H	F	I	I	P	V	C	L	I	A	Y	T	Y	G	S	L	V	L	T	V	K	A	A	A	A	S	Q	Q	D	S	A	S	T	Q	K	A	E	K	E	250
RH2B	N	E	S	Y	V	I	Y	M	F	T	C	H	F	C	V	P	V	V	T	I	F	F	T	Y	G	S	L	V	L	T	V	K	A	A	A	A	S	Q	Q	D	S	A	S	T	Q	K	A	E	K	E	250
RH2A	V	T	R	M	C	I	L	M	V	C	G	F	M	V	A	W	T	P	Y	A	T	L	A	A	Y	I	F	F	N	K	G	I	A	F	S	A	Q	S	M	A	I	P	A	F	F	S	K	S	S	A	300
RH2B	V	T	R	M	C	F	L	M	V	C	G	F	L	I	A	W	T	P	Y	A	S	F	A	A	Y	I	F	F	N	K	G	A	A	F	T	A	T	A	M	A	I	P	A	F	F	S	K	S	S	A	300
RH2A	L	F	N	P	I	I	Y	V	L	M	N	K	Q	F	R	C	M	L	A	A	V	G	M	K	A	-	E	E	G	E	T	S	V	S	T	S	K	T	E	V	S	S	A	G	P	A	346				
RH2B	I	F	N	P	V	I	Y	V	L	M	N	K	Q	F	R	S	C	M	L	A	A	V	G	I	S	S	G	A	E	D	E	T	S	V	S	A	S	K	T	E	V	S	S	V	G	P	A	347			

Figure 3.11. Alignment between the amino acid sequences of the coho (*Oncorhynchus kisutch*, Walbaum) RH2A (Dann *et al.*, 2004) and RH2B deduced from coho MWS opsin cDNA show 86.1 percent amino acid sequence identity. Conserved residues in positions analogous to bovine I113, C187, and K296 are in red. The 48 amino acid differences between the RH2A and RH2B are highlighted in yellow. The E123Q substitution, (analogous to position 122 in bovine rod opsin) that may be responsible for the hypsochromic shift of the RH2B relative to RH2A, is in red and highlighted.

are known to form a disulphide bond (Karnik *et al.*, 1988). There were also three amino acids that are located in cytoplasmic loop three that are conserved in all opsins, these were S240, T243, and V250 (Archer, 1995). In addition, RH2B had an E to Q substitution at position 123 analogous to position 122 in bovine rod opsin. This E122Q substitution is known to cause a hypsochromic (short wavelength) shift in λ_{\max} of RH2 opsins (Chinen *et al.*, 2003; Sakmar *et al.*, 1989; Yokoyama *et al.*, 1999).

Summary of results

The key findings of this series of experiments were: (i) that TH induced smoltification like transitions in pre-smolt coho under all rearing conditions and at all times of the year tested; (ii) in all five experiments TH caused an increase in rod λ_{\max} and the observed λ_{\max} range for rods was from 503 to 533, consistent with a shift in A_1/A_2 ratio; (iii) both rods and cones shifted λ_{\max} concomitantly; (iv) the range of λ_{\max} values observed for MWS cones extended from 495 to 548 nm, which was greater than predicted for a shift in A_1/A_2 ratio within a single opsin; and (v) a second putative RH2 opsin subtype was isolated, cloned and sequenced, which possessed an E123Q substitution that could explain the shift in MWS cone λ_{\max} .

Discussion

Effects of TH on coho rod visual pigments

Exogenous TH caused increases in ROS λ_{\max} under all rearing conditions and at all times of year tested (Table 3.1). The increase, though greater than previously reported for this species (see below), was consistent with a shift from A₁ to A₂. In all previous studies with teleosts, except one, the effect of exogenous TH, thyroid stimulating hormone or prolactin, has always been to increase the proportion of A₂ in the VPs (Alexander *et al.*, 1998; Allen, 1971, 1977; Allison *et al.*, 2004; Beatty, 1969a; Cristy, 1974; Jacquest & Beatty, 1972; McFarland & Allen, 1977; Munz & Swanson, 1965; Tsin & Beatty, 1979). Only two of these studies (Alexander *et al.*, 1998; Cristy, 1974) investigated interactions between exogenous hormones and rearing conditions or time of year.

Cristy (1974) found that rainbow trout shifted to a higher proportion of A₂ when treated in summer compared to winter. In light of the present findings, and Allen's (1977) observation that TH dosage affects the degree of chromophore shift, the observations reported by Cristy (1974) may have been confounded by the different TH concentrations used (1 mg/litre in summer and 0.001mg/litre in winter).

The second study (Alexander *et al.*, 1998), showed the classic increase in A₂ when coho reared under summer like conditions (warm, 15 °C and bright light) were treated with exogenous TH. However, Alexander *et al.* (1998) also reported a small but significant decrease in A₂ when coho reared under winter like conditions (cold, 5 °C and dim light) were treated with TH. This result was used to support Alexander *et*

al.'s (1994) previous hypothesis that coho shift to A_1 coincident with the peak in plasma TH in spring prior to seaward migration (migration/metamorphosis hypothesis). The results of my experiments, which encompassed the time of year when coho migrate to sea, did not concur with Alexander *et al.*'s (1998) findings, but instead fit the classic model of TH increasing A_2 .

There are at least two possible reasons why my results differed from those of Alexander *et al.* (1998). Firstly, the cold temperature and natural sun light (Exp III) and the cold temperature and dim light (Exp IV) treatments (Table 3.1) did not result in increased A_2 content prior to the TH treatments. This was surprising since previous reports have shown clear effects of temperature and light intensity on A_1/A_2 ratio in teleosts (Allen & McFarland, 1973; Tsin & Beatty, 1977; Tsin *et al.*, 1981). It may be that increases in A_2 in response to changes in rearing conditions are acute and temporary. Since my fish were acclimated to the novel conditions for several weeks before the experiment began, they may have returned to their natural seasonal fluctuations (Chapter 1, Temple *et al.*, 2006). As Alexander *et al.* (1998) suggest, the reverse effect of TH (causing a shift from A_2 to A_1) may only occur when the retina is in a state of A_2 dominance.

Secondly, Alexander *et al.*'s (1998) TH treatments were performed in September – October, while my winter experiment (Exp I) took place in February – March and my winter-like experiments (Exps III and IV) took place in January – April. There may be underlying seasonal variability in physiological regulators of A_1/A_2 ratio. Variability in deiodinase and TH receptor levels could potentially affect the response to exogenous TH. Likewise, variability in the, as yet unidentified, 3,4-

dehydrogenase, which is proposed to convert A₁ to A₂ in the RPE (Bridges & Yoshikami, 1970b; Dartnall, 1964; Naito & Wilt, 1962) might also affect the response to exogenous TH. In order to confirm the unique finding of a reverse response to TH in coho by Alexander *et al.* (1998) further investigation would be required.

The magnitude of change in A₁/A₂ ratio differed between Experiments I & II and III – V. This may have been due to the use of different TH delivery systems. To accommodate the large number of fish and water required in Experiments I & II, TH was delivered via the food, whereas TH bath treatment was used for Experiments III – V in an attempt to reduce between fish variation. Both techniques have been shown to be effective at increasing percent A₂ in rods, measured primarily by ESP (Alexander *et al.*, 1998; Allison *et al.*, 2004; Beatty, 1969a, 1972; Crim, 1975; Cristy, 1974; Jacquest & Beatty, 1972; McFarland & Allen, 1977). Alexander *et al.* (1998) used 12 ppm T₃ sprayed on the food and achieved an increase in A₂ from approximately 25 – 75 percent in coho kept at 15 °C over the same duration (4 – 6 weeks). It would seem that using food as the delivery system may not be as effective as the bath treatment, since both Alexander *et al.*'s (1998) and my present experiments did not produce a complete conversion to A₂. The TH bath treatment, on the other hand, resulted in rod λ_{\max} values that were the most bathochromically shifted yet recorded for this species.

The mean rod λ_{\max} values recorded in the treatment groups from Experiments III – V were all above 530 nm, at least 3 nm higher than previously reported for coho rods with A₂-based VPs (Alexander, 1998; Alexander *et al.*, 1994; Beatty, 1972;

Munz & Beatty, 1965; Temple *et al.*, 2006). The λ_{\max} range for individual rods was 500 – 540 nm (Fig. 3.5). If I subtract a measurement error of ± 3 nm, the change in λ_{\max} for rods appears to extend from 503 – 537 nm. There are a few possible reasons why my λ_{\max} measurements extend higher than previously reported. This is the first study to have used a bath treatment for delivery of TH to coho salmon. Previous studies used either: (i) untreated fish that were under relatively natural conditions (Alexander *et al.*, 1994; Temple *et al.*, 2006); (ii) fish that were treated with TH added to their food (Alexander *et al.*, 1998); or (iii) fish that received multiple intraperitoneal injections of TH but that were measured after no more than 14 days (Beatty, 1972). Therefore, it is possible that none of these previous methods resulted in a transition to 100 percent A₂. I suggest that my values may be a more accurate estimate of the actual λ_{\max} for the A₂ state of coho rods. This contention is supported by the fact that the λ_{\max} of rods measured in the ventral retina did not differ from that measured in the dorsal retina after 4 - 6 weeks of TH bath treatment in Experiments III – V. These results suggest that the maximum λ_{\max} value had been attained since the ventral retina is typically higher in A₂ than the dorsal retina in most fishes (Bowmaker, 1991; Muntz & Northmore, 1971), including coho (Temple *et al.*, 2006).

Another possible reason for the higher λ_{\max} value recorded from rods in my experiments was that I used MSP to measure individual photoreceptors instead of using ESP to measure a mean λ_{\max} from large sections or whole retina (Alexander *et al.*, 1994; Alexander *et al.*, 1998; Beatty, 1966; Munz & Beatty, 1965). The mean values obtained by ESP may mask the extreme long or short wavelength states of rod VPs. What is not clear, is why partial bleaching (Beatty, 1966; Munz & Beatty, 1965)

was not able to provide a similar estimate to that recorded here. Again, perhaps the previous experiments did not attain 100 percent A_2 levels in the retinas.

Finally, a third possibility is that the longer rod λ_{\max} values observed in this study were due to population differences. Previous studies all used different stocks of coho: (i) Alexander *et al.* (1994, 1998) used a stock from the Fraser River on the mainland, Southern British Columbia; (ii) Beatty (1972) used fish from Crammond, Alberta; and (iii) Munz and Beatty (1965) used wild fish collected from waters up and down the west coast, from British Columbia to California. My study used two stocks of fish, one that originated from the Kitimat River in central British Columbia and the other that came from a river on the west coast of Vancouver Island in Southern British Columbia. Given the diversity of stocks used in previous studies, it seems unlikely that the two stocks I chose would be the only two that differed. Although I can not exclude this possibility, I am inclined to think that the bathochromically shifted λ_{\max} value I obtained for the rods was likely due to the TH bath treatment combined with the use of MSP rather than ESP.

Concomitant shift in λ_{\max} of rods and cones

The trend of MWS and LWS λ_{\max} increasing with rod λ_{\max} (Fig. 3.9) was consistent with previous findings of rods and cones shifting A_1/A_2 ratio at the same time (Loew & Dartnall, 1976). The explanation for this simultaneous shift in organisms exposed to light:dark cycles is that both rods and cones use the same source of chromophores, the RPE, for VP regeneration (Liebman & Entine, 1968; Loew & Dartnall, 1976). Work with bullfrogs (Reuter *et al.*, 1971), demonstrated that

when a bleached retina was placed on RPE it would acquire the A_1/A_2 ratio that was present in that portion of the eye (high A_2 dorsal and high A_1 ventral) regardless of the origin of the piece of retina. The same result was attained when the retina of one species was placed on the RPE of another (Bridges, 1973). Comparable studies using these techniques for investigating the A_1/A_2 ratio in cones, using MSP, have not been reported. The recent suggestion by Mata *et al.* (2002) that cones may use a different VP regeneration pathway, involving Müller cells, might imply that such a relationship may not necessarily hold. However, the Müller cell pathway has only been observed in cone dominated retinas, and there is no information yet about the interaction between the two regeneration pathways with respect to A_1/A_2 ratios. The general trend in mean λ_{\max} of rods and cones observed here suggests that, whatever the regulatory mechanism, the A_1/A_2 ratio is consistent for both rods and cones. Unfortunately, the observed variance in MWS cone λ_{\max} , which was greater than could be explained by a shift in chromophore alone, prevented an accurate estimate of A_1/A_2 ratio for comparison among rods and cones.

MWS cones express two RH2 opsin subtypes

A second novel RH2 opsin subtype was isolated and sequenced from coho salmon, which I have named RH2B. When compared to the existing sequence (RH2A, Dann *et al.*, 2004) I found 48 amino acid differences (Fig. 3.11). Of particular interest was the substitution of glutamate for glutamine at position 123, which is analogous to 122 in bovine rod opsin. An E122Q substitution has been shown to hypsochromically shift the λ_{\max} of bovine RH1 by 20 – 25 nm (Sakmar *et*

al., 1989). It has since been recognized that this particular site is likely to play an important role in spectral tuning of both RH1 and RH2 opsins (Yokoyama *et al.*, 1999). Three of the four RH2 opsin subtypes in zebrafish possess the glutamate at position 122 and all three absorb maximally at shorter wavelengths than the fourth (17 - 38 nm, Chinen *et al.*, 2003; Chinen *et al.*, 2005a). It is therefore likely that the coho RH2B opsin subtype is short wavelength shifted relative to RH2A. Current efforts are aimed at characterizing the RH2B opsin and determining its spatio-temporal expression patterns. The results of these experiments suggest that TH may play a role in regulating expression levels of RH2A and RH2B opsin subtypes, which is consistent with the function of TH as a signaling mechanism in vertebrate metamorphosis (Power *et al.*, 2001) and in retinal development (Allison *et al.*, 2003; Browman & Hawryshyn, 1992; Harpavat & Cepko, 2003).

Evidence for LWS cones expressing more than one opsin

LWS cones, like the MWS cones, showed a greater range in λ_{\max} than could be explained by a chromophore substitution in a single opsin. When λ_{\max} of all LWS cones from the five experiments were plotted as a frequency histogram (Fig. 3.12) the variation extended from approximately 550 nm to over 635 nm. While a second copy of the LWS opsin has not yet been isolated, other evidence suggests that salmonids underwent a complete genome duplication event early in their evolutionary history, as observed in several other teleosts (Taylor *et al.*, 2003). Given that the ancestor of all vertebrates is thought to have had at least one copy of each opsin class (Collin & Trezise, 2004; Yokoyama & Yokoyama, 1996), the potential exists for coho to

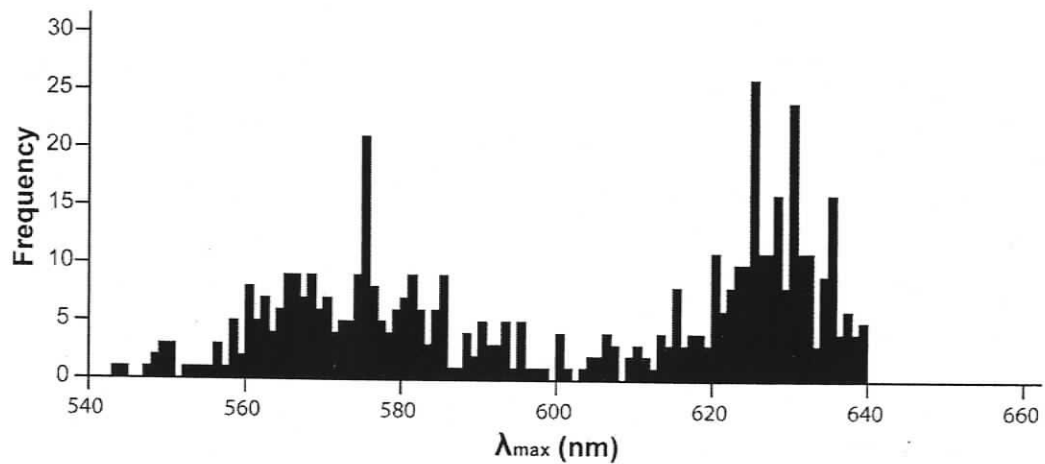


Figure 3.12. Frequency histogram of the λ_{max} values of all LWS cones recorded from both the dorsal and ventral retinal hemispheres of all coho salmon (*Oncorhynchus kisutch*, Walbaum) used in Experiments I - V. The distribution of λ_{max} values for the LWS cone class is greater than can be explained by the model of a single LWS opsin combined with a range of A_1/A_2 values (see text).

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Summary

- 1) The λ_{\max} of coho rods was found to vary from approximately 503₁ to over 534₂ nm, a range that is extended by at least 7 nm into the long wavelength end of the spectrum compared to previous estimates of 503₁ – 527₂ nm.
- 2) The application of exogenous TH resulted in increases in rod λ_{\max} indicative of a shift to A₂ dominance under all rearing conditions and times of year. This finding suggests that TH is not involved in the shift from A₂ – A₁ that occurs in spring prior to smoltification and seaward migration. Because of the role of TH in other physiological changes that occur at smoltification, it is suggested that the A₂ – A₁ shift is not linked to smoltification in coho salmon.
- 3) The shift in MWS cone λ_{\max} was greater than could be explained by a shift in chromophore ratio. However, a putative second RH2 opsin subtype was discovered that had an E123Q substitution relative to the already identified RH2, which could account for the spectral shift in this cone class.

Chapter 4 : Ontogenetic changes in cone photoreceptor spectral absorption in coho salmon (*Oncorhynchus kisutch*)

This chapter is based on a collaboration with the following authors and is in preparation for submission to *Vision Research* as cited below:

S. E. Temple, K. M. Veldhoen T. J. Haimberger, J. T. Phelan, N. J. Veldhoen, and C. W. Hawryshyn. (In Prep.). Ontogenetic changes in cone photoreceptor spectral absorption and opsin expression in coho salmon (*Oncorhynchus kisutch*). *Vision Research*.

Introduction

The early life history of anadromous salmonids is characterized by a migration event from their natal freshwater streams and rivers to the open ocean. This movement from one habitat to another is accompanied by a change in spectral environment and visual tasks. For many fishes such changes are accompanied by alterations in spectral sensitivity (Allison *et al.*, 2003; Browman & Hawryshyn, 1994; Loew *et al.*, 2002; Shand, 1993; Shand *et al.*, 2002; Shand *et al.*, 1988; Takechi & Kawamura, 2005). In vertebrates, several mechanisms exist to alter spectral sensitivity including: (i) gain or loss of a photoreceptor class; (ii) expression of different opsin classes or subtypes within a photoreceptor class; and (iii) changes in chromophore type (A_1 or A_2). Previous studies have shown that all three of these mechanisms are present in members of the genus *Oncorhynchus* (Chapter 3 and Beatty, 1966; Bowmaker & Kunz, 1987; Cheng & Novales Flamarique, 2004).

Of the Pacific salmonids, coho are of particular interest for examining the timing of changes in spectral sensitivity because they normally spend over a year in fresh water before migrating to sea. This extended period of freshwater residency necessitates a visual system that is well adapted to the spectral environment and visual tasks at hand. It has been thought, for some time, that changes in chromophore ratio were an adaptation to alter the spectral sensitivity of coho salmon and other salmonids to match the two different spectral environments. Most research has supported the hypothesis that A_2 -based VPs are matched to the long-wave shifted fresh water habitat, while A_1 -based VPs are matched to the short-wave shifted open

ocean habitat (Alexander *et al.*, 1994; Alexander *et al.*, 1998; Beatty, 1975a; Bridges, 1972; Novales Flamarique, 2005; Wald, 1941). However, a recent study that followed the A_1/A_2 ratio, over an extended period of time in different age classes of coho, found that shifts in A_1/A_2 ratio were closely correlated with seasonal changes in environmental variables (Chapter 2 and Temple *et al.*, 2006). Subsequent examination into the effects of TH on VP composition in coho salmon uncovered evidence for the existence of more than one opsin subtype in the MWS and LWS cone classes. These findings were supported by the isolation and sequencing of a second RH2 opsin subtype (Chapter 3).

In the present study, I sought to determine if coho salmon alter their spectral sensitivity when they change habitats by changing opsin expression rather than changing chromophore ratio. I used MSP to compare the λ_{\max} values of ROSs to both outer segment members of the double cones in coho salmon of different age classes. I also tested whether TH would alter the λ_{\max} of double cones in a manner similar to that which occurs naturally at smoltification.

Materials and Methods

Animals and Care

Coho salmon were obtained from Target Marine salmon farm and hatchery (Sechelt, British Columbia, Canada). Fish were transported in coolers to the aquatic facilities at the University of Victoria in April and May of 2005 and maintained in

conditions similar to those at the hatchery (matched temperatures and natural daylight) until used in experiments (less than 5 days, except for a subgroup of alevins that were treated with TH for 4 weeks). The alevins, which had hatched in February 2005, were approximately four months of age when examined and were 5.5 ± 0.2 cm in length and 2.0 ± 0.3 g in weight. Their yolk sacs were not apparent and they readily fed on dry commercial salmon food. The parr had hatched in February, 2004 (~16 months old) and had begun the parr-smolt transformation, evident by the presence of silvering, greenish-blue dorsal pigmentation and darkened fins. They were 8.6 ± 0.5 cm in length and 6.9 ± 1.0 g in weight. Ocean smolts were from the 2003 hatch and were approximately 28 months of age. They were 45 ± 12 cm in length and between 1.5 – 2.0 kg in weight.

Thyroid hormone treatment

To determine if ontogenetic shifts in VP λ_{\max} could be stimulated with exogenous TH, I measured the difference in λ_{\max} of rod and cone OS's in both control and TH treated alevins. Two groups of 25 alevins were kept outdoors under natural, partly shaded, daylight in 15 l tanks with static water kept at 11.0 ± 1.0 °C. Tank water was changed three times weekly. The treatment tank received sufficient T_4 dissolved in 1.5 ml of 0.1 M NaOH to bring the final concentration to $300 \mu\text{g l}^{-1} T_4$, while the control tank received the vehicle only (1.5 ml of 0.1 M NaOH). Care and treatment of fish was in accordance with the University of Victoria's Animal Care Committee, under the auspices of the Canadian Council for Animal Care.

Microspectrophotometry

Fish were dark adapted for at least one hour prior to sacrifice by an overdose of Eugenol 100 mg l⁻¹ (ICN Biomedicals Inc., Irvine, CA.), followed by cervical transection. The right eye was enucleated and hemisected along an anterior posterior axis. A piece of retina 1 - 2 mm² was cut out of the dorsal most section of the dorsal hemisphere of the retina. The retinal sample was teased apart on a glass cover slip and a drop of MEM (Sigma; pH adjusted to 7.4 - 7.6) was applied to the sample. A second cover slip was placed over the sample and sealed with paraffin. All procedures were performed under deep red illumination (>650 nm) or using a dissecting scope equipped with infrared LED (800 nm) illumination and monitored with a CCD-camera.

I used a CCD-MSP device that has been described previously (Hawryshyn *et al.*, 2001), to measure spectral absorbance of individual rod and cone photoreceptors. The CCD-MSP device delivered a short flash (0.05 - 0.5 seconds)* of full spectrum light (300-800 nm; 150 W xenon light source – intensity regulated, Oriel) to the photoreceptor outer segment. The beam size was approximately 2 x 3 μm at the focal plane of the outer segment. The transmitted beam passed through a spectrometer (300 nm blazed grating, Acton Research Corporation) and onto a 1340 x 400 pixel, Peltier cooled (-45°C), back-illuminated CCD-detector (Princeton Instruments, Roper Scientific, Inc., Trenton, NJ, USA). Photoreceptor absorbance ($\log_{10} (1/T)$) was calculated by comparing the transmitted intensity through the photoreceptor (I_M) to

* Duration was dependent on intensity and was set to deliver an optimum number of photons per exposure time = total counts see Appendix B

the transmitted intensity through an area clear of debris adjacent to the photoreceptor (reference (I_R)) thus, $T = I_M/I_R$). If acceptable, the record was then stored for later analysis.

The retinal sample was examined under infrared illumination (Schott RG850 filter, Ealing Optics) and monitored by an infrared camera (Canadian Photonics Laboratory). The search image and infrared filtered beam (Schott RG850 filter) were displayed on a computer monitor. The position of the ROS relative to the measurement beam was controlled by a motorized stage (Marhauser-Wetzlar GmbH & Co., Germany). The path of the motorized stage was plotted on-screen to prevent repeated measurements of ROSs.

Criteria for acceptance of absorbance spectra were: (i) presence of a baseline on the long wavelength arm (Harosi & MacNichol, 1974); (ii) peak absorbance near the expected wavelength (~500 - 530 nm for coho rod photoreceptors); (iii) minimal absorbance by photoproduct and; (iv) signal to noise ratio of the α absorption band greater than 5 to 1. These criteria rejected approximately ten percent of MSP recordings. Determinations of λ_{max} , and percent A_2 from absorbance records were performed offline subsequent to initial sampling.

A custom designed analysis program was used to determine the λ_{max} from absorbance records using existing templates. Each MSP record consisted of 1340 points between 300 and 750 nm. Each record was linear detrended if necessary (Harosi, 1987). A nine-point adjacent averaging function was used for line smoothing. The smoothed curve was normalized to zero at the baseline on the long wavelength arm and to one at the center of the α -peak. The fit of the normalized curve

was compared with a nonlinear least-squares routine to the upper 20 percent of the weighted A_1/A_2 averaged Govardovskii *et al.* (2000) template (based on the center of the α -peak ± 40 nm).

For some rods, I also obtained a second estimate of λ_{\max} based on a template created by Munz and Beatty (1965) for rod pigments of coho salmon. The absorbance curves were compared (minimum variance fit) to the Munz and Beatty (1965) template, which extends from the λ_{\max} to a point at 20 percent of the maximum on the long wavelength arm. The Munz and Beatty (1965) template assumes that the λ_{\max} of coho rods varies from 503 – 527 (nm). Their model is in close agreement with Harosi's (1994), which predicts that an A_1 -based VP with a λ_{\max} of 503 nm would shift to 529 nm if the A_1 chromophore were replaced with an A_2 chromophore (see results). However, many of the rods measured here had λ_{\max} values that exceeded 527 nm and therefore could not be fit to the Munz and Beatty (1965) template. In such cases I used the fit to the Govardovskii *et al.* (2000) template.

Data Analysis

MSP records were collected from individual photoreceptors from the dorsal retina. For each receptor class of interest, a mean λ_{\max} value was calculated for each fish (fish mean λ_{\max}). For comparisons between groups (age classes and TH treated), I calculated a mean λ_{\max} for each group (group mean λ_{\max}), which was the mean of all fish mean λ_{\max} values for each group. Comparisons among group mean λ_{\max} values were made using a one way analysis of variance (ANOVA) with $\alpha = 0.05$. Tukey's HSD post hoc analysis was used for pair-wise comparisons among groups.

Results

Age classes and TH treatment

The group mean λ_{\max} of rods differed significantly ($F_{(3, 16)} = 571.5$, $p < 0.001$) among the four groups of coho sampled (Fig. 4.1). However, there were no differences ($p > 0.262$, Tukey HSD pair wise comparisons) among the three age classes (alevin, parr and ocean smolts), which had a combined mean of 509.6 nm. There was a significant difference ($p < 0.001$) between the TH treated alevins and all other groups. The mean λ_{\max} of rods in the TH treated group was 533.0 nm, consistent with a shift in chromophore ratio, which shifts the λ_{\max} of coho rods from 503 – 534 nm (Chapter 3, Temple *et al.*, In Prep).

Variation in the group mean λ_{\max} of MWS cones supported the finding of a second RH2 opsin subtype in this cone class. The group mean λ_{\max} of the MWS cones differed significantly ($F_{(3, 16)} = 41.0$, $p < 0.001$) among the four groups (alevin 501.5 ± 2.2 ; parr 511.6 ± 9.0 ; ocean going smolts 507.1 ± 7.8 ; TH treated alevin 547.7 ± 4.9 ; Fig 4.1). However, as observed for rods, only the TH treated alevins had a mean λ_{\max} significantly higher ($p < 0.001$) than the other three groups. The high degree of variance in MWS cone λ_{\max} observed in alevin and parr age classes was consistent with my previous findings (Chapter 3, Temple *et al.*, In Prep), which found evidence for the existence of more than one RH2 opsin subtype in the MWS cones. The expression of more than one opsin subtype meant that the λ_{\max} values should not be treated as originating from a single normally distributed population. Therefore,

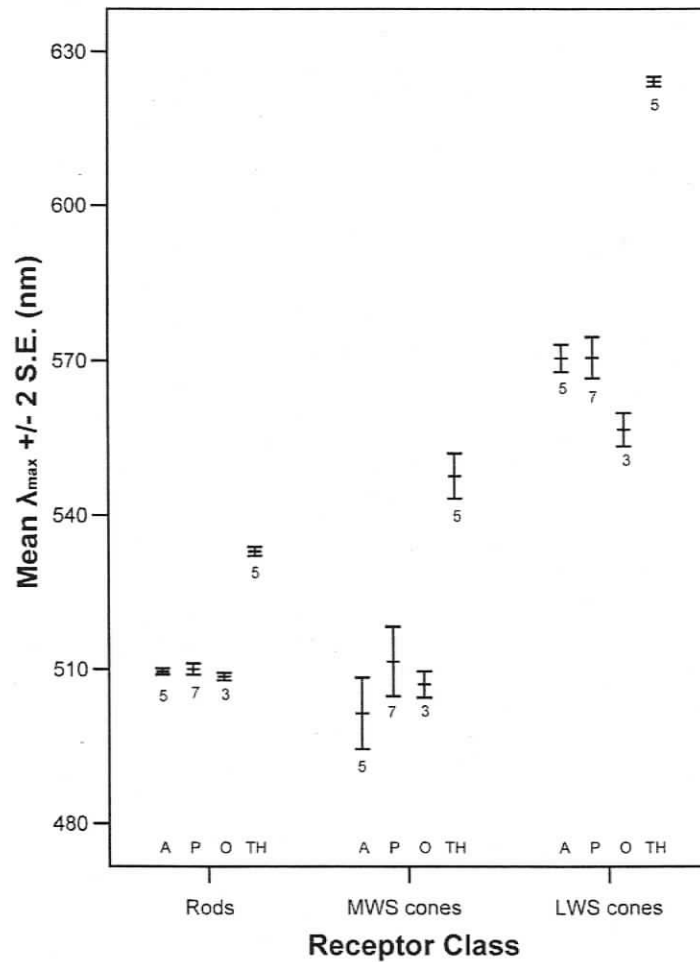


Figure 4.1. Mean $\lambda_{max} \pm 2$ S.E. for rods, MWS and LWS cones from alevin (A), parr (P), ocean smolts (O) and TH treated alevin (TH) coho (*Oncorhynchus kisutch*, Walbaum). The mean for each age group or treatment group is based on the λ_{max} values obtained for that receptor class for each fish (n-values below the error bars). The λ_{max} values for each fish are the mean of all receptors of that class measured from the dorsal retina.

ANOVA was not an appropriate statistical approach for comparing λ_{\max} values, and a different type of analysis was necessary (see below).

The group mean λ_{\max} of LWS cones was significantly ($F_{(3, 16)} = 298.0$, $p < 0.001$) different among the four groups. There was no difference in λ_{\max} between the alevin and parr ($p = 1.000$), but the ocean smolts were significantly lower than the other three groups ($p \leq 0.001$, Tukey HSD pair wise comparisons) and the TH treated alevins were significantly higher ($p < 0.001$; Fig. 4.1). The group mean λ_{\max} of the LWS cones varied from a low of 556.6 nm (ocean smolts) to a high of 624.2 nm (TH treated alevins), a range that could not be accounted for by a single opsin combined with A₁ and A₂ chromophores (see below).

Analysis of the variation in λ_{\max} of MWS and LWS cone classes

To address the variation in λ_{\max} of MWS cones, which was due to changes in chromophore ratio together with the proposed change in opsin subtype expression, I looked at the frequency distribution of λ_{\max} values of all MWS cones. The range of λ_{\max} values recorded from individual MWS cones from all four groups extended from below 490 nm to above 550 nm (Fig. 4.2A). This variance in λ_{\max} was greater than could be explained by a change in chromophore ratio (Chapter 3). When MWS cone λ_{\max} values were plotted for each age class, the variation recorded in the alevin and parr was still greater than could be accounted for by a shift in A₁/A₂ ratio (Figs. 4.2B, and D). This extended range of MWS cone λ_{\max} values was evident within individual fish (Fig. 4.2F).

The frequency distribution of λ_{\max} values from individual LWS cones from all age classes and the TH treated alevins is displayed in Figures 4.3A. The variance in λ_{\max} when all LWS cones are compared together was greater than could be explained by a change in chromophore ratio. However, the variance within each age class (Figs. 4.3B, D, and E), and within the TH treated alevin group (Fig. 4.3C) were not greater than could be explained by a change in A_1/A_2 ratio. In all cases except the ocean smolts, the spectral spread of the LWS cone λ_{\max} values was less than that observed for the MWS cones.

Plotting λ_{\max} of LWS against MWS cone OS's for both members of individual double cones (Fig. 4.4) provided further evidence that more than one opsin subtype was being expressed in these photoreceptor classes. By placing lines at the upper and lower limits of the horizontal and vertical scatter in this data set (Fig. 4.4), and allowing for a ± 3 nm measurement error, it was possible to estimate the λ_{\max} when one of the opsin subtypes was combined with A_1 and when the other was combined with A_2 (lower and upper extremes respectively). For MWS cones (horizontal axis, Fig. 4.4), the short end of the distribution ended at approximately 490 nm suggesting that one of the opsins when combined with A_1 had an observed λ_{\max} at 490₁. The upper limit provided an estimate of yet another opsin when combined with A_2 , which had an observed λ_{\max} of approximately 548₂.

There are several published models that predict the spectral shift in λ_{\max} that results from exchanging A_1 and A_2 in the same opsin (see Table 3.2 in Chapter 3 and Temple *et al.*, In Prep). Inserting my observed MWS cone λ_{\max} values obtained from Figure 4.4 into these models provided estimates of predicted λ_{\max} values for the A_1

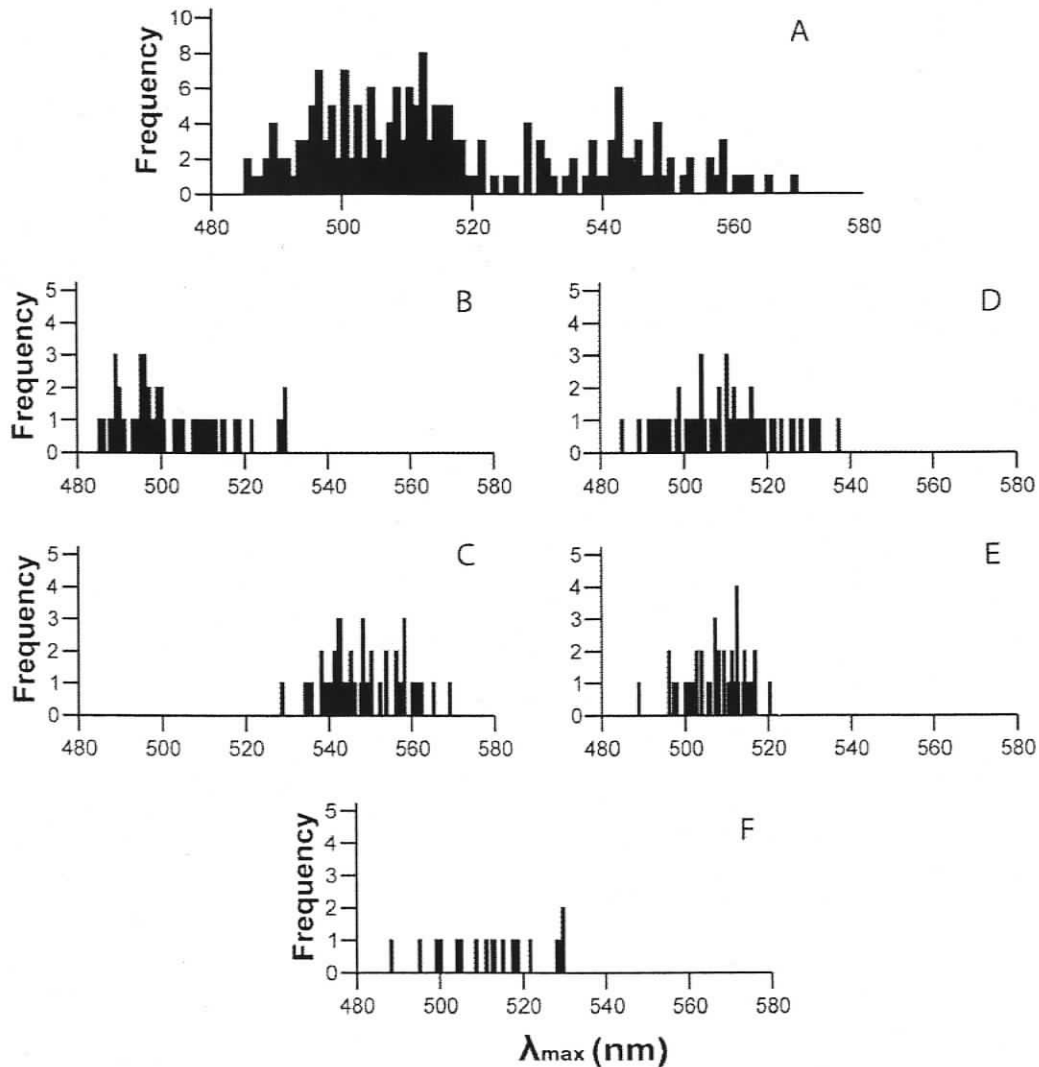


Figure 4.2. Frequency histograms of λ_{\max} values of individual MWS cones from coho salmon (*Oncorhynchus kisutch*, Walbaum): (A) all age classes combined, (B) alevins, (C) TH treated alevins, (D) parr, (E) ocean going smolts, and (F) an individual alevin. Bin size = 1 nm. The λ_{\max} values were obtained by MSP of individual MWS cones from the dorsal retina of coho salmon obtained from Target Marine salmon farms in April-May, 2005. There are progressively fewer MWS cones with λ_{\max} values below 500 nm as the coho increase in size from alevin (B) to parr (D) and ocean going smolt (E). Treatment with exogenous TH (C) resulted in a significant increase in the λ_{\max} of MWS cones, mostly as a result of a conversion from predominantly A_1 to A_2 based chromophores

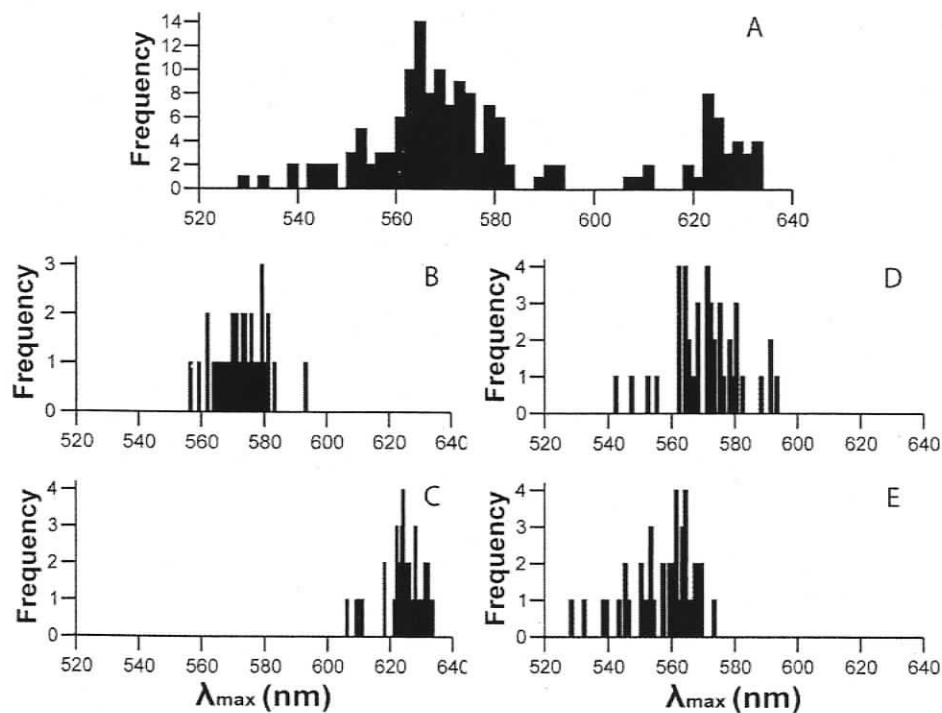


Figure 4.3. Frequency histograms of λ_{\max} values of individual LWS cones from coho salmon (*Oncorhynchus kisutch*, Walbaum): (A) all age classes combined, (B) alevins, (C) TH treated alevins, (D) parr, and (E) ocean going smolts. Bin size = 2 nm. The λ_{\max} values were obtained by MSP of individual LWS cones from the dorsal retina of coho salmon obtained from Target Marine salmon farms in April-May, 2005. Treatment with exogenous TH resulted in a significant increase in the λ_{\max} of LWS cones, mostly as a result of a conversion from predominantly A₁ to A₂ based chromophores

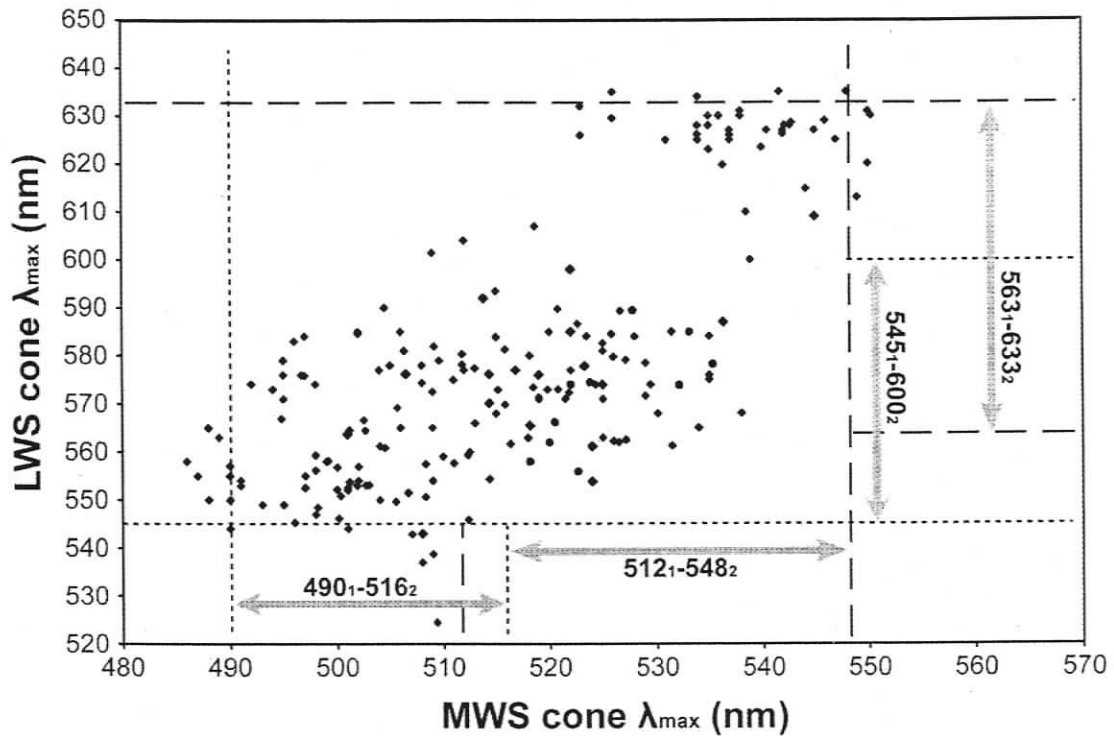


Figure 4.4. Scatter plot showing λ_{\max} values for MWS and LWS outer segments of individual double cones measured in coho salmon (*Oncorhynchus kisutch*, Walbaum). Vertical dotted lines predict the range of λ_{\max} values for the MWS cones at the short wavelength range of the data set. Using the lower value of 490₁ nm as the A₁ observed value I used the Whitmore and Bowmaker (1989) model to calculate that the same opsin would have a λ_{\max} of 516₂ nm if combined with an A₂ chromophore. Vertical dashed lines indicate the range for the longer of the two opsins, with a pigment pair that would have a range of 512₁ to 548₂ nm. The horizontal dotted lines predict the range for the shorter of the two proposed LWS cone opsins, having a pigment pair that extends from 545₁ to 600₂ nm based on Harosi's (1994) model. Likewise, the horizontal dashed lines indicate the range for the longer of the two proposed LWS opsins, with a pigment pair that has a range of 563₁ to 633₂ nm. All data points fit between these vertical and horizontal limits within the measurement error of the MSP device (± 3 nm), except those in the lower range of the LWS data set (see text).

and A₂ counterparts for each of these opsins. In order to reduce the probability of suggesting that there was a second opsin when in fact there was not (type I error), I chose the most conservative model, *i.e.* one that predicted the largest shift in λ_{\max} . For the range of values encompassed by the MWS cones, the most conservative model (Whitmore & Bowmaker, 1989) predicted that the 490₁ VP would be paired with a 516₂ and the 548₂ paired with a 512₁ VP (Fig 4.4).

Performing the same analysis on the observed LWS cone λ_{\max} values required that I invoke the hypothesis that the LWS cones expressed a second opsin as well. From figure 4.4, the two LWS VPs and their A₁/A₂ pigment pairs were estimated to have λ_{\max} values of 545₁ – 600₂ nm and 563₁ – 633₂ nm.

Further evidence to support the hypothesis that more than one opsin subtype was being expressed in MWS cones was provided by the fit of the absorbance curves to A₁ and A₂ based templates. I found that absorbance curves of MWS cones with λ_{\max} values ranging anywhere from 490 to 512 nm were often better fit by the A₁ template (Figs. 4.5A, B and C), while MWS cones with λ_{\max} values ranging from 518 to 550 nm were often better fit by the A₂ template (Figs. 4.5D, E, and F). If there were only one opsin present in the MWS cones then only the A₁ template should have fit the absorbance curves with λ_{\max} values at the shorter end of the range and conversely only the A₂ template should have fit at the longer wavelength end of the MWS cone λ_{\max} distribution. The good fit of the A₁ template for MWS cones ranging from 490 – 512 nm and the A₂ template for MWS cones ranging from 518 – 550 nm suggests that both the opsin and the chromophore were changing.

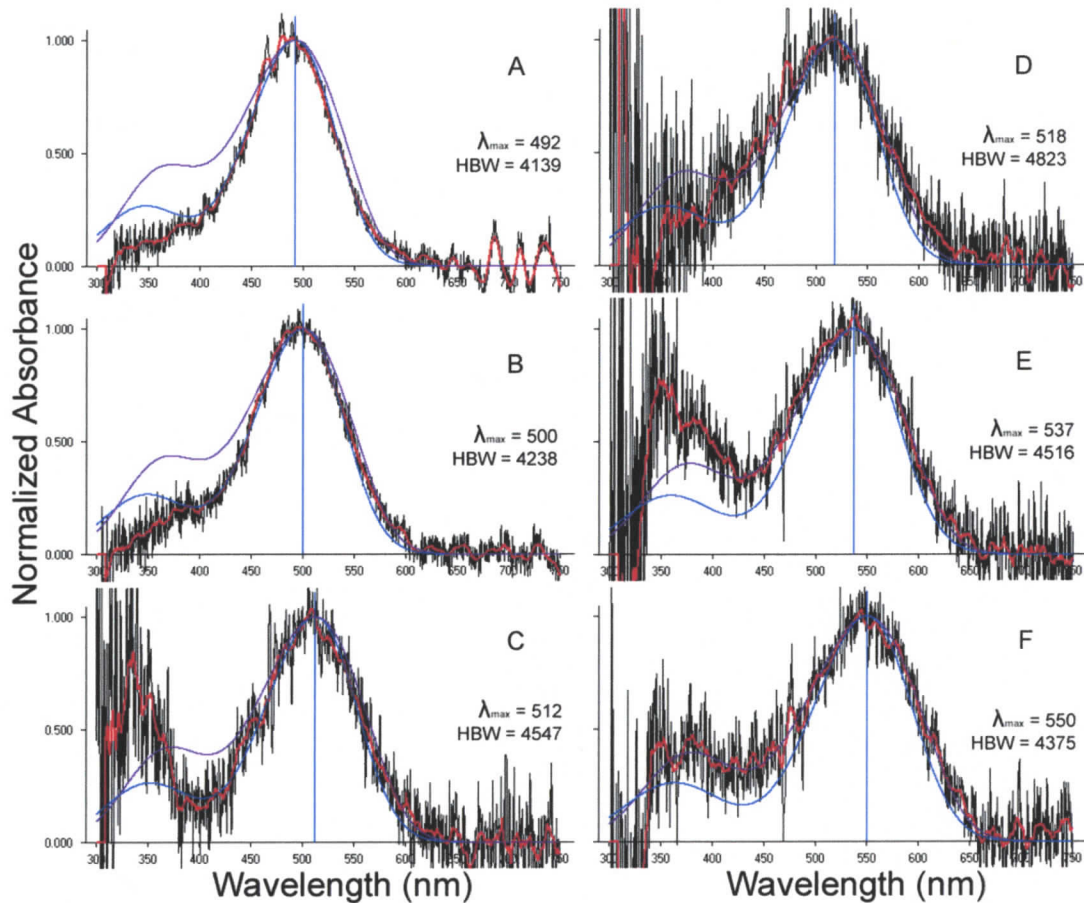


Figure 4.5. Representative absorbance spectra collected using microspectrophotometry of individual MWS cones from coho salmon (*Oncorhynchus kisutch*, Walbaum). The linearly detrended and normalized raw data are represented by the noisy black lines, the red lines through the centre of the noisy black lines are the nine point smoothing functions. The thin blue and purple lines are the A₁ and A₂ templates for the specified wavelength of maximum absorbance (λ_{max}). The thin blue vertical lines extend downward directly from the λ_{max} to facilitate reading the scale on the abscissa. The absorbance spectra of the MWS cones represented in A, B and C were best fit by A₁ templates, while those in D, E, and F were best fit by the A₂ template.

Summary of results

In summary, these results show that: (i) the A_1/A_2 ratio was not significantly different among the three age classes regardless of habitat; (ii) the distribution of λ_{\max} values for MWS and LWS cones differed among the age classes; (iii) the spectral range of λ_{\max} values for both MWS and LWS cones extended over a much greater range than previously reported; and (iv) the most likely explanation for the range in λ_{\max} of the MWS and LWS cones was the expression of more than one opsin in these cone classes.

Discussion

Rods

The λ_{\max} of rods in this study varied from 503₁ – 533₂ nm, a range that is consistent with a shift in A_1/A_2 ratio combined with the expression of a single opsin. The predicted λ_{\max} value for an A_1 -based VP with a λ_{\max} of 503₁ nm when combined with A_2 is between 521.9₂ – 534.1₂ depending on which model is used (Bridges, 1965; Dartnall & Lythgoe, 1965; Harosi, 1994; Parry & Bowmaker, 2000; Tsin *et al.*, 1981; Whitmore & Bowmaker, 1989). To date, only one RH1 (rod) opsin has been found in coho and other pacific salmonids (Dann *et al.*, 2004), and most teleosts for that matter (reviewed in Yokoyama, 2000). Therefore, my data strongly supports the contention that the variation in coho rod λ_{\max} was the result of a shift in A_1/A_2 ratio.

The similarity in rod λ_{\max} among all three age classes (fresh water alevin, parr, and ocean going smolts) measured at the same time of year supports the seasonal hypothesis for the shift in chromophore ratio. Numerous studies have demonstrated that the A_1/A_2 ratio can be measured by changes in λ_{\max} (Allen, 1971; Beatty, 1975a; Bridges, 1964a; Dartnall *et al.*, 1961; Harosi, 1975; Parry & Bowmaker, 2000; Tsin *et al.*, 1981; Whitmore & Bowmaker, 1989), but it must be added that this assumes that only one opsin is expressed in the photoreceptors of interest. For coho rods this appears to be true, and I therefore concluded that all three age classes had similar A_1/A_2 ratios in their rods.

MWS and LWS cones

The range of λ_{\max} values observed for both MWS and LWS cones was greater than could be explained by a shift in chromophore ratio, according to published models (Table 3.2, Chapter 3). As an explanation for this observation, I proposed that more than one opsin subtype was being expressed in both the MWS and LWS cone classes (Fig 4.4). To test this hypothesis, I plotted the λ_{\max} values of MWS OS's against LWS OS's from individual double cones (Fig. 4.4). The upper and lower limits for the MWS and LWS cones from this plot were used to calculate the estimated λ_{\max} of the possible opsins when combined with A_1 - and A_2 -based chromophores. This plot was effective because it was expected that both OS members of a double cone should have similar A_1/A_2 ratios. While the regulation of the A_1/A_2 ratio in the retina is poorly understood, both proposed sources of 11-cis chromophore for VP regeneration (RPE and Müller cells, Bridges & Yoshikami, 1970b; Mata *et al.*,

2002) would be expected to provide neighbouring OS's with similar A_1/A_2 ratios. Furthermore, differences in A_1/A_2 ratio are not expected between adjacent OS's, because vertebrate photoreceptors and their opsins do not differentiate among chromophore isomers (Chen & Liu, 1996; Makino *et al.*, 1990; Parry & Bowmaker, 2000).

Additional support for the hypothesis that more than one opsin was being expressed in the MWS cones came from the discovery of a second putative RH2 opsin subtype in coho salmon. In a previous study (Chapter 3 and Temple *et al.*, In Prep), a novel RH2 opsin subtype was isolated, cloned and sequenced. Based on amino acid sequence, the RH2B opsin subtype was predicted to be a functional opsin. The RH2B possessed an E123Q substitution, which would be expected to shift the λ_{\max} to shorter wavelengths relative to the previously identified RH2A (Dann *et al.*, 2004). I have not yet verified the existence of a second LWS opsin subtype in this species, but recent evidence suggests the potential for all fishes to possess a second LWS opsin subtype.

Many fish have been found to possess more than one copy of the RH2 and LWS opsin classes, which is compatible with recent models about the evolution of opsins in fishes. Early vertebrates are believed to have possessed copies of all five vertebrate opsin classes (Collin & Trezise, 2004). By combining this, with the proposal that there was a complete genome duplication event early in the phylogeny of fishes (Taylor *et al.*, 2003), it is reasonable to infer that all fishes have the potential to possess at least two copies of all five opsin classes. Certainly, several fish species that have been studied in detail have been found to possess and express two or more

copies of the RH2 opsin gene (Hisatomi *et al.*, 1994; Johnson *et al.*, 1993; Mader & Cameron, 2004; Minamoto & Shimizu, 2005; Neafsey & Hartl, 2005; Shand *et al.*, 2002). There are two SWS2 opsin subtypes expressed in some cichlids (Carleton & Kocher, 2001), and zebrafish express four RH2 and two LWS opsin subtypes (Chinen *et al.*, 2003).

Significance of visual pigment changes

Changes in A₁/A₂ ratio and opsin expression appear to occur on different temporal scales in coho salmon (unpublished results showed that opsin expression levels of RH2A and RH2B, measured using real time quantitative RT-PCR, differed among the alevin, parr and TH treated alevin). I have demonstrated that coho salmon at various life history stages shift their A₁/A₂ ratio in correlation with changes in season (Chapter 2, Temple *et al.* 2006) a finding that was corroborated in this study. The proposed shift in opsin expression for the MWS and LWS cones occurs sometime between the alevin and ocean smolt stages. Therefore, it is possible that the changes in opsin expression occur at the time of smoltification prior to seaward migration. Most, if not all, species in the genus *Oncorhynchus* lose most of their UV cone population at the time of smoltification, and these changes can be induced with the application of exogenous TH (Allison *et al.*, 2003; Browman & Hawryshyn, 1992; Hawryshyn *et al.*, 1989). In several other fish species, changes in opsin expression have been proposed to account for ontogenetic changes in VP λ_{\max} for example in: (i) eels (*Anguilla spp.*) by Beatty (1975b), Carlisle and Denton (1959), Wood and Partidge (1993), and Wood *et al.* (1992); (ii) cardinal fish (*Apogon*

brachygrammus, Jenkins) by Munz and McFarland (1973); (iii) pollock (*Pollachius pollachius*, L), goatfish (*Upeneus tragula*, Richardson), and black bream (*Acanthopagrus butcheri*, Munro) by Shand and others (Shand, 1993; Shand *et al.*, 2002; Shand *et al.*, 1988); and (iv) yellowfin tuna (*Thunnus albacares*, Bonnaterre) by Loew *et al.* (2002). Therefore, opsin shifting within a photoreceptor class is not a novelty as was recently proposed by Cheng and Novales Flamarique (2004). If the changes in MWS and LWS opsin expression are linked to smoltification in coho, then the two modes of shifting spectral sensitivity in coho salmon (seasonal A₁/A₂ shift and ontogenetic change in opsin expression) might fit a recent model in which changes in VPs are classified as either reversible (responding to habitat changes on a daily, seasonal, or migratory cycle) or irreversible (shifting with metamorphosis or ontogeny) (Evans, 2004).

The dynamic and additive nature of A₁/A₂ shifts and changes in opsin expression provide coho salmon with extremely flexible spectral tuning mechanisms. The two mechanisms together may allow for a shift of approximately 60 nm in the MWS cones and nearly 90 nm in the LWS cones. This flexibility might permit precise spectral tuning to the highly variable photic environments which salmonids inhabit (Novales-Flamarique & Hawryshyn, 1993; Novales-Flamarique *et al.*, 1992) while maintaining some optimum signal to noise ratio in the face of temperature variation. Based on these findings, coho possess one of the most naturally flexible vertebrate VP systems discovered to date.

The short wavelength shift in LWS cone λ_{\max} observed between the fresh water and oceanic life history stages matches the blue shift in photic environment

when coho migrate from fresh water to sea. The spectral distribution of light in freshwater environments is typically richer in long wavelength light than the open ocean (Jerlov, 1976; Lythgoe & Partridge, 1989; Tyler & Smith, 1970). Therefore, the proposed change in opsin expression that would shift LWS cones to a shorter λ_{\max} may fit the hypothesis that double cones match the background photic environment in fishes (Levine & MacNichol, 1979; Loew & Lythgoe, 1978; Lythgoe, 1984).

The adaptive significance of the ontogenetic long wavelength shift in MWS cone λ_{\max} is not as clear. The observed shift in MWS cones was attributed to a decrease in variance with a reduction in the number of cones that had λ_{\max} values below 500 nm (Fig. 4.2) in the oceanic juveniles. One possibility is that the MWS cone is acting as an offset detector for horizontal light and a matched receptor for downwelling light once the fish reach the ocean. This would require a λ_{\max} at slightly longer wavelengths (see description of matched and offset pigments in Munz & McFarland, 1975).

A similar shift in coho MWS cone λ_{\max} was observed by Novales Flamarique (2005), however his hypothesis of an A_1/A_2 shift in a single cone class was erroneous. Novales Flamarique (2005) recorded a shift from 490 nm to 553 nm in the MWS cones of coho salmon, which he claimed was due to a change in chromophore ratio, and was “compensatory” for the loss of UV cones during smoltification. Apart from not explaining how shifting the MWS cone compensates for the loss of UV cones, there are two concerns with the conclusion drawn by Novales Flamarique (2005). First, there is no published model that predicts a shift in λ_{\max} of over 60 nm due to a change of chromophore ratio for a VP absorbing maximally around 490 nm.

Thus at least one other opsin would need to be expressed in MWS cones to explain his observations. Secondly, his conclusion implies that the MWS and LWS members of the double cones would have different A_1/A_2 ratios. To date, there is no evidence to suggest that the two outer segment members of a double cone can differentially uptake A_1 or A_2 chromophores. In fact, most studies have demonstrated that neighbouring receptors use a common pool of chromophore (Bridges, 1973; Loew & Dartnall, 1976; Reuter *et al.*, 1971). Furthermore, photoreceptor OS's, and the opsins within, non-selectively accept several different forms of chromophore including 9-cis and 6-cis isomers (Chen & Liu, 1996; Makino *et al.*, 1990; Parry & Bowmaker, 2000). Given these observations, it is probable that the shift in λ_{\max} observed by Novales Flamarique (2005), was in fact due to a change in MWS opsin expression, the ontogenetic timing of which matched that observed here.

To conclude, the present findings further support the seasonal hypothesis for explaining the timing of the A_1/A_2 shifting and increase the evidence for the expression of more than one opsin subtype in the MWS and LWS cones in coho salmon. My research has demonstrated that coho possess an extremely variable VP spectral tuning mechanism that can be attributed to changes in A_1/A_2 ratio combined, most likely, with changes in opsin expression. Furthermore, I suggest that this potential flexibility in VP λ_{\max} is probably more common than previously thought and that considerable effort will be required to elucidate the functional significance of this plasticity in the visual system.

Chapter 5 Visual pigment composition in zebrafish (*Danio rerio*): Evidence for an A₁ – A₂ interchange system

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Introduction

Zebrafish have become an important model for visual neuroscience (reviewed in Bilotta & Saszik, 2001; Goldsmith & Harris, 2003; Li, 2001; Taylor *et al.*, 2000). Knowledge of zebrafish photoreceptor spectral absorbance properties have important implications for methodologies that employ spectral sensitivity measures or spectral stimuli to elicit innate behaviours for the purposes of screening and evaluating the visual system of mutant fish (Bilotta *et al.*, 2001; Krauss & Neumeyer, 2003; Neuhauss, 2003; Neuhauss *et al.*, 1999; Neumeyer, 2003; Van Epps *et al.*, 2001). A primary determinant of visual sensitivity is variation in the opsin amino acid sequence, but in some vertebrates an additional determinant is the vitamin A₁-A₂ VP interchange system.

Most marine and terrestrial vertebrates possess a vitamin A₁-based VP composition, whereas many freshwater and euryhaline fishes, amphibians and some reptiles possess an A₁-A₂ VP pair system (*e.g.* Loew, 1995). In paired pigment fishes and amphibians, the retinal and pineal photoreceptors generally contain mixtures of the A₁-A₂ based VPs, and the ratio of the two can change based on environmental variables such as light regime, temperature, or hormone manipulations (reviewed in Beatty, 1984; Bridges, 1972; Levine & MacNichol, 1979; Loew, 1995). Changes in the A₁-A₂ VP composition affect the spectral properties of photoreceptors: A₂-based VPs have λ_{\max} values at longer wavelengths than A₁-based VPs, for a given opsin protein, (Bridges, 1965; Harosi, 1994; Koskelainen *et al.*, 2000; Loew & Dartnall, 1976; Parry & Bowmaker, 2000; Whitmore & Bowmaker, 1989).

Zebrafish rod and cone opsin proteins can be classified based on amino acid similarity and, like those of many other teleosts (Allison *et al.*, 2003; Carleton & Kocher, 2001; Dann *et al.*, 2004; Hisatomi *et al.*, 1997), represent examples of all five vertebrate opsin classes (Raymond *et al.*, 1993; Raymond *et al.*, 1996; Vihtelic *et al.*, 1999). All copies of rod and cone opsins have been isolated from the zebrafish genome, and the λ_{\max} values of each have been estimated by *in vitro* expression (Chinen *et al.*, 2003). I adopt the nomenclature of Hunt *et al.* (2001a) for cone classes, *i.e.* ultraviolet-, short-, medium- and long-wavelength sensitive (UVS, SWS, MWS, and LWS) cones express SWS1, SWS2, RH2, and LWS opsin genes respectively, and rods express rod opsin RH1.

Over the past four decades, a variety of studies have concluded that zebrafish and their congener, the giant danio (*D. aequipinnatus*, McClelland); possess solely A₁-based VPs (Cameron, 2002; Chinen *et al.*, 2003; Levine & MacNichol, 1979; Nawrocki, 1985; Palacios *et al.*, 1996; Schwanzara, 1967). These results come from a diverse assortment of methods, including pigment extractions, partial bleaching, and high performance liquid chromatography (HPLC). Furthermore, the λ_{\max} of *in vitro* expressed opsin proteins reconstituted with A₁-based chromophore match the λ_{\max} observed in suction electrode recordings and MSP performed on isolated photoreceptors (Allen, 1971; Bridges, 1972; Kusmic & Gualtieri, 2000; Schwanzara, 1967; Tsin & Beatty, 1978). However, many other closely related cyprinids have been found to possess an A₁-A₂ interchange system (Levine and MacNichol, 1979; Palacios *et al.*, 1996; Chinen *et al.*, 2003). Because there are a variety of underlying factors driving the A₁-A₂ interchange system, there could conceivably be problems

related to identifying this attribute for a particular species (for examples from cyprinids, see Allen, 1971; Tsin & Beatty, 1978; Tsin *et al.*, 1981). A recent study by Saszik & Bilotta, (1999) suggested that zebrafish might possess A₂-based pigments in situations where the holding temperature is varied.

In order to determine if zebrafish possess an A₁-A₂ interchange system, I used TH treatment, known to produce a shift to A₂ VP dominance in the photoreceptors of other fishes. I used MSP to measure the λ_{\max} and other spectral properties of each photoreceptor class. I observed a long wavelength shift in rod and cone photoreceptor λ_{\max} , matching properties predicted for A₂-based VPs. Therefore, my data suggest zebrafish possess a paired pigment system that allows them to shift between A₁ and A₂-based VPs.

Materials and Methods

Zebrafish were purchased from a local pet shop and maintained in non-circulating dechlorinated municipal water in 15 L plastic tanks. Experiments were carried out during September-October and again in November-December, 2002. A 12L:12D photoperiod was provided by standard fluorescent lights (colour temperature 3500°K). Fish were fed standard flake food and trout pellets. Ten fish were maintained in each of three different treatments: (i) cold (water temperature 15 ± 1°C), (ii) warm (water temperature 28 ± 1°C, normal holding temperature for zebrafish), and (iii) warm plus TH. TH treatment was administered by adding L-thyroxine (Sigma, St. Louis, MO), dissolved in 1.5 mL of 0.1 M NaOH, to a final concentration of 300 µg L⁻¹ L-thyroxine. Fish were transferred to fresh TH treated

water daily, and fish not receiving TH treatment received the vehicle only (1.5 mL of 0.1 M NaOH). Fish were maintained under these treatment conditions for three to four weeks prior to being sacrificed. To sample the retinae, fish were sacrificed by prolonged anaesthesia with 100 mg L⁻¹ Eugenol (ICN Biomedicals Inc., Irvine, CA.) until euthanized (~15 mins. exposure). Care of fish and all procedures were in accordance with and approved by the University of Victoria Animal Care Committee under the auspices of the Canadian Council for Animal Care and thus conformed to the principles regarding the care and use of animals adopted by the American Physiological Society and the Society for Neuroscience.

Microspectrophotometry

The spectral absorbance of individual photoreceptors was measured using MSP to determine the characteristics (λ_{\max} , maximum absorbance (A_{\max}), half-band width (HBW), specific density, A_1/A_2 ratio) of zebrafish photoreceptors in the experimental design outlined above. All MSP procedures described below were carried out at 17°C, in a dark room and under dim red illumination. Zebrafish were dark adapted for at least one hour, euthanized, and the right eyes were surgically removed and hemisected in MEM (Sigma). All dissection procedures were performed under infrared light. For consistency of sampling location, retinal tissue from the dorsal half of the eye was used. Pieces of retina from the dorsal retina were teased apart, placed on a 35 x 50 mm (No. 1) glass microscope cover slip and macerated. I used a CCD-MSP device that has been described in detail previously (Hawryshyn *et al.*, 2001), for the measurement of spectral absorbance. In brief, this device delivered

short duration flashes (500 msec) of full spectrum (300 - 800 nm, 150 W xenon light source – intensity regulated, Oriel), unpolarized light through the photoreceptor outer segment (beam size approx. $2 \times 3 \mu\text{m}$). The transmitted beam passed through a spectrometer (300 nm blazed grating, Acton Research Corporation, MA) onto a Peltier cooled (-45°C), back-illuminated CCD-detector (NTE / CCD-1340/400-EMB Princeton Instruments, Roper Scientific, Inc., Trenton, NJ). Photoreceptor absorbance ($\log_{10} T^{-1}$) was calculated by comparing the transmitted intensity through the photoreceptor (I_M , measurement intensity) to the transmitted intensity through an area clear of debris and in media adjacent to the photoreceptor (I_R , reference intensity) thus, $T = I_M/I_R$.

Real time imaging of the retinal sample under infrared illumination employed a CCD camera (Canadian Photonics Laboratory, Minnedosa, MB, Canada). A Pentium computer was used as a central control unit for the CCD-MSP system, data acquisition, on-line analysis, and data storage. Photoreceptor types were identified based on their distinct morphology, and secondarily confirmed using the spectral position of the λ_{max} of the main absorbance band (α -absorption band) and the difference spectra calculated subsequent to photoreceptor bleaching. Difference spectra were used to verify that the α -absorption band was indeed a photolabile pigment and were calculated by subtracting the bleached absorbance curve (full spectrum bleach for 2 - 5 seconds) from the initial absorbance curve.

Absorbance spectra were examined online and were retained for subsequent analysis based on strict compliance with the following criteria: (i) shape of the absorbance curve had the expected shape based on known templates (see below) and

the λ_{\max} was near the expected wavelength based on cone morphology; (ii) presence of a clear baseline on the long wavelength arm; (iii) absence or minimal presence of photobleaching *i.e.* absorption due to photoproduct; and (iv) baseline noise less than 15 - 20 percent of A_{\max} . These criteria rejected approximately ten percent of MSP recordings.

Determination of λ_{\max} from absorbance spectra was performed off-line. Absorbance data were subjected to linear detrending and normalized relative to A_{\max} . However, the main estimate of λ_{\max} was determined by a minimum variance fit to the upper 20 percent of the absorption spectrum based on the centre of the α -peak \pm 40 nm of the Govardovskii *et al.* (2000) template. Photoreceptor outer segment diameters were measured from several of the photoreceptors examined using Northern Eclipse 5.0 image analysis software (Empix Imaging Inc., Mississauga, ON, Canada). In the case of cones, measurements were completed on the portion of the outer segment that was immediately distal to the inner segment. I noted that there was no difference in outer segment diameter of photoreceptors from the different experimental treatments, and thus the diameter measurements were pooled.

For cone photoreceptors, I estimated λ_{\max} based on calculating a mean for all photoreceptors collected. Thus, for comparisons between treatments each cone was considered to be an independent observation. In each case, the mean λ_{\max} was calculated based on photoreceptors from multiple individuals. I used this approach for the comparison of cone photoreceptor λ_{\max} values between treatments because the number of cones collected per fish was highly variable. For rods, where I had more samples per individual, I was also able to compare treatments using individual fish as

sampling units. A comparison between treatments was made using a one-way ANOVA ($\alpha = 0.05$) followed by a Tukey's HSD post hoc test to determine which groups differed. To compare my results with previously reported values, I calculated mean estimates of λ_{\max} for each photoreceptor class (UVS = 360; SWS = 412; MWS = 480, LWS = 560, Rod = 502.4) based on the values reported in the literature (Cameron, 2002; Chinen *et al.*, 2003; Harosi, 1994; Nawrocki *et al.*, 1985; Robinson *et al.*, 1993). These mean values were used as the predicted A₁-based λ_{\max} values.

Substituting these predicted values of A₁-based λ_{\max} into Harosi's (1994) equation (1)

$$y = 27.914 - 2.3598x + 0.0505x^2. \quad (1)$$

where $x = A_1$ -based λ_{\max} in nm, and $y = A_2$ -based λ_{\max} in nm, I was able to predict λ_{\max} values for the A₂-based VPs based on the same opsins for each photoreceptor class. Harosi's equation was chosen as a representative model that describes the general trend of increased shift in λ_{\max} with increased wavelength, above approximately 400 nm (*e.g.* see also models proposed by Bridges, 1965; Dartnall & Lythgoe, 1965; Parry & Bowmaker, 2000; Tsin *et al.*, 1981; Whitmore & Bowmaker, 1989). The predicted and observed values for both the A₁- and A₂-based pigments were plotted for direct comparison.

As a second line of inquiry regarding the possible presence of A₂-based VPs, I compared the HBW of the absorbance spectra with that predicted from the A₁- (2) and A₂-based (3) HBW equations provided by Harosi (1994):

$$y = 10.17945 + 1.20985x - 0.0241x^2. \quad (2)$$

$$y = 20.74607 + 2.31334x - 0.0499x^2. \quad (3)$$

where y is the predicted HBW in 1000 cm^{-1} and x is the reciprocal λ_{max} in 1000 cm^{-1} . These equations predict a parabolic function when comparing A_1 - and A_2 -based pigments, with a greater difference in the width of the HBW for VPs with λ_{max} near 434 nm (approx. 23000 cm^{-1}) than for VPs with longer and shorter λ_{max} .

Results

Microspectrophotometry was used to examine the spectral absorption properties of zebrafish photoreceptors. The rods and four cone classes have been shown to be morphologically distinct. UVS and SWS cones are single cones that differ in size and axial position, whereas the MWS and LWS cones form the accessory and principle members of double cones, respectively (Cameron, 2002; Nawrocki *et al.*, 1985; Raymond *et al.*, 1993; Robinson *et al.*, 1993; Vihtelic *et al.*, 1999). Figure 5.1 shows the absorbance spectra of these photoreceptors (see Table 5.1 for descriptive statistics), which appear to be in good agreement with the current literature (Nawrocki *et al.*, 1985; Raymond *et al.*, 1993; Robinson *et al.*, 1993; Vihtelic *et al.*, 1999).

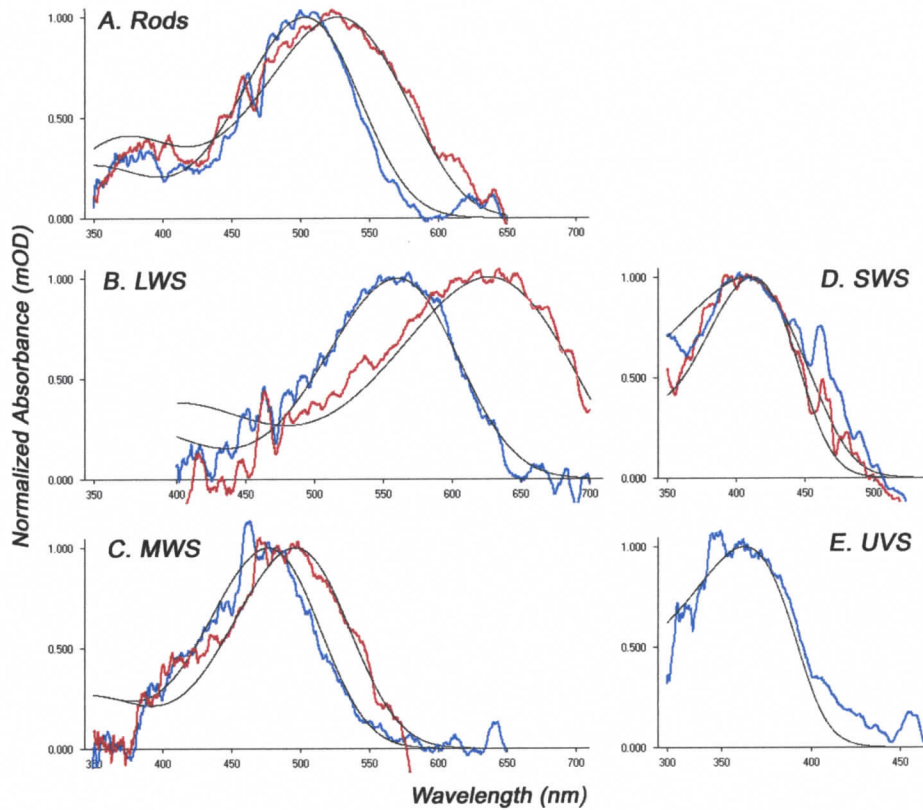


Figure 5.1. Representative absorbance spectra collected using microspectrophotometry of individual photoreceptors isolated from zebrafish. Normalized data from control and TH treated fish are represented by blue and red jagged lines, respectively, and the templates used to fit the data are represented by the smooth black lines. The data from control fish were similar to previous findings from zebrafish, whereas TH treatment resulted in photoreceptors utilizing A_2 -based pigments. The latter was determined by the absorption maxima being shifted to longer wavelengths, and broader half-band widths; the latter was especially apparent in the LWS cones and the rods, as would be expected in chromophore switching. A. rod photoreceptors; B. to E. represent long-, medium-, short-, and ultraviolet-wavelength sensitive (LWS, MWS, SWS, UVS) cones, respectively. In panel E only zebrafish not treated with TH are represented. Note that the scale of the abscissa changes between some panels.

Table 5.1 Spectral data for zebrafish (*Danio rerio*) photoreceptors examined in this study.

Photoreceptor type	Mean λ_{max} in nm \pm SD ¹ (N)	Mean HBW ² in cm ⁻¹ \pm SD (N)	Difference in HBW ³ (cm ⁻¹)	Mean $A_{\text{max}}\pm$ SD (N)	Mean OSD in $\mu\text{m}\pm$ SD (N)	Specific absorbance ⁴ (μm^{-1})
Rods						
Control (A ₁ -based)	503 \pm 5 (194)	4007 \pm 743 (106)	378	0.019 \pm 0.0006 (186)	2.4 \pm 0.3 (53)	0.0079
TH-treated (A ₂ -based)	527 \pm 8 (212)	4385 \pm 680 (212)		0.015 \pm 0.0007 (160)		0.0063
Cones						
LWS-double cones						
Control (A ₁ -based)	565 \pm 10 (14)	4109 \pm 258 (14)	55	0.019 \pm 0.0004 (14)	2.6 \pm 0.3 (16)	0.0073
TH-treated (A ₂ -based)	613 \pm 11 (20)	4164 \pm 492 (20)		0.014 \pm 0.0006 (15)		0.0054
MWS-double cones						
Control (A ₁ -based)	482 \pm 6 (9)	4291 \pm 128 (8)	261	0.015 \pm 0.0005 (9)	2.4 \pm 0.2 (12)	0.0063
TH-treated (A ₂ -based)	504 \pm 19 (3)	4552 \pm 198 (2)		0.014 \pm 0.0006 (3)		0.0058
SWS-single cones						
Control (A ₁ -based)	411 \pm 5 (31)	4478 \pm 665 (29)	967	0.019 \pm 0.0006 (31)	2.8 \pm 0.8 (8)	0.0070
TH-treated (A ₂ -based)	412 \pm 4 (4)	5445 \pm 616 (4)		0.014 \pm 0.0006 (4)		0.0052
UVS-single cones						
Control (A ₁ -based)	361 \pm 3 (6)	n/a ⁵		0.021 \pm 0.0005 (6)	3.9 \pm 0.3 (6)	0.0054

¹ Mean wavelength of maximum absorbance values (λ_{max}). N is sample size.

² Half-band widths.

³ Calculated by subtracting the A₁-based HBW from the A₂-based HBW (in cm⁻¹).

⁴ Calculated by taking ratio of mean A_{max} over mean outer segment diameter.

⁵ UVS cone HBW were not calculated due to the unreliability of the short wavelength arm of the absorbance curve, which goes below 330 nm. In this portion of the spectrum the quantal flux of the xenon light source is limited.

HBW, Half-band widths; A_{max} , maximum absorbance; OSD, Outer segment diameter; TH, thyroid hormone; LWS, MWS, SWS, & UVS are long-, medium-, short-, & ultraviolet-wavelength-sensitive cones, respectively.

Visual pigment content in rod photoreceptors

In order to examine the VP content of rod photoreceptors, I first assumed that each photoreceptor was a statistically independent entity. This is a conventional assumption for MSP analyses and is consistent with the relevant literature (Cameron, 2002; Chinen *et al.*, 2003; Levine & MacNichol, 1979; Nawrocki *et al.*, 1985; Palacios *et al.*, 1996; Schwanzara, 1967). The mean $\lambda_{\max} \pm 1$ SD values of rod photoreceptors of fish held in warm (28°C) and cold (15°C) treatments were 503 ± 5 nm (N = 106) and 504 ± 6 nm (N = 88), respectively, while zebrafish treated with TH in warm water (28°C) had a mean rod λ_{\max} at 527 ± 8 nm (N = 212). A one-way ANOVA comparing the three treatments found a significant effect ($p < 0.001$, $df = 2$) and a multiple comparison Tukey HSD post hoc test showed that the two temperature treatments did not differ ($p = 0.744$) from one another, but that both differed ($p < 0.001$) from the TH treated group.

I collected a large number of rod absorbance spectra from several individual fish (>18 rods/fish), which permitted me to use each fish as the sampling unit. This eliminates the issue of pseudoreplication, *i.e.* that multiple photoreceptors sampled from the same individual are not independent observations. Thus, in a second step of data analysis, I focused on the results of 12 fish that were examined over a four day period (4 fish at 28°C, 5 fish at 28°C + TH treated, and 3 fish held at 15°C). This allowed me to calculate a mean rod λ_{\max} for each fish, and to use each fish as a statistically independent entity (see Fig. 5.2). Fish held at 28°C (not TH-treated) had a mean $\lambda_{\max} \pm 1$ SD of 503.5 ± 1.14 nm (N = 4), those held at 15°C had a mean $\lambda_{\max} \pm 1$ SD of 504.0 ± 1.18 nm (N = 3), while the mean λ_{\max} of the TH treated fish (at 28°C)

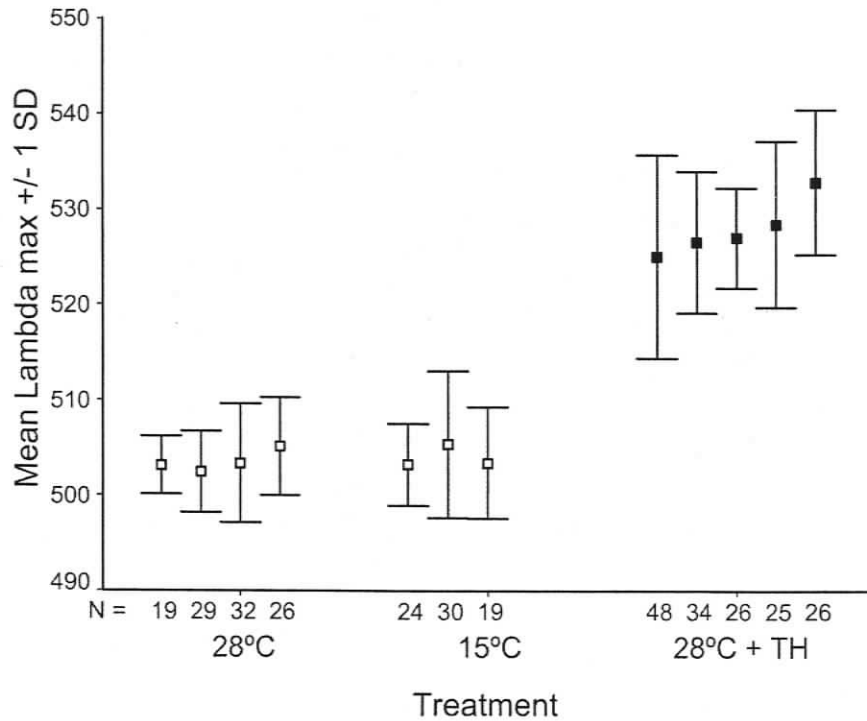


Figure 5.2. Mean wavelength of maximum absorbance values (λ_{max}) of the rod photoreceptors from 12 individual zebrafish as determined by microspectrophotometry. Zebrafish from the same cohort were maintained in one of three conditions: at 28°C, 15°C, or at 28°C and receiving TH treatment. Temperature did not significantly affect mean rod λ_{max} values, whereas TH treatment shifted the means to significantly longer wavelengths compared to untreated fish at 28°C ($p < 0.001$, $df = 2$). Means from untreated fish are represented by the empty symbols, and fish receiving TH treatment are represented by filled symbols. The number of rods measured per individual fish appears below the abscissa.

was 527.9 ± 2.9 nm ($N = 5$, Fig. 5.2). A one-way ANOVA showed that there was a significant effect ($p < 0.001$, $df = 2$) of treatment on the rod λ_{\max} values and the Tukey's post hoc test again showed that the temperature treatments did not differ ($p = 0.953$) while both warm and cold treatments differed ($p < 0.001$) from the warm TH treated treatment. Rod λ_{\max} values in TH treated fish matched the value I had predicted for a shift from an A_1 to an A_2 -based rod opsin pigment based on Harosi's (1994) formula solved using previously determined zebrafish A_1 -based rod opsin λ_{\max} values (Fig. 5.3).

Zebrafish rod absorbance spectra collected from fish held at 28°C (not TH treated) had a mean HBW similar to the values reported for giant danio rods, which were A_1 -based (Harosi, 1994) (Table 5.1). The mean HBW of rods from TH-treated zebrafish was larger than that of untreated zebrafish, which matches the shift predicted by Harosi's (1994) equations (2 and 3). The mean A_{\max} of rods from TH treated zebrafish (0.015 ± 0.0007) was lower than untreated zebrafish (0.019 ± 0.0006 ; Table 5.1), consistent with the lower molar extinction coefficient of A_2 -based VPs (Brown *et al.*, 1963). Measurements of rod outer segment diameter for both TH treated and control groups (Table 5.1) were similar to previously reported values (Connaughton & Dowling, 1998).

Visual pigment composition in cone photoreceptors

MSP on cone photoreceptors from fish that did not receive TH revealed λ_{\max} values that closely matched the average λ_{\max} from previous MSP studies (Fig. 5.3; Nawrocki *et al.*, 1985; Cameron, 2002). In particular, these results were comparable

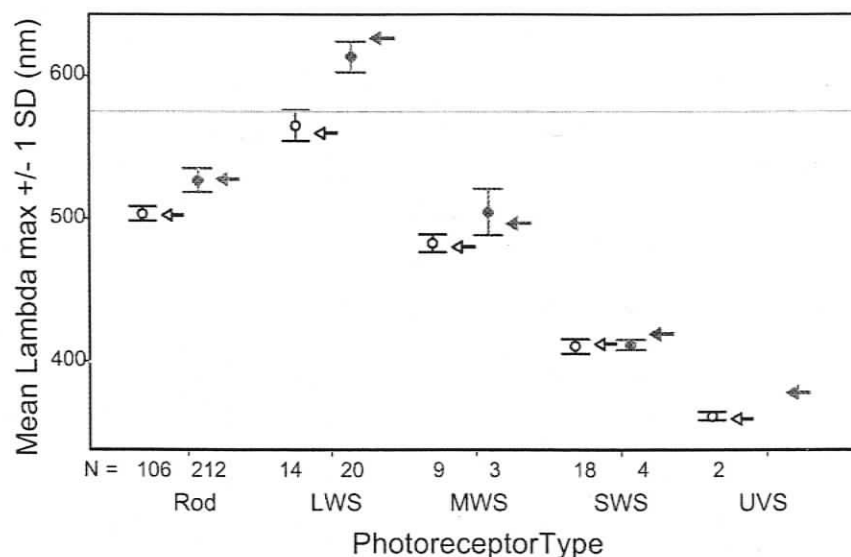


Figure 5.3. Mean wavelength of maximum absorbance values (λ_{max}) of photoreceptors measured from control and TH zebrafish maintained at 28°C. Data was grouped into five photoreceptor classes: rods and long-, medium, short, and ultraviolet-wavelength sensitive (LWS, MWS, SWS, UVS) cones. The λ_{max} values determined for photoreceptors from untreated zebrafish (empty circles) closely matched the mean of results from previous studies (indicated by empty arrows). Photoreceptors from TH treated zebrafish had mean λ_{max} (filled circles) that were shifted to longer wavelengths. These closely matched values one predicts when photoreceptors switch from an A₁- to A₂-based chromophore (filled arrows). The difference observed in this switch was greater at longer wavelengths, as expected. The difference was found to be significant for rods, LWS, and MWS cones. The shifts could not be explained by changes in opsin expression as all rod and cone opsins and their associated λ_{max} have been identified from zebrafish genome (Chinen *et al.*, 2003). Furthermore, the λ_{max} determined for LWS cones from TH-treated fish exceeded the largest possible value for an A₁-based pigment (thin horizontal line at 575 nm, see text), and thus it represents an A₂-based pigment. The data for UVS cones from TH treated fish did not meet my criteria for acceptance and thus are not presented. The sample size per treatment for each photoreceptor class appears below the abscissa.

to those from a recent study examining zebrafish opsins expressed *in vitro* and reconstituted with A₁-based chromophore (Chinen *et al.*, 2003). The LWS cone λ_{\max} I measured (565 ± 10 , N = 14) was similar to that reported by Cameron (2002) and longer than that reported by Nawrocki *et al.* (1985). The values I obtained for SWS cone λ_{\max} fell between those found by these authors.

The LWS cone photoreceptors from zebrafish treated with TH had a mean λ_{\max} value (613 ± 11 nm, N = 20) that was significantly higher ($p < 0.001$, $df = 32$, t-test) than observed in zebrafish not treated with TH (565 ± 10 nm, N = 14). The λ_{\max} of the MWS cones from TH treated fish were also shifted to significantly ($p = 0.005$, $df = 10$, t-test) longer wavelengths from a mean of 482 ± 6 nm (N = 9) for the control fish to a mean of 505 ± 16 nm (N = 3) for the TH treated fish (Table 5.1, Fig. 5.3). The small change in λ_{\max} that one expects when comparing A₁- and A₂-based VPs for the SWS cone (Bridges, 1965; Harosi, 1994; Parry & Bowmaker, 2000; Tsing *et al.*, 1981; Whitmore & Bowmaker, 1989) was confirmed in the present study with no difference evident between the control (411 ± 5 nm, N = 18) and the TH-treated fish (412 ± 4 nm, N = 4) (Table 5.1, Fig. 5.3).

Cone absorbance spectra collected from zebrafish at 28°C (TH untreated) had mean HBW values (Table 5.1) that were broader for cone classes with shorter λ_{\max} , as expected (Harosi, 1994; Hawryshyn *et al.*, 2001). The HBW of the cone spectra from TH-treated fish were broader than the HBW of untreated zebrafish for each cone class. This change was greater at shorter wavelengths (Table 5.1), as would have been expected if the TH-treated fish possessed A₂-based pigments (Harosi, 1994).

Differences in HBW values were predicted by taking the difference of values based

on equations 2 & 3. The difference in HBW I observed at the short and long wavelength spectral extremes examined in this study (55 and 967 cm^{-1} for LWS and SWS cones, respectively) closely matched the values predicted by Harosi's equations (25 and 1012 cm^{-1}). A_{max} was lower in cones from TH treated zebrafish (Table 5.1), as would have been predicted for A_2 -based pigments (Hawryshyn *et al.*, 2001) which have a molar extinction $\frac{3}{4}$ that of A_1 -based pigments (Brown *et al.*, 1963). The measurements of cone outer segment diameter (Table 5.1) were similar to previously reported values for cones (Connaughton & Dowling, 1998), however my measurements extend the previous data set to differentiate between UVS and SWS cones.

Discussion

Zebrafish have become an important model organism for many aspects of visual neuroscience, and knowledge of the potential variation in photoreceptor spectral sensitivity is required to permit effective experimental design and appropriate interpretation of results. In the present study, I provide data supporting the hypotheses that zebrafish have a paired pigment system and therefore can have a substantially broader range of spectral sensitivity than was previously believed. My MSP results on photoreceptors from fish that did not receive TH treatment showed λ_{max} values that closely match the average λ_{max} from previous MSP experiments (Fig. 5.3). However, even the small (statistically insignificant) differences observed here are of note because data such as this improve the ability to model spectral sensitivity (Cameron, 2002). The criteria for accepting spectra in my analysis were relatively strict and my

sample sizes, in most cases, were larger than previous reports. The reported differences between all studies including this one could be biologically relevant, particularly in the case of MWS and LWS cones where the expression of different opsin variants (Chinen *et al.*, 2003) could account for the observed differences.

Previous results for zebrafish photoreceptor λ_{\max} are known to represent A₁-based VPs based on partial bleaching experiments (Schwanzara, 1967; Nawrocki *et al.*, 1985), HBW comparisons (Nawrocki *et al.*, 1985; Harosi, 1994; Cameron, 2002), and HPLC analysis (Palacios *et al.*, 1996; Taylor *et al.*, 2000). My results from fish not receiving TH are consistent with these findings, as are experiments where zebrafish opsins were expressed *in vitro* and reconstituted with A₁-based chromophore (Chinen *et al.*, 2003).

Temperature

Temperature did not significantly affect the λ_{\max} of rods in this study: the fish maintained at low temperatures appeared to possess primarily A₁-based VPs (Fig. 5.2). An earlier experiment comparing fish maintained at 28°C and 19°C also showed that temperature did not significantly affect the proportion of A₁- and A₂-based VPs (*i.e.* the fish had predominantly A₁-based VPs) in rods ($p = 0.175$, $df = 284$). On the other hand, the same cohort of fish (receiving identical light regime, water, treatment vehicle and diet) showed evidence of A₂-based pigments when treated with TH. Previous studies have shown that giant danio raised at low temperatures also possess A₁-based VPs (Levine & MacNichol, 1979; Palacios *et al.*, 1996).

My study does not show any evidence of temperature effect on VP composition. The temperature treatments effectively enveloped the temperatures experienced by zebrafish in their natural environment, although other factors such as day length could have additive effects. It is noteworthy that the previous reports regarding temperature effects have focused on temperate species (see Beatty, 1984), and the tropical, thermally stable environment of zebrafish may result in temperature being an irrelevant cue for chromophore switching.

Thyroid Hormone

TH treatment resulted in significantly longer λ_{\max} values in the rods, MWS, and LWS cones (Fig. 5.2 & 5.3), consistent with zebrafish possessing a paired pigment system. The shifts seen in this study were consistent with those seen during TH treatment of another cyprinid, the goldfish (Tsin & Beatty, 1978). The degree of λ_{\max} shift is described by well-defined functions (Bridges, 1965; Dartnall & Lythgoe, 1965; Harosi, 1994; Parry & Bowmaker, 2000; Tsin *et al.*, 1981; Whitmore & Bowmaker, 1989); VPs with larger λ_{\max} produce a greater shift towards longer wavelengths. As expected from these functions, I saw little change in the λ_{\max} of SWS cones, an increase of 20 - 25 nm in the λ_{\max} of rods and MWS cones, and an increase of about 60 nm in the LWS cone. Further, the result for rods matched the classic 503_1 - 527_2 pigment pair of many vertebrates with labile VPs (Schwanzara, 1967; Levine & MacNichol, 1979; Beatty, 1984; Harosi, 1994). Thus the difference in λ_{\max} between control and TH treated fish matched that expected for a shift from A_1 - to A_2 -based chromophores.

The shifts in λ_{\max} I observed cannot be explained solely by changes in opsin expression, because all copies of the rod and cone opsins, along with their A₁-based λ_{\max} , have been identified from zebrafish genome (Chinen *et al.*, 2003). This is true for at least the rods and LWS cones, where the mean λ_{\max} values observed were 527 and 613 nm, respectively. These values were substantially longer than the longest λ_{\max} reported for these VPs reconstituted with A₁-based chromophore (501 and 558 nm, respectively) (Chinen *et al.*, 2003).

Other aspects of these results add strong support to the conclusion that the TH-treated zebrafish had A₂-based pigments in their rods and cones. Templates required to fit the absorbance spectra of TH-treated fish were broader, *i.e.* they had larger HBW as measured in wave-numbers (see Table 5.1), than those for untreated fish. The effect was most apparent for the SWS cone, where I observed little change in λ_{\max} , but a substantial broadening of the HBW. The differences in HBW between treatments were smaller in photoreceptor classes with longer λ_{\max} ; this was also consistent with the established relationship of HBW for A₁- and A₂-based pigments (see Table 5.1, Harosi, 1994). Furthermore, each photoreceptor class from TH-treated fish had lower A_{\max} values, consistent with lower molar extinction coefficient of A₂-based pigments (Brown *et al.*, 1963). Finally, I note that biophysical considerations indicate that the longest possible λ_{\max} for an A₁-based pigment, regardless of opsin sequence, is near 580 nm (Blatz & Liebman, 1973). Indeed, no A₁-based pigment has been observed with a λ_{\max} greater than approximately 570 nm. Reconstitution of *in vitro* expressed opsins and their mutated variants from a variety of vertebrates with A₁-based chromophore do not achieve λ_{\max} greater than approximately 565 nm

(Yokoyama & Radlwimmer, 2001). Therefore, even if one speculates that every copy of the LWS opsin has not been found in the zebrafish genome, which is unlikely (see Chinen *et al.*, 2003), my observation that the λ_{max} of LWS cones was 613 ± 11 nm ($n = 20$) allows me to confirm that the TH-treated fish possessed A₂-based pigments.

Conclusion

Despite their importance to visual ecology, the genes underlying the chromophore interchange system remain unknown. The shift from A₁- to A₂-based pigments, which entails the addition of a double bond to the terminal ring of the chromophore, is presumed to require an unidentified 3,4-dehydrogenase. Considering the expanding genetic tools available for zebrafish, mutational screens or analyses of differential gene expression could provide promising methodologies to discover this pathway.

My results demonstrate that A₂-based VPs can be present in zebrafish. This should be carefully considered when assessing their visual sensitivity. The presence of A₂-based pigments would be expected to have substantial effects on both scotopic and photopic functional measurements, broadening the absorption band, lowering the absolute sensitivity and shifting the spectral sensitivity to longer wavelengths (Allen *et al.*, 1973; Allen & Munz, 1983; Kennedy, 1957; Whitmore & Bowmaker, 1989). Thus my results could be relevant to mutational screens, toxicology screens, hormone treatments, and functional assessments of zebrafish retinal development and regeneration.

Chapter 6 : General Conclusions and Future Directions

A₁-A₂ shifting

Measurements of rod λ_{\max} , from different age classes over time, in three sets of experiments (Chapters 2, 3 and 4), support the hypothesis that A_1/A_2 ratio varies seasonally in coho, and demonstrates that these shifts are not exclusively linked to smoltification (migration/metamorphic hypothesis). This conclusion extends earlier findings by Beatty (1966), who observed that chromophore shifting was correlated with season in coho, but only during the fresh water phase. My findings contradict the existing paradigm that explains the timing of chromophore shifts in euryhaline fishes and amphibians, which I have called the migration/metamorphosis hypothesis. This hypothesis suggests that the shift in chromophore ratio is an adaptation that matches spectral sensitivity to changes in spectral environment resulting from migration and metamorphic events, and it has been supported by several observations (Beatty, 1966; Crescitelli, 1958, 1991; Munz & Beatty, 1965; Wald, 1939, 1941, 1960) including some more recent works (Alexander, 1998; Alexander *et al.*, 1994; Alexander *et al.*, 1998; Novales Flamarique, 2005). However, in Chapter 2, my results clearly show that shifts from A_2 to A_1 in rods were not restricted to the smoltification event that precedes migration. Rather, shifts in A_1/A_2 ratio occurred in fish of all ages and were correlated with seasonal changes in day length and temperature. In Chapter 3, I observed that exogenous TH, which stimulates smoltification-like events, caused increases in A_2 in rods and cones, not decreases as would be predicted if the chromophore shift were linked to smoltification. In Chapter 4, I recorded A_1/A_2 ratios that were not significantly different among three different age classes of coho salmon

(alevin, parr-smolts and ocean going smolts), all of which were sampled at the same time of year.

My findings in coho salmon contradicted most literature (Alexander *et al.*, 1994; Beatty, 1975a; Crescitelli, 1958; Munz & Beatty, 1965; Wald, 1939, 1941, 1957), which has concluded that the A_1/A_2 shift in salmonids is linked to migration/metamorphic events despite some of these studies having clear seasonal patterns. Beatty (1966) saw the same seasonal cycle I did, when he followed the A_1/A_2 ratio for two years in pacific salmonids. While he came to the conclusion that chromophore shifts were not connected to metamorphosis, he maintained that they were somehow linked to migration, based on his observations in returning spawners. Likewise, Alexander (1998) provided a full year data set in his thesis that extended the data set he had previously published (Alexander *et al.*, 1994), and which showed a clear seasonal pattern identical to mine. However, because he only followed a single age class, he had a different interpretation of the seasonal pattern that he observed. Alexander (1998) suggested that the smolts were reverting back to their freshwater (A_2) VP.

My observations support the interpretation that A_1/A_2 shifts are an adaptation to minimize thermally induced photoreceptor noise while maximizing long wavelength sensitivity for organisms confronted with seasonal temperature variations (Ala-Laurila *et al.*, 2003; Archer, 1999; Baylor *et al.*, 1980; Donner *et al.*, 1990; Fyhrquist, 1999; Whitmore & Bowmaker, 1989; Williams & Milby, 1968). Coho normally spend over a year in fresh water followed by several months in near-shore coastal waters; environments that are rich in long wavelength light (Jerlov, 1968;

Novales-Flamarique & Hawryshyn, 1993). Maximizing sensitivity above 570 nm requires that coho use A_2 -based VPs. However, A_2 -based VPs are less thermally stable (Ala-Laurila *et al.*, 2004a; Ala-Laurila *et al.*, 2004b; Baylor *et al.*, 1980; Donner *et al.*, 1990; Fyhrquist, 1999; Koskelainen *et al.*, 2000; Williams & Milby, 1968). As temperature rises during summer months, thermally induced noise may place a limit on the effectiveness of A_2 -based VPs, thus selecting for a shift to the more thermally stable but slightly blue shifted A_1 -based VPs.

The signal to noise ratio model can also be used to explain the correlation of chromophore shifts with day length. Water temperatures fluctuate on the scale of hours and days, due to the effects of sun angle during the day, cloud cover, weather patterns and changes in hydrology. It may not be energetically effective to be constantly changing the A_1/A_2 ratio to accommodate these higher frequency fluctuations. Instead, the A_1/A_2 ratio could be tuned to larger scale (monthly) temperature variations by being controlled by an endogenous circannual rhythm tuned to day length, which is the most widespread zeitgeber for biological seasonal patterns (Gwinner, 2003; Nisimura & Numata, 2001; Palmer, 1976). This would explain why in the absence of temperature variations, coho maintained a seasonal pattern at the Kispiox hatchery. However, this endogenous rhythm may still possess a temperature override (Allen & McFarland, 1973), which would allow for increases or decreases in A_1/A_2 ratio under extenuating circumstances, such as an unusually warm summer. Changes in A_1/A_2 ratio may therefore be an interesting feature to observe in relation to global climate change patterns.

Further clues as to the functional significance of the A_1/A_2 labile pigment pair system may come from investigations with species that are exceptions to the rule. I think that future research might benefit from looking at populations of coho salmon that migrate to sea at different times of year, such as a populations in Washington State that migrate seaward in the fall instead of the spring, or those at more northern latitudes that spend as much as nine years in fresh water before seaward migration. Likewise, American and European eels (*A. anguilla* and *A. rostrata*) are believed to shift A_1/A_2 ratio at the time of migration (Beatty, 1975b), but have never been examined for seasonal patterns in chromophore ratio. Both species of eel are catadromous and migrate from sea to fresh water in summer and return to the sea in late winter early spring (Froese & Pauly, 2005). We might also learn from species that move back and forth between fresh and salt water without regard to season, such as sticklebacks (*Gasterosteus* spp.) or ayu (*Plecoglossus altivelis*, Temminck & Schlegel).

Another aspect of chromophore shifting that needs to be explored is the existence of the proposed 3,4-dehydrogenase that converts A_1 to A_2 . My finding of a labile pigment pair system in zebrafish (Chapter 5 and Allison *et al.*, 2004) may assist in this endeavour since the zebrafish is a model species for which the entire genome will be sequenced in the near future (Sanger Institute, 2005). If the 3,4-dehydrogenase could be isolated and sequenced, we might be able to learn more about its function and the timing of A_1/A_2 shifting, as well as which species possess the protein and therefore the ability to synthesize the A_2 chromophore.

Shifts in opsin expression

The unexpected finding of a second RH2 opsin subtype in the MWS cones of coho salmon opened up new avenues of exploration for my research. However, the finding came late in my program and there are outstanding questions I would have liked to address. Firstly, it would be interesting to examine the spatial pattern of expression of the two RH2 opsin subtypes across the retina. *In situ* hybridization techniques could be used to elucidate any patterns and determine whether they change with ontogeny as was recently observed in zebrafish (Takechi & Kawamura, 2005). At present, there are no proposed explanations for the interesting expression pattern of concentric rings that has been observed for the four zebrafish RH2 opsins. I predict that this will be a whole new area of exploration for visual scientists; using comparative studies among teleosts possessing different life history strategies to shed light on the adaptive function of these temporally and spatially variable expression patterns.

The observation that the expression levels of the two RH2s varied with the application of exogenous TH could be used to test whether control of expression can be turned off and on in a reversible manner. The current hypothesis for explaining temporal changes in opsin expression is that the resultant shift in VP λ_{\max} and spectral sensitivity, matches changes in visual tasks and photic environment that occur over the life history of the organism (Archer, 1999; Carleton & Kocher, 2001; Loew *et al.*, 2002; Loew & Sillman, 1993; Shand, 1993; Shand *et al.*, 2002; Shand *et al.*, 1988; Wood *et al.*, 1992). The implication has been that these changes coincide with ontogenetic events and are unidirectional. However, as was observed with

populations of UV cones in rainbow trout (Allison *et al.*, 2003; Browman & Hawryshyn, 1992, 1994), it may be that the expression levels can be turned up or down reversibly. I speculate that these opsin subtypes may provide some organisms with a means to modify spectral sensitivity in a dynamic fashion.

Given the range of λ_{\max} values observed in the LWS cone class, I proposed in Chapters 3 and 4 that it is likely that a second opsin subtype exists for the LWS opsin class. This hypothesis remains to be confirmed by isolating and sequencing of the putative LWS opsin subtype. Recent work with zebrafish (Chinen *et al.*, 2003) has found four RH2 and two LWS opsin subtypes. These findings support the contention that there was a complete genome duplication event early in the evolution of fishes (Taylor *et al.*, 2003). Combined with the hypothesis that the vertebrate ancestor possessed a copy of all five retinal opsin classes (Collin & Trezise, 2004; Yokoyama & Yokoyama, 1996) this means that all fishes have the potential for at least two copies of all opsin classes in the absence of mutational decay of duplicated copies into pseudogenes (as in pufferfish, Neafsey & Hartl, 2005). It would be interesting to investigate how widespread opsin subtypes are among fishes. Certainly, they are widespread among Euteleosts, being present in at least three of five Superorders (Protacanthopterygii, Acanthopterygii, and Ostariophysii) (Parry *et al.*, 2005).

A new model for predicting the λ_{\max} associated with A_1 - A_2 shifting

In Chapters 3, 4 and 5, I used published models for predicting the shift in λ_{\max} of a VP when one chromophore (A_1 or A_2) was substituted for the other (Bridges, 1965; Dartnall & Lythgoe, 1965; Harosi, 1994; Parry & Bowmaker, 2000; Tsin *et al.*,

1981; Whitmore & Bowmaker, 1989). All of these models were created from observed ESP or MSP measurements of photoreceptors believed to have a predominance of A₁- or A₂-based VPs, *i.e.* under the assumption that only the chromophore differed, and that this difference alone was responsible for the change in λ_{\max} . This assumption was warranted under the paradigm that each receptor class expresses only a single opsin. However, this is not always the case, and changes in opsin expression both temporally (with ontogeny) and spatially (across the retina) may be wide spread among vertebrates. There is considerable variation among the six predictive models, some of which is undoubtedly due to the use of different measurement techniques (ESP and MSP) and the focus on different cell types (rods, then cones with λ_{\max} values over 400, and then all cone types). I propose that the variation among the three most recent models (that were all based on MSP and included all cone types) may be the result of variable opsin expression within the species used. Most of the species used to create these models were fish, and recent evidence suggests that the expression of multiple opsin subtypes is more common than previously thought.

Of the three most recent models, Parry and Bowmaker's (2000) may be the most accurate since they employed a reconstitution technique in which they exchanged A₁ for the native A₂ within the same photoreceptors in goldfish. This technique was least likely to be affected by a change in opsin expression and their model predicts the least difference between the λ_{\max} values of A₁- and A₂-based VPs using the same opsin (Fig. 3.8).

Here, I propose an experiment that has the potential to more accurately describe the relationship between the λ_{\max} values of VPs when they are combined with A_1 and A_2 . The procedure requires isolating, cloning and expressing a selection of opsins from the five vertebrate opsin classes, followed by spectrophotometric measurements of these opsins reconstituted with both A_1 and A_2 . A mathematical fit to a plot of $\Delta\lambda_{\max}$ against the $A_1 \lambda_{\max}$ would then hopefully provide a universal equation that could be used to predict the shift in λ_{\max} in any opsin resulting from a change in A_1/A_2 ratio. However, even this proposed model may not provide a single line of best fit. It is possible that the interaction of the two different chromophores with different opsins may not result in a predictable shift relative to the λ_{\max} of the A_1 -based VP. Unpredictability has been observed in the minimum energy for photoactivation (E_a) of different opsins combined with A_1 and A_2 , such that the Stiles-Lewis-Barlow relationship ($E_a = \text{constant} \times (1/\lambda_{\max})$) is not equally well fit when changes in both chromophore and opsin are incorporated into the same data set (Ala-Laurila *et al.*, 2004b). If there is unpredictability in the relationship of E_a when A_1 and A_2 are combined within a sample of opsins, then there would also be unpredictability in the resultant λ_{\max} values. A further cautionary note in regards to this approach is that the reconstituted VPs may not have the same λ_{\max} values as the native VP. This difference in λ_{\max} may occur because interactions between the opsin and the lipid membrane may differ and also because slight differences in protein folding might exist between *in vitro* reconstituted and native VPs.

In conclusion

The two mechanisms of spectral tuning studied in this thesis, A₁-A₂ shifting and changes in opsin expression, may act over very similar temporal and spatial scales and both should be carefully considered when measurements of photoreceptor λ_{\max} and spectral sensitivity of an organism are being made. There is considerable evidence to show that the A₁/A₂ labile pigment pair system offers species a rapid and reversible mechanism to adjust spectral sensitivity and possibly also the temperature dependent signal to noise ratio. The change in opsin expression has not yet been adequately studied to know if these changes are reversible. At present, the understanding is that changes in opsin expression are probably linked to metamorphic events and that these are less likely to be reversible (Archer, 1999; Evans, 2004). However, I would not presume that such a labile and reversible mechanism does not exist, as it would certainly offer species that do not have an A₁/A₂ labile pigment pair the opportunity to modify spectral sensitivity and signal to noise ratio in a dynamic manner.

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Appendix A

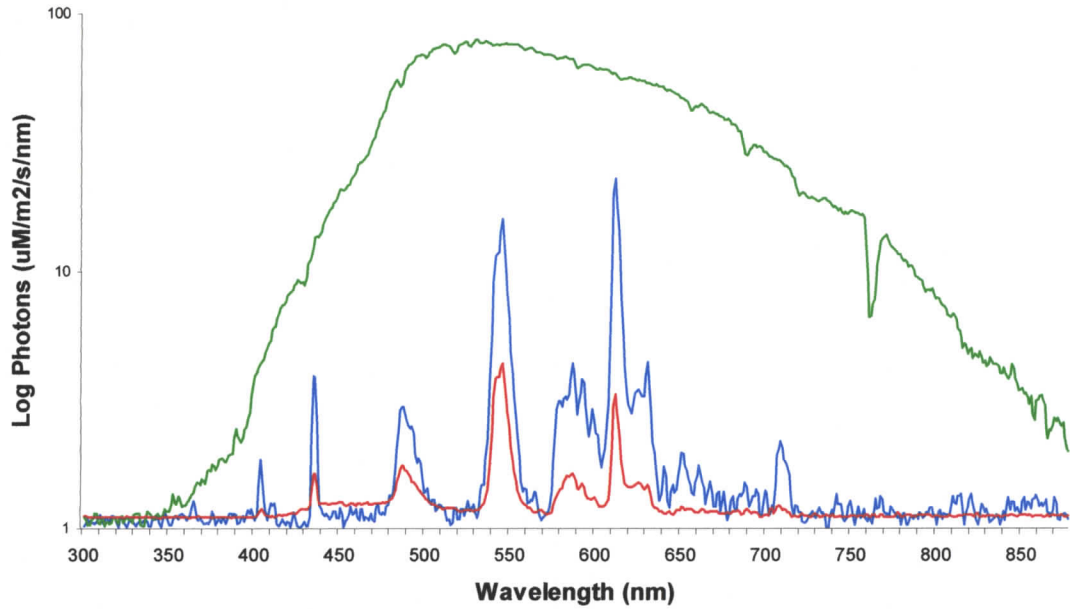


Figure A0.1. Spectral irradiance measurements at the waters surface of the three lighting conditions used in the thyroid hormone treatment experiments described in Chapters 3 and 4: full sunlight at midday under clear plastic lid of tank (green), compact fluorescent in fridge (blue) and fluorescent room lighting under translucent chloroplast tank cover (red). Measurements were made using an Ocean Optics USB2000 (Dunedin, FL, USA) spectroradiometer connected to a laptop computer.

Appendix B

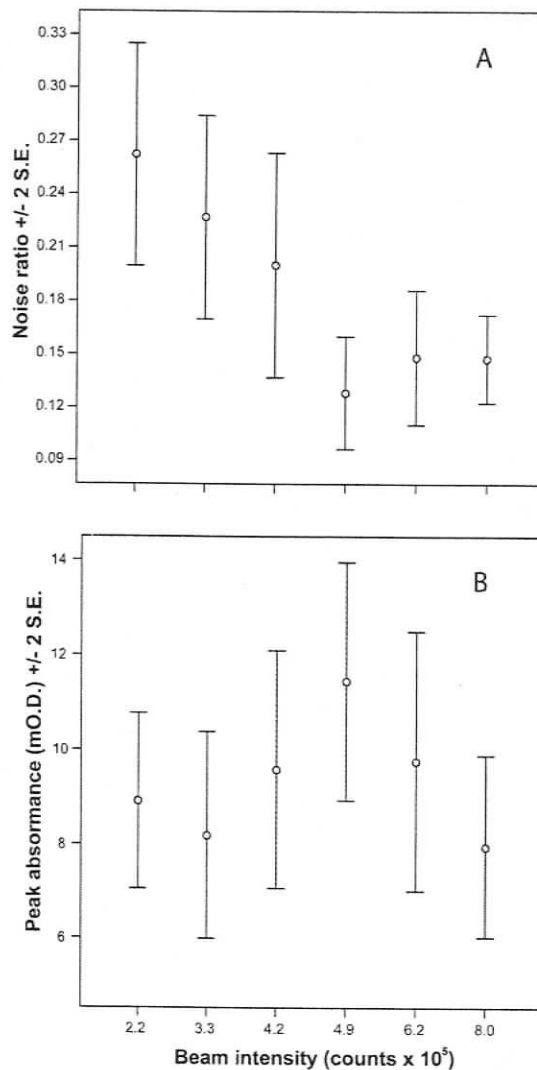


Figure B0.1. Peak absorbance (A_{max}) and noise ratio versus beam intensity measured as total counts over the duration of the beam flash (0.025 – 0.1 secs). The noise ratio (A) was calculated as the average variation in the raw data divided by the A_{max} . A_{max} (B) is measured from the baseline of the spectral absorbance curve to the peak of the main absorbance peak (peak of the α -band).

Appendix C

RH2B. ORF OkRH2A	ATGCAGAACGGCACTGAAGGAAACAACCTTCTACATCCCATGTCCAACAGGACTGGACTT 60 ATGCAGAACGGCAGAGGAAGCAACTTCTACATCCCATGTCCAACCGGACTGGGCTT 60 *****
RH2B. ORF OkRH2A	GTAAGAAGTCCCTTTGAATACCCCTCAGTACTACTTGGCGGATCCATGGCAATTCTATCTG 120 GTAAGGAGTCCCTTTCTATACCAACAATACTACTTGGCGGCTCCATGGCAATCTATTGT 120 *****
RH2B. ORF OkRH2A	CTTGCTGTCTACATGTTTTCTGATCTGTTTTGGATTCCCATCAACGGTCTGACCTTG 180 CTTGCTGTCTATACGTTTCTTCTGATCTGCTTTGGATTCCCATCAACGGTCTGACCTTG 180 *****
RH2B. ORF OkRH2A	TACGTCACAGCGACAACAGAAAGCTCCAGCAACCCCTCAACTTCATTCTGGCCAACCTG 240 TATGTCACAGCGACAACAGAAAGCTCCAGCAACCCCTCAACTTCATTCTGGTCAACTTG 240 *****
RH2B. ORF OkRH2A	GCTGCAGCTGGAATGATCATGTAATGTTTGGATTCCCATCAACATTACATCTGCTGTC 300 GCTGCAGCTGGAATGATCATGTAATGTTTGGATTCCCATCAACATTACATCTGCTGTC 300 *****
RH2B. ORF OkRH2A	AATGGCTACTTCGCTTTGGACCTATGGGCTGTGCCATCGAGGGTTTCATGGCTACACTT 360 AATGGTACTTCATCTGGGGACCATTTGGGCTGTGCCATCGAGGGTTTCATGGCTACACTT 360 *****
RH2B. ORF OkRH2A	GGAGGTCAGGTTGCTCTGTGGTCTCTGTGGTGTGGCCATTGAGAGATACATTGTGGTC 420 GGAGGTCAGGTTGCTCTGTGGTCTCTGTGGTGTGGCCATTGAGAGATACATTGTGGTC 420 *****
RH2B. ORF OkRH2A	TGCAAGCCATGGGCGAGCTTCACATTCACTACCAACCCAGCTGGTGGCGGATGTGCATTC 480 TGCAAGCCATGGGCGAGCTTCACATTCACTACCAACCCAGCTGGTGGCGGATGTGCATTC 480 *****
RH2B. ORF OkRH2A	ACCTGGGTATGGCAATGGCCTGTGCTGCCCCCATTTGGTTGGCTGGTCTAGATATATC 540 ACCTGGGTATGGCAATGGCCTGTGCTGCCCCCATTTGGTTGGCTGGTCTAGATATATC 540 *****
RH2B. ORF OkRH2A	CCAGAGGATGCACTGCTCATGTGGACCTGATTACTACACCTTGGCCGAAGGCTTCAAC 600 CCAGAGGATGCACTGCTCATGTGGACCTGATTACTACACCTTGGCCGAAGGCTTCAAC 600 *****
RH2B. ORF OkRH2A	AATGAATCATATGTGATCTACATGTTCACTGCCACTTCTGTGTTCTGTCTGCTCACCATC 660 AATGAATCATATGTGATCTACATGTTCACTGCCACTTCTGTGTTCTGTCTGCTCACCATC 660 *****
RH2B. ORF OkRH2A	TTCTTCACTACCGAAGCCTTGTCTTCCAGTCAAGGCGGCTGCAGCTTCCAGCAGGAC 720 GCTTACACTTATGGAAGTCTTGTCTTCCAGTCAAGGCGGCTGCAGCTTCCAGCAGGAC 720 *****
RH2B. ORF OkRH2A	TCAGCATCTACCCAGAAAGCTGAGAAGGAAGTGACAGTATGTGCTTCTGATGGTTGT 780 TCAGCATCTACCCAGAAAGCTGAGAAGGAAGTGACAGTATGTGCTTCTGATGGTTGT 780 *****
RH2B. ORF OkRH2A	GGTTTCTGATGCTTGGACCCCTATGCCAGCTTTGCTGCTGGATTTTCTTCAACAAA 840 GGTTTCTGATGCTTGGACCCCTATGCCAGCTTTGCTGCTGGATTTTCTTCAACAAA 840 *****
RH2B. ORF OkRH2A	GGAGTGCCTTTACAGCCACAGCCATGGCTATTCAGCCTTCTTCTCAAGAGCTCAGCC 900 GGAATTGCCTTACAGCCACAGCCATGGCTATTCAGCCTTCTTCTCAAGAGCTCAGCC 900 *****
RH2B. ORF OkRH2A	ATCTTCAACCCAGTCTATATGTGTTGATGAACAACAGTTCCGTAGCTGCATGTTGGCT 960 TTGTTCAACCCCATATCTATGTGTTGATGAACAACAGTTCCGTAGCTGCATGTTGGCT 960 *****
RH2B. ORF OkRH2A	GCTGTAGGAATTAGTTCAGGAGCTGAAGATGAGACCTCTGTTTCAGCAAGCAAGACAGAA 1020 GCTGTAGGAATTAGTTCAGGAGCTGAAGATGAGACCTCTGTTTCAGCAAGCAAGACAGAA 1017 *****
RH2B. ORF OkRH2A	GTGTCTCTGTGGGCCCTGCATAA 1044 GTGTCTCTGTGGGCCCTGCATAG 1041 *****

Figure C0.1. Alignment of RH2A and RH2B nucleotide sequences. Similarities are marked by an asterisk below the sequences which demonstrate 86.9 percent nucleotide identity compared to the 86.1 percent amino acid identity (see Fig. 3.11).