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Ontogenetic changes in photoreceptor opsin gene expression in coho salmon (*Oncorhynchus kisutch*, Walbaum)

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SUMMARY

Pacific salmonids start life in fresh water then migrate to the sea, after a metamorphic event called smoltification, later returning to their natal freshwater streams to spawn and die. To accommodate changes in visual environments throughout life history, salmon may adjust their spectral sensitivity. We investigated this possibility by examining ontogenetic and thyroid hormone (TH)-induced changes in visual pigments in coho salmon (*Oncorhynchus kisutch*, Walbaum). Using microspectrophotometry, we measured the spectral absorbance (quantified by λ_{max}) of rods, and middle and long wavelength-sensitive (MWS and LWS) cones in three age classes of coho, representing both freshwater and marine phases. The λ_{max} of MWS and LWS cones differed among freshwater (alevin and parr) and ocean (smolt) phases. The λ_{max} of rods, on the other hand, did not vary, which is evidence that vitamin A_1/A_2 visual pigment chromophore ratios were similar among freshwater and ocean phases when sampled at the same time of year. Exogenous TH treatment long wavelength shifted the λ_{max} of rods, consistent with an increase in A_2. However, shifts in cones were greater than predicted for a change in chromophore ratio. Real-time quantitative RT-PCR demonstrated that at least two RH2 opsin subtypes were expressed in MWS cones, and these were differentially expressed among alevin, parr and TH-treated alevin groups. Combined with changes in A_1/A_2 ratio, differential expression of opsin subtypes allows coho to alter the spectral absorbance of their MWS and LWS cones by as much as 60 and 90 nm, respectively. To our knowledge, this is the largest spectral shift reported in a vertebrate photoreceptor.

Key words: development, retina, fish, teleost, rhodopsin, porphyropsin, isoform, retinal, eye.

INTRODUCTION

Movements of an organism from one habitat to another are often coupled with changes in spectral environment and visual tasks (Lythgoe, 1988). This is particularly true in aquatic environments where the spectral transmittance of water itself may change drastically both spatially and temporally. To compensate for these changes, some vertebrates are able to use mechanisms that alter spectral sensitivity including: (i) gain or loss of a photoreceptor class (Allison et al., 2003; Allison et al., 2006a); (ii) changes in chromophore type [retinal (A_1) or 3,4-dehydroretinal (A_2)] (reviewed by Temple et al., 2006); and (iii) expression of different opsin classes or subtypes within a photoreceptor class (reviewed by Bowmaker and Loew, 2008). All of these mechanisms are present among Pacific salmonids (*Oncorhynchus* spp.), which migrate from cold freshwater streams and lakes to the open ocean within days to months of hatching and then migrate back to their natal freshwater streams and lakes a few years later to spawn and die (Beatty, 1966; Bowmaker and Kunz, 1987; Browman and Hawryshyn, 1992; Allison et al., 2003; Hawryshyn et al., 2003; Cheng and Novales Flamarique, 2004; Temple et al., 2008).

Of the seven species of Pacific salmonids, coho salmon (*Oncorhynchus kisutch*, Walbaum) are appropriate for examining the timing of changes in spectral sensitivity because they typically reside in fresh water for over a year before undergoing metamorphosis (smoltification) prior to migrating to the sea (reviewed by Groot and Margolis, 1991). This extended period of freshwater residency necessitates a visual system that is well adapted to the spectral environment and visual tasks at hand. Furthermore, they have been the subject of debate concerning the timing of changes in visual pigment (VP) A_1/A_2 chromophore ratio (reviewed by Temple et al., 2006), which, combined with a large body of work on other Pacific salmonids, has provided considerable background information about their visual system (Alexander et al., 1994; Alexander et al., 1998; Alexander et al., 2001; Beatty, 1966; Beatty, 1972; Novales Flamarique, 2005; Temple et al., 2006; Temple et al., 2008).

Coho salmon possess rod photoreceptors and four classes of cone photoreceptors: ultraviolet, short wavelength, medium wavelength and long wavelength sensitive (UVS, SWS, MWS and LWS). These photoreceptors express all five vertebrate opsin classes: RH1, UVS, SWS, RH2 and LWS, respectively (Dann et al., 2004), and we have recently shown (Temple et al., 2008) that coho express at least two subtypes of the RH2 opsin (RH2A and RH2B). Preliminary evidence suggests that the expression of these two RH2 opsin subtypes may vary throughout life history (Temple et al., 2008).

In the present study, we investigated whether coho salmon alter expression levels of RH2A and RH2B opsin subtypes with ontogeny, and whether exogenous thyroid hormone (TH) could induce a change in RH2A/B opsin subtype expression during the freshwater alevin stage. Real-time quantitative RT-PCR (QPCR) was used to measure relative changes in expression levels of RH2A and RH2B opsin subtypes, and microspectrophotometry was used to compare the λ_{max} values (wavelength of maximum absorbance) of MWS and LWS cones with those of rods in different age classes and fish treated with TH.

MATERIALS AND METHODS

Animals and care

Three age classes of coho salmon (alevin, parr and ocean smolt) were obtained from a local salmon hatchery (Target Marine Products, Sechelt, British Columbia, Canada). Live specimens were transported to the University of Victoria aquatic facilities in April and May of 2005. Here they were maintained under conditions matched to those at the hatchery (natural daylight and 11.0±1.0°C) until used in experiments (less than 5 days, except for a subgroup of alevins that were treated with TH for 4 weeks).

Alevins were 4 months old, 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 food. Parr were 16 months old and in the initial stages of smoltification (parr–smolt transformation). Parr were 8.6±0.5 cm in length and 6.9±1.0 g in weight. Ocean smolts were 28 months old, 45±12 cm in length, and between 1.5 and 2.0 kg in weight.

Thyroid hormone treatment

Treatment with exogenous TH was used to test the hypothesis that RH2 opsin subtype expression levels vary at smoltification. We compared expression levels of RH2 opsin subtypes and photoreceptor λ_{max} in control and TH-treated coho. Two groups of 25 alevins were maintained outdoors under natural, partly shaded, daylight in 15 l tanks with static water kept at 11.0±1.0°C using a thermostatically controlled water bath. TH was delivered by adding L-thyroxine (Sigma, St Louis, MO, USA) dissolved in 1.5 ml of 0.1 mol l^{-1} NaOH to the tank water to a final concentration of 300 μg l^{-1} L-thyroxine. The control tank received the vehicle only (1.5 ml of 0.1 mol l^{-1} NaOH). Tank water was changed three times per week. Care and treatment of fish were 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 1 h prior to being killed with an overdose of Euganol (100 mg l^{-1}; ICN Biomedicals, Irvine, CA, USA), followed by cervical transection. The right eye was enucleated and hemisected along an anterior–posterior axis. A piece of retina 1–2 mm^2 was cut out of the dorsal-most section of the dorsal hemisphere. The dorsal retina was used because the A_1/A_2 VP chromophore ratio varies across the retina in coho salmon (Temple et al., 2006), therefore standardizing the sampling location reduced inter-fish variability. The retinal sample was teased apart on a glass coverslip and a drop of minimum essential medium (Sigma, Oakville, Ontario, Canada; pH adjusted to 7.4–7.6) was applied to the sample. A second coverslip was placed over the sample and sealed with paraffin. All procedures were performed under deep red illumination (>650 nm) or using a dissecting microscope equipped with infrared light-emitting diode (800 nm) illumination and monitored with a charge-coupled device (CCD)–camera.

A CCD-microspectrophotometer (MSP), that has been described previously (Hawryshyn et al., 2001), was used to measure spectral absorbance of individual rod and cone photoreceptors. The CCD–MSP device delivered a short flash [0.05–0.5 s; duration was dependent on intensity and was set to deliver an optimum number of photons per exposure time = total counts (500,000 counts)] of full spectrum light (300–800 nm; 150 W xenon light source – intensity regulated; Oriel, Stratford, CT, USA) to the photoreceptor outer segment. Beam size was approximately 2 μm×3 μm. After passing through the sample, the transmitted beam was directed through a spectrometer (300 nm blazed grating; Acton Research Corporation, Acton, MA, USA) and onto a 1340 pixel×400 pixel, Peltier-cooled (–45°C), back-illuminated CCD-detector (Princeton Instruments, Roper Scientific, Trenton, NJ, USA). Photoreceptor absorbance [$\log_{10}(1/T)$] was calculated by comparing the transmitted intensity through the photoreceptor (I_M) with the transmitted intensity through an area clear of debris adjacent to the photoreceptor (I_R); thus, $T=I_M/I_R$.

Retinal samples were examined under infrared illumination (Schott RG850 filter; Ealing Optics, London, UK) and monitored by an infrared camera (Canadian Photonics Laboratory, Minnedosa, Manitoba, Canada). The search image and infrared filtered beam (Schott RG850 filter) were displayed on a computer monitor. A motorized *X*–*Y* stage (Marhauser-Wetzlar GmbH & Co., KG, Steindorf, Germany) was used to position the photoreceptor outer segment relative to the measurement beam. The path of the motorized stage was recorded to prevent repeated measurements of photoreceptor outer segment. Difference spectra were used to verify that the α-absorption band was due to the presence of a photolabile pigment and were calculated by subtracting the bleached absorbance curve (full spectrum bleach 2–5 s) from the initial absorbance curve.

Criteria for acceptance of absorbance spectra were: (i) presence of a baseline on the long wavelength limb (Harosi and MacNichol, 1974); (ii) λ_{max} near the expected wavelength for known *Oncorhynchus* spp. photoreceptors UVS ~350–380 nm, SWS ~420–450 nm, MWS ~490–550 nm, LWS ~540–630 nm and rod ~500–530 nm (Hawryshyn et al., 2001; Hawryshyn and Harosi, 1994); (iii) minimal absorbance by photoproduct and; (iv) signal-to-noise ratio of the main absorption band (α-band) greater than 5:1. Determinations of λ_{max}, and percentage A_2 from acceptable absorbance records were performed offline subsequent to initial sampling.

A custom-designed analysis program was used to determine λ_{max} from absorbance records using existing templates. Each MSP record consisted of over 1000 points collected 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, and the smoothed curve was normalized to zero at baseline on the long wavelength arm and to one at the centre of the α-band. The fit of the normalized curve was compared with a non-linear least-squares routine to the upper 20% of the weighted A_1/A_2 averaged Govardovskii et al. template (Govardovskii et al., 2000) (based on the centre of the α-peak ±40 nm).

For some rods, we also obtained a second estimate of λ_{max} based on a template created by Munz and Beatty for coho rod pigments (Munz and Beatty, 1965). Rod absorbance curves were compared (minimum variance fit) to the Munz and Beatty template (Munz and Beatty, 1965), which extends from λ_{max} to a point at 20% of the maximum on the long wavelength arm. The Munz and Beatty template assumes that λ_{max} values of coho rods vary from 503 to 527 nm, which is in close agreement with published models that predict the shift that occurs when A_1 is replaced by A_2 in the same opsin (Bridges, 1965; Dartnall and Lythgoe, 1965; Harosi, 1994; Parry and Bowmaker, 2000) (reviewed by Temple et al., 2008; Tsin et al., 1981; Whitmore and Bowmaker, 1989). However, many of the rods we measured had λ_{max} values that exceeded 527 nm and therefore were not fitted by the Munz and Beatty template (Munz and Beatty, 1965). In these cases, we used the estimate obtained by the fit to the Govardovskii et al. template (Govardovskii et al., 2000).

Real-time quantitative RT-PCR

Retinal isolation

Fish were dark adapted for 1 h and then killed by immersion in 100 mg l^{-1} euganol for 10 min, followed by cervical transection.

Under deep red illumination (>650 nm), the right eye was enucleated and hemisected along an anterior–posterior axis. The neural retina was then dissected free of pigmented epithelium. The entire dorsal retinal hemisphere was used in the following procedures. Immediately after dissection, each isolated retina was preserved in 0.5 ml RNA*later* (Ambion, Austin, TX, USA) and stored at 4°C.

Preparation of retinal total RNA and cDNA

Total RNA was isolated from the retina using TRIzol reagent (Invitrogen Canada, Burlington, Ontario, Canada) as per the manufacturer's recommended protocol. Each retinal sample was placed in a 1.5 ml microcentrifuge tube containing TRIzol reagent (100 µl for alevin retina and 200 µl for parr retina) and was homogenized using a disposable Kontes® Pellet Pestle® with cordless motor tissue grinder (Kimble Kontes, Vineland, NJ, USA). Due to the small amount of tissue, 20 µg of glycogen (Roche Diagnostics, Laval, Québec, Canada) was used as a nucleic acid carrier during preparation of total RNA from alevin retinal samples. Isolated RNA was re-suspended in 20 µl 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 µg 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. The cDNA samples were diluted 20-fold for QPCR analysis.

Primer design

Primers were designed against *O. kisutch* RH2A and RH2B open reading frame sequences (GenBank accession numbers AY214147 and DQ309027, respectively) using Primer Premier V4.1 software (Premier Biosoft International, Palo Alto, CA, USA) and were synthesized by Operon Biotechnologies (Huntsville, AL, USA) (Table 1). Primer pairs were diluted and combined in an equimolar ratio to a final concentration of 10 µmol l^{-1}. We chose β-actin as our normalization reference for gene expression across samples because, in this study, its expression did not vary significantly either spatially within the retina (i.e. dorsal *vs* ventral) or following TH treatment (data not shown). We utilized primers designed for rainbow trout cytoplasmic β-actin to PCR amplify and clone a partial β-actin ORF sequence from coho retinal cDNA (GenBank accession number EU262946).

The specificity of each QPCR primer pair was tested by amplifying target gene sequences present within cDNA synthesized from 1 µg parr retinal total RNA. Amplified DNA products were separated in a 1.5% agarose gel and visualized by ethidium bromide staining. If the amplified product obtained from each primer pair consisted of a single DNA band and was of the correct size, it was excised from the gel and extracted by freeze–thaw centrifugation (Smith, 1980). Extracted DNA was cloned into PCR2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen). Plasmid DNA was purified using a QIAprep Spin miniprep kit (Qiagen, Mississauga, Ontario, Canada) and sequenced (Centre for Biomedical Research DNA Sequencing Facility, University of Victoria). Positive identification of cloned DNA amplicons (three independent clones for each primer pair) served to confirm that each gene-specific primer pair was amplifying the correct cDNA target sequence from coho retinal samples.

Real-time quantitative RT-PCR

QPCR analysis of individual retinal cDNA samples was carried out using β-actin, RH2A and RH2B primer sets. Each 15 µl reaction contained 10 mmol l^{-1} Tris HCl, 50 mmol l^{-1} KCl, 3 mmol l^{-1} $MgCl_2$, 0.01% Tween 20, 0.8% glycerol, 40,000-fold dilution of SYBR Green I (Molecular Probes, Eugene, OR, USA), 200 µmol l^{-1} dNTPs, 83 nmol l^{-1} ROX reference dye (Stratagene, La Jolla, CA, USA), 10 pmol of each primer, 2 µl of cDNA diluted 20-fold, and 1.0 U Platinum Taq DNA polymerase (Invitrogen). DNA amplification was carried out using an MX4000 real-time quantitative PCR system (Stratagene). The thermocycle program was 95°C for 9 min, followed by 40 cycles of 95°C for 15 s, 62°C for 30 s and 72°C for 45 s. Controls included a reaction lacking cDNA template and one lacking Taq DNA polymerase. The potential for genomic DNA contamination was assessed by comparison of amplification patterns generated from cDNA and genomic DNA using the RH2A primer set. No genomic DNA contamination was evident in the cDNA samples used for QPCR. Opsin gene expression for each retinal sample was analysed in quadruplicate, averaged, and normalized to expression of the β-actin control. Cycle threshold values were converted to copy number using standard plots generated for each target DNA sequence using known amounts of serially diluted plasmid DNA containing the amplicon of interest.

Data analysis

MSP records were collected from individual photoreceptors from the dorsal retina of the right eye. Photoreceptors were assigned to classes based on morphology and λ_{max}. We collected a sufficient number of records from each fish to perform our statistical analysis on λ_{max} values from rods, and MWS and LWS cone types. For MWS and LWS cones it was possible to use λ_{max} to assign outer segments to MWS and LWS cone classes as there was no overlap in λ_{max} values measured from these two cone classes within any single fish or within a group of fish (age class or TH treated). For each fish, a mean λ_{max} value ±1 s.d. was calculated for all three photoreceptor classes (we refer to these as fish mean λ_{max} values). For comparisons between age classes/groups (alevin, parr, smolt and TH-treated alevin), we calculated a 'group mean λ_{max}' ±1 s.d., which was the mean of all individual fish mean λ_{max} values for a specific receptor type within that age class/group. Except when specified otherwise, all mean λ_{max} values reported hereafter refer to group mean λ_{max} values. Our approach of using individual fish as the sample unit is appropriate since photoreceptors from a single fish are not independent observations (Temple et al., 2008).

Comparisons among group mean λ_{max} values, and relative expression (copy number) of RH2A and RH2B, were made using a one-way analysis of variance (ANOVA) with α=0.05. Tukey's HSD *post hoc* analysis was used for pair-wise comparisons among groups.

Table 1. Gene-specific primer sequences used in QPCR

Gene	Forward primer	Reverse primer	Amplicon size (bp); linear R^2 value
RH2A	TTGCATTCACCTGGATAGCT	CTTTCTGGGTAGATGCTGA	267; 0.9975
RH2B	CCATTGGTTGGCTGGTCT	TTTGAGAAGAAGGCTGGA	377; 0.9944
β-Actin	ATCGCCGCACTGGTTGTT	TCTCCCTGTTGGCTTTGG	340; 0.9995

QPCR, quantitative RT-PCR. Linear R^2 value is a measure of PCR efficiency, calculated from the slope of the standard plots generated for each target sequence.

RESULTS

Photoreceptor λ_{max} differs among age classes and TH-treated fish

The λ_{max} values recorded from rods, MWS and LWS cones varied, not only between fish and between fish in different age classes/treatments, but also within a single fish. This variation is expected in a species with a variable A_1/A_2 chromophore ratio since the A_1/A_2 ratio varies across the retina. The mean standard deviation in rod λ_{max} for individual fish from all age classes and the TH-treated group was ±3.6 nm. This variation in λ_{max} is equivalent to a change in A_1/A_2 chromophore ratio of nearly 40%. To account for high within-fish variability in λ_{max} and to avoid pseudoreplication (Temple et al., 2008), comparisons between groups were made using group mean λ_{max} values for each photoreceptor class.

The mean λ_{max} of rods measured from each group of fish, which is an estimate of the proportion of vitamin A_1- to A_2-based VPs in rods, did not differ significantly (P>0.262) among age classes (alevin, parr and ocean smolts). The combined mean λ_{max} for these three groups was 509.6±1.2 nm, equivalent to a chromophore ratio of 36.6% A_2. However, the mean λ_{max} of TH-treated alevins was 533.0±1.0 nm, equivalent to a chromophore ratio of 100% A_2, and was significantly long wavelength shifted (P<0.001) relative to all three untreated groups (Fig. 1).

The mean λ_{max} of MWS cones did not differ significantly (P>0.119) among age classes (alevin 501.5±2.2 nm; parr 511.6±9.0 nm; ocean smolts 507.1±7.8 nm; Fig. 1). However, MWS cones in TH-treated alevins (547.7±4.9 nm) were significantly long wavelength shifted (P<0.001) relative to all three untreated groups. The variance in λ_{max} values of MWS cones in alevin and parr (error bars in Fig. 1) was greater than that observed in rods and was consistent with previous findings indicating the presence of more than one RH2 opsin subtype in MWS cones in coho parr (Temple et al., 2008). The frequency distribution of λ_{max} values of individual MWS cones from the different groups (Fig. 2) shows a decrease in the number of MWS cones with λ_{max} values at shorter wavelengths as the fish transition from alevin (Fig. 2B) to parr (Fig. 2D) to smolt (Fig. 2E).

Mean λ_{max} of LWS cones differed significantly (P<0.001) among the four groups (Fig. 1). Ocean smolts (556.6±1.6 nm) were significantly short wavelength shifted (P≤0.001) and TH-treated alevins (624.2±0.5 nm) were significantly long wavelength shifted

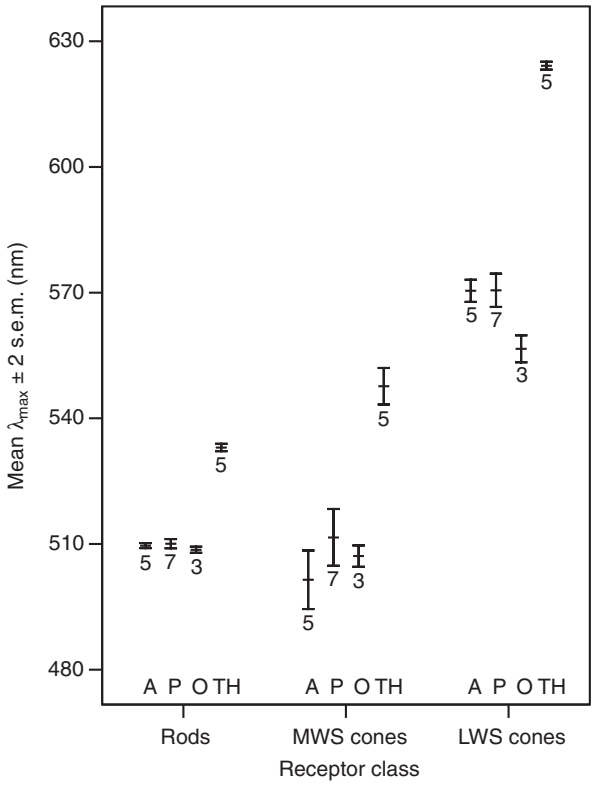

Fig. 1. Mean λ_{max} ±2 s.e.m. for rods, middle wavelength-sensitive (MWS) cones and long wavelength-sensitive (LWS) cones from alevin (A), parr (P), ocean smolt (O) and TH-treated alevin (TH) coho (*Oncorhynchus kisutch*, Walbaum). The mean values for each group were calculated from the means of all individual fish in each group (*N*-values below the error bars). The means for each fish were based on the λ_{max} values obtained for that receptor class (see text for details).

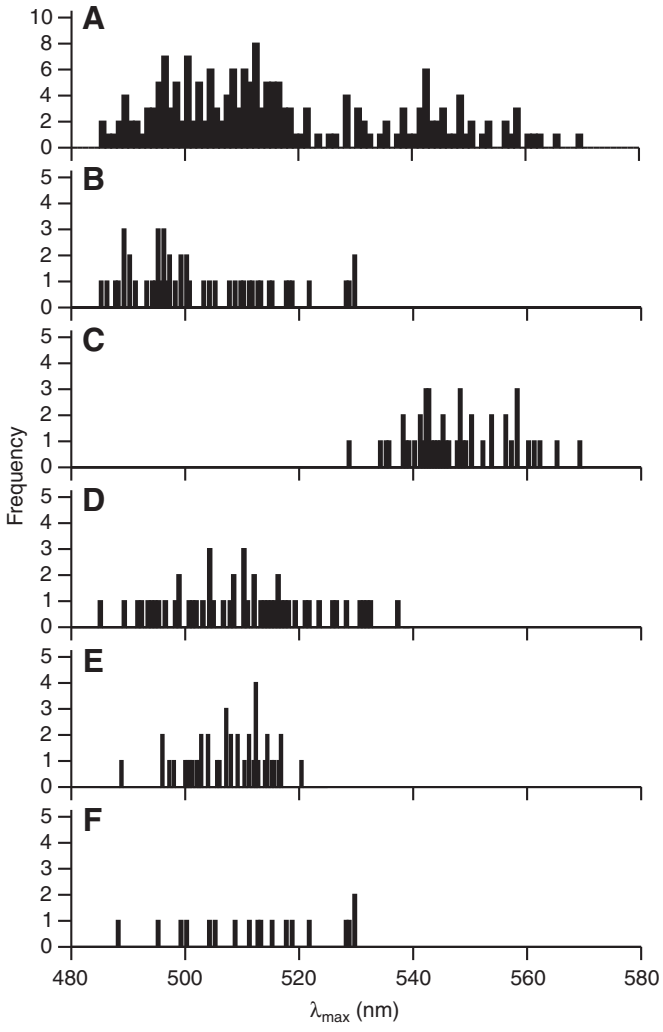

Fig. 2. Frequency histograms of λ_{max} values of individual MWS cones from coho salmon (*Oncorhynchus kisutch*, Walbaum): (A) all groups combined, (B) alevins, (C) TH-treated alevins, (D) parr, (E) ocean smolts and (F) an individual alevin. Bin size=1 nm. The λ_{max} values were obtained by microspectrophotometry (MSP) of individual MWS cones from the dorsal retina of coho salmon obtained from Target Marine Products 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 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.

Table 2. Models for calculating A_2 and A_1 λ_{max} values from known A_1 and A_2 λ_{max} values and the predicted values based on our MSP observations

Literature source	Receptor class	Opsin class	A_1 λ_{max} (nm)	Equation for A_1–A_2, where λ=$A_1\lambda_{max}$ in nm*	Calculated A_2 λ_{max} (nm)	A_2 λ_{max} (nm)	Inverse equation for A_2–A_1, where λ=$A_2\lambda_{max}$ in nm*	Calculated A_1 λ_{max} (nm)
Bridges, 1965	Rod	RH1	503.0		527.8	534.0		506.8
	LWS	LWSA	545.0	$=1.6187\times(\lambda_1-286.42)$	595.8	600.0	$=(\lambda_2+286.42)/1.6187$	547.6
	MWS	LWSB	563.0		624.9	633.0		568.0
		RH2A	490.0		506.7	522.0‡		499.4
		RH2B	512.0†		542.4	548.0		515.5
Dartnall and Lythgoe, 1965	Rod	RH1	503.0		529.1	534.0		506.1
	LWS	LWSA	545.0	$=-263.1382+1.57505\times\lambda_1$	595.3	600.0	$=(\lambda_2+263.182)/1.57505$	548.0
	MWS	LWSB	563.0		623.6	633.0		569.0
		RH2A	490.0		508.6	522.0‡		498.5
		RH2B	512.0†		543.3	548.0		515.0
Tsin et al., 1981	Rod	RH1	503.0		530.0	534.0		506.2
	LWS	LWSA	545.0	$=(\lambda_1-79)/0.8$	582.5	600.0	$=(\lambda_2\times1.8)+79$	559.0
	MWS	LWSB	563.0		605.0	633.0		585.4
		RH2A	490.0		513.8	522.0‡		496.6
		RH2B	512.0†		541.3	548.0		517.4
Whitmore and Bowmaker, 1989	Rod	RH1	503.0		**534.1**	534.0		502.9
	LWS	LWSA	545.0	$=e^{[\ln(\lambda1/52.5)/0.4]+250}$	597.2	600.0	$=(\lambda_2-250)^{0.4}\times52.5$	546.8
	MWS	LWSB	563.0		626.5	633.0		566.8
		RH2A	490.0		**516.0**	522.0‡		494.3
		RH2B	512.0†		548.0	548.0		**512.7**
Harosi, 1994*	Rod	RH1	503.0		528.9	534.0		506.4
	LWS	LWSA	545.0	$=\lambda_1-(27.91483-2.35989\times\lambda_1+0.05054\times\lambda_1^2)$*	**598.1**	600.0	$=\{3.35989-\sqrt{}[11.28886081-0.20216\times(\lambda_2+27.91483)]\}/0.10108$	546.0
	MWS	LWSB	563.0		632.2	633.0		**563.4**
		RH2A	490.0		510.1	522.0‡		498.3
		RH2B	512.0†		542.6	548.0		515.4
Parry and Bowmaker, 2000	Rod	RH1	503.0		521.9	534.0		512.2
	LWS	LWSA	545.0	$=e^{(\lambda1/400+5)}$	579.7	600.0	$=400\times[\ln(\lambda_2)-5]$	558.8
	MWS	LWSB	563.0		606.4	633.0		580.2
		RH2A	490.0		505.2	522.0‡		503.0
		RH2B	512.0†		533.8	548.0		522.5

*In Harosi's equation (Harosi, 1994), λ_{max} values are given as reciprocal λ_{max} values so λ=10,000/λ_{max} in nm.

Values marked in bold are calculated values that were chosen because the model predicted the greatest difference in λ_{max} between the A_1 and A_2 states (see text for details).

†The λ_{max} values of the paired MWS (RH2B) or LWS cone opsins when combined with A_1 are unknown; these values simply represent possible values derived from back calculating from the observed values for the upper limits of the MWS/LWS cone λ_{max} values in Fig. 4 using Whitmore and Bowmaker's formula (Whitmore and Bowmaker, 1989) for the MWS cones and Harosi's formula (Harosi, 1994) for the LWS cones.

‡The λ_{max} values of the paired MWS (RH2A) or LWS cone opsins when combined with A_2 are unknown; these values simply represent possible values derived from back calculating from the observed values for the lower limit of the MWS/LWS cone λ_{max} values in Fig. 4 using Whitmore and Bowmaker's formula (Whitmore and Bowmaker, 1989) for the MWS cones and Harosi's formula (Harosi, 1994) for the LWS cones.

MSP, microspectrophotometer; LWS, long wavelength sensitive; MWS, middle wavelength sensitive.

(P<0.001) relative to the other groups (Fig. 1). However, there was no significant difference (P=1.000) between the two age classes found in fresh water (alevin 570.4±1.3 nm and parr 570.5±1.9 nm).

It was not possible to estimate the A_1/A_2 ratio from MWS or LWS cones because half-bandwidth, which is used as an estimate of A_1/A_2 content, would also have been affected by co-expression of multiple opsins in photoreceptor outer segments. The half-bandwidth of the absorbance curve of A_2-based VPs is wider than that of A_1-based VPs (Govardovskii et al., 2000; Harosi, 1994); however, the expression of more than one opsin in a single photoreceptor will also broaden the half-bandwidth (Archer and Lythgoe, 1990). For this reason we devised a different approach to interpret our MWS and LWS data (see below) (see also Temple et al., 2008).

Distribution of MWS and LWS cone λ_{max} values

Plotted as frequency histograms, the broad distribution of λ_{max} values indicated the presence of multiple opsin subtypes in MWS and LWS cones. The λ_{max} values from individual MWS cones from all four groups extended from below 490 nm to above 550 nm (Fig. 2A). 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 (Bridges, 1965; Dartnall and Lythgoe, 1965; Harosi, 1994; Parry and Bowmaker, 2000; Tsin et al., 1981; Whitmore and Bowmaker, 1989). When compared with these models, the observed variation in MWS cone λ_{max} was greater than could be explained by a change in A_1/A_2 chromophore ratio in a single opsin (Table 2). When we plotted the frequency histograms for MWS cones for each age class separately, the variation recorded in alevin and parr groups was still greater in both cases than could be explained by a change in A_1/A_2 chromophore ratio in a single opsin (Fig. 2B,D). However, the variance did not mask the clear differences between the TH-treated alevin (Fig. 2C) and the control alevin groups (Fig. 2B). Comparatively, there was less variance in MWS cone λ_{max} values in ocean smolts (Fig. 2E) than in alevin and parr. The broad distribution of MWS cone λ_{max} values recorded in the alevin group

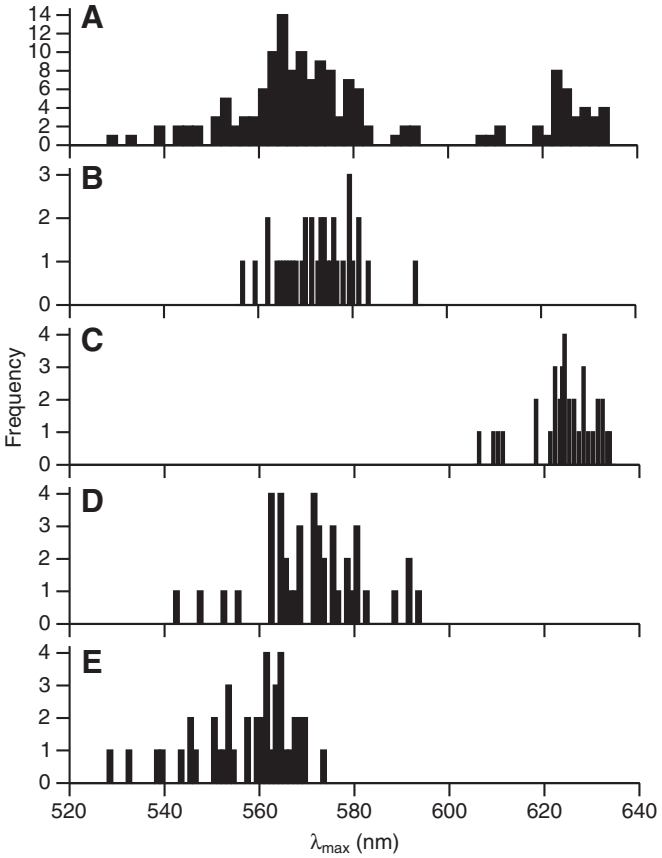

Fig. 3. Frequency histograms of λ_{max} values of individual LWS cones from coho salmon (*Oncorhynchus kisutch*, Walbaum): (A) all groups combined, (B) alevins, (C) TH-treated alevins, (D) parr and (E) ocean smolts. Bin size=2 nm. The λ_{max} values were obtained by MSP of individual LWS cones from the dorsal retina of coho salmon obtained from Target Marine Products 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 A_1- to A_2-based chromophores.

was also evident within an individual fish (Fig. 2F). The simplest model to explain the breadth of the distribution of λ_{max} values observed in MWS cones requires that coho salmon express at least two RH2 opsin subtypes.

The frequency distribution of λ_{max} values from individual LWS cones from all four groups of coho is displayed in Fig. 3A. The variance observed in LWS cones was also greater than could be explained by a change in A_1/A_2 ratio in a single opsin (Table 1). However, the variance within each age class (Fig. 3B,D,E), and within the TH-treated alevin group (Fig. 3C) was not as large as that observed in MWS cones.

Estimating λ_{max} of opsin subtypes in MWS and LWS cones

To estimate λ_{max} values of MWS and LWS opsin subtypes, when both chromophore and opsin subtype expression were variable, we plotted LWS *vs* MWS cone λ_{max} values from measurements made on approximately 100 double cones from which both outer segments had been recorded (Fig. 4). The distribution of points in Fig. 4 demonstrates that more than one opsin subtype was being expressed in both MWS and LWS cones [see analysis in our previous publication (Temple et al., 2008)]. By placing lines at the upper and lower limits of the horizontal and vertical scatter in this data set (Fig. 4), and allowing for a measurement error of ±3 nm, we obtained estimates of λ_{max} values when one opsin subtype was combined with A_1 and the other was combined with A_2 (lower and upper limits, respectively). MWS cone λ_{max} distribution extended from 490 to 548 nm indicating that one opsin subtype combined with A_1 had an observed λ_{max} at approximately 490_1 nm (subscript denotes the chromophore associated with this λ_{max}: subscript 1, A_1; subscript 2, A_2). The upper limit provided an estimate of yet another opsin subtype combined with A_2, which had an observed λ_{max} of approximately 548_2 nm.

Using existing models that predict the change in λ_{max} ($\Delta\lambda_{max}=A_2\lambda_{max}-A_1\lambda_{max}$) that occurs when A_1 and A_2 chromophores are exchanged in a single VP opsin (Table 2), we calculated predicted λ_{max} values for A_1 and A_2 counterparts for each opsin subtype based on the values obtained from Fig. 4. As a conservative measure, to reduce the probability of type I error, we compared our observed data set with the model that predicted the largest shift in λ_{max} for the given A_1–A_2 VP pair. For the range of λ_{max} values encompassed by MWS cones, the most conservative model (Whitmore and Bowmaker, 1989) predicted that the 490_1 nm VP would be paired with a 516_2 nm VP and that the 548_2 nm VP would be paired with a 512_1 nm VP (Fig. 4). The same analysis performed on the observed LWS cone λ_{max} values predicted two pairs of pigments with λ_{max} values at 545_1–600_2 nm and 563_1–633_2 nm.

Change in opsin subtype expression levels

Expression levels of the two RH2 opsin subtypes differed among the groups of fish tested (alevin, parr and TH-treated alevin). Due to technical difficulties, we did not obtain retinal material of sufficient quality for PCR from ocean smolts so they are not included in these analyses. RH2A expression levels were significantly higher in TH-treated alevin than in both control alevin (P=0.013) and parr (P=0.041), while RH2B expression levels were significantly higher in control alevin than in both parr (P=0.015) and TH-treated alevin (P<0.001; Fig. 5).

DISCUSSION

The VP system of coho salmon is highly flexible allowing them to alter spectral sensitivity by independently varying both VP chromophore ratio and opsin subtype expression (Temple et al., 2008). Previously, we have demonstrated that the VP A_1/A_2 chromophore ratio follows a seasonal pattern (Temple et al., 2006), and that coho express a second subtype of the RH2 opsin (Temple et al., 2008). In this study our objective was to determine whether there was an ontogenetic shift in the pattern of expression of RH2A and RH2B opsin subtypes and whether this shift could be induced with TH, which is associated with smoltification. We compared λ_{max} values of rods, MWS cones and LWS cones in three age classes and for one age class we treated a subset of fish with exogenous TH. We found no difference in mean λ_{max} of rods in untreated groups indicating that A_1/A_2 chromophore ratios did not differ among freshwater and ocean-going life history stages. However, there were differences in the frequency distribution of λ_{max} values of MWS and LWS cones, for which we proposed changes in opsin subtype expression. To support this hypothesis, we found that the pattern of expression of RH2A and RH2B opsin subtypes mirrored the differences in λ_{max} values measured in MWS cones in alevin, parr and TH-treated alevin groups. A similar comparison was not possible for the change in LWS cones as we have not yet identified a second subtype for the LWS opsin.

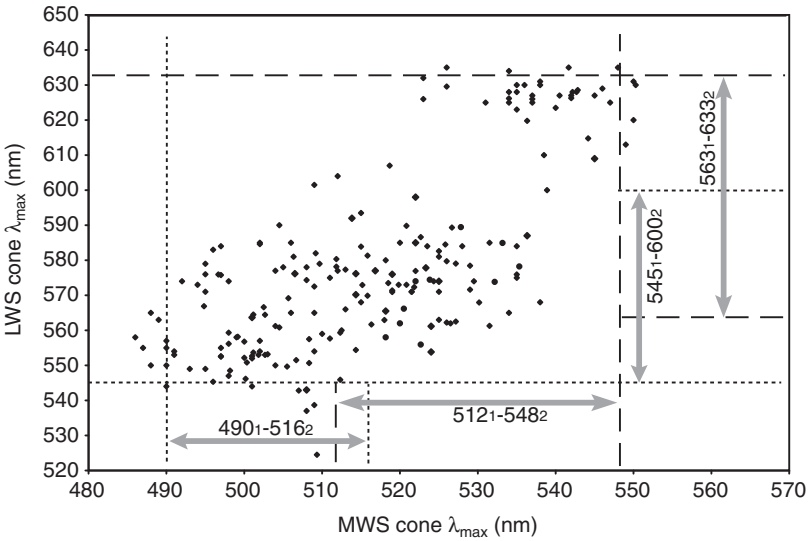

Fig. 4. Scatter plot showing λ_{max} values for MWS and LWS outer segments of individual double cones measured in coho salmon (*Oncorhynchus kisutch*, Walbaum). Vertical dotted lines predict the range of λ_{max} values for the MWS cones at the short wavelength range of the data set. Taking the lower value of 490_1 nm as the A_1 observed value, we used the Whitmore and Bowmaker model (Whitmore and Bowmaker, 1989) to calculate that the same opsin would have a λ_{max} of 516_2 nm if combined with an A_2 chromophore. Vertical dashed lines indicate the range for the longer of the two opsins, with a pigment pair that would have a range of 512_1 to 548_2 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 545_1 to 600_2 nm based on Harosi's model (Harosi, 1994). 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 563_1 to 633_2 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).

Rods

The fish mean λ_{max} values of rods varied from 506_1 to 534_2 nm, a range that is consistent with previous observations in coho salmon (Alexander et al., 1994; Alexander et al., 1998; Alexander et al., 2001; Beatty, 1966; Beatty, 1972; Novales Flamarique, 2005; Temple et al., 2006; Temple et al., 2008). The coho rod VP when combined with A_1 has been shown to have a λ_{max} of 503_1 nm; when combined with A_2 it is predicted to have a λ_{max} of between 521.9_2 and 534.1_2 nm, depending on the model used (Bridges, 1965; Dartnall and Lythgoe, 1965; Harosi, 1994; Parry and Bowmaker, 2000; Tsin et al., 1981; Whitmore and Bowmaker, 1989). To date, only one RH1 (rod) opsin has been found in coho (Dann et al., 2004); however, there is some evidence for the existence of a second RH1 opsin subtype in the congener *O. mykiss*, Walbaum (Allison et al., 2006b). The distribution of λ_{max} values recorded from individual rods, from all fish used in this study, ranged from 503 to 540 nm. This range is greater than would be expected for a single opsin combining with A_1- and A_2-based chromophores. Examining the data set in this way suggests that more than one RH1 opsin subtype may also be present in coho salmon. Further work toward isolating and cloning RH1 opsin subtypes from this species would be useful.

That all three age classes (alevin, parr and ocean smolt), measured at the same time of year, had mean rod λ_{max} values that did not differ significantly, supports previous findings of a seasonal shift in chromophore ratio in coho salmon (Temple et al., 2006). The seasonal shift is further supported by the fact that the mean λ_{max} of rods in this study (509.6±1.2 nm) did not differ significantly (P=0.532) from measurements that we reported in a previous study that sampled three different age classes of coho salmon from three different locations (510.4±4.3 nm) in the same month in two consecutive years (Temple et al., 2006).

A close correlation between the timing of changes in A_1/A_2 ratio and seasonal changes in temperature and day length is not restricted to coho salmon; similar observations have been made recently in Japanese dace (*Tribolodon hakonensis* Günther) by Ueno and colleagues (Ueno et al., 2005) as well as in several other vertebrates and an invertebrate [see Table 1 in Temple et al. (Temple et al., 2006)]. That seasonal shifts in A_1/A_2 VP ratio are found in such a diverse range of species suggests that vitamin A_1/A_2 VP ratio is not linked directly to migration and metamorphic events as was previously thought (Crescitelli, 1958; Crescitelli, 1991; Munz and Beatty, 1965; Wald, 1939; Wald, 1941; Wald, 1960), particularly when the seasonal timing of these events is taken into consideration.

MWS and LWS cones

The spectral distribution of λ_{max} values observed in both MWS and LWS cones was greater than predicted for a shift in chromophore ratio (Bridges, 1965; Dartnall and Lythgoe, 1965; Harosi, 1994; Parry and Bowmaker, 2000) (reviewed by Temple et al., 2008; Tsin et al., 1981; Whitmore and Bowmaker, 1989). As an explanation for this observation, we proposed that more than one opsin subtype was being expressed in both MWS and LWS cone classes. To test this hypothesis, we plotted the λ_{max} of individual MWS cone outer segments against the λ_{max} of the other member of the double cone pair, in this case LWS cone outer segments (Fig. 4). The resultant scatter plot was effective because the two outer segment members of a double cone should have similar A_1/A_2 VP ratios. Though regulation of A_1/A_2 VP ratio in the retina is poorly understood, the two proposed sources of 11-cis chromophore for VP regeneration (retinal pigmented epithelium and Müller cells) (Bridges and Yoshikami, 1970; Mata et al., 2002) would be expected to provide neighbouring photoreceptor outer segments with similar A_1/A_2 ratios. Furthermore, differences in A_1/A_2 ratio are not expected between adjacent outer segments because vertebrate photoreceptors and their opsins do not differentiate among chromophore isomers (Chen and Liu, 1996; Makino et al., 1990; Parry and Bowmaker, 2000). Given the assumption that A_1/A_2 ratios are not dissimilar in individual double cone outer segments, it follows that the distribution of λ_{max} values observed in MWS and LWS cones is best explained by the expression of more than one opsin in each of these cone classes.

The distribution of MWS and LWS cone λ_{max} values in Fig. 4 shows that at least four different opsins must be expressed in MWS and LWS cones in order to explain the spread of λ_{max} values observed in coho double cones. The λ_{max} values of MWS and LWS cone outer segments fall into a spectral range that extends from approximately 490 to 633 nm. Using the most conservative models (Table 2) to predict the shift in λ_{max} resulting from a change in chromophore ratio in a single opsin, this spectral range can only be explained by the presence of at least four different opsins (490_1–516_2 nm; 512_1–548_2 nm; 545_1–600_2 nm; 563_1–633_2 nm). As

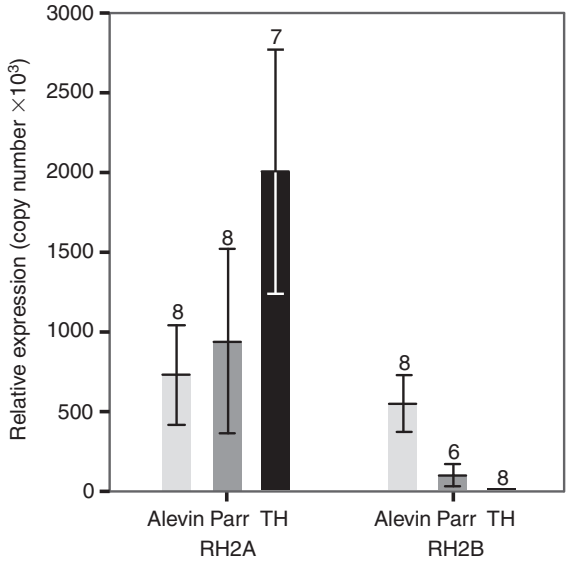

Fig. 5. RH2 opsin subtype expression in dorsal retinas of alevin, parr and TH-treated alevin coho salmon (*Oncorhynchus kisutch*, Walbaum) measured using real-time quantitative RT-PCR. Expression levels are the mean of three replicates per fish (*N*-values above error bars represent the number of fish) and were normalized to the β-actin gene. Error bars represent 2 s.e.m.

the data set used to generate these estimates is based on measurements made from both outer segments of individual double cones where one member always had a higher λ_{max} than the other (Fig. 4), and because there is little overlap in the spectral range of the four proposed opsins, we suggest that there are at least two different opsins expressed in MWS cones and another two different opsins expressed in LWS cones.

We found differences in expression levels of RH2 opsin subtypes and mean λ_{max} values of MWS cones among age classes and TH-treated fish, which indicated that the timing of the change in VPs in MWS cones occurs prior to smoltification. This timing of change is consistent with previous MSP records in coho (Novales Flamarique, 2005), which showed a shift from 490 nm to 553 nm for parr to smolts. However, in that study it was hypothesized that the shift in λ_{max} was the result of a change in chromophore ratio, despite the fact that none of the published models (Table 2) predict a shift in λ_{max} as large as 60 nm for a change in chromophore ratio in a VP with a λ_{max} of 490 nm. We have demonstrated that the ontogenetic shift in MWS cone λ_{max} can be explained by a change in opsin expression.

Our hypothesis, that more than one opsin was being expressed in MWS cones, was supported by the discovery of a second RH2 opsin subtype in coho salmon (Temple et al., 2008). Based on amino acid sequence, the new opsin subtype, named RH2B, had 48 amino acid differences from the previously sequenced coho RH2A opsin (Dann et al., 2004) and was predicted to be a functional opsin. The RH2B opsin subtype possessed a substitution of glutamate for glutamine at position 123 (analogous to position 122 in bovine rod opsin), which would be expected to shift the λ_{max} to shorter wavelengths relative to RH2A (Sakmar et al., 1989; Temple et al., 2008). Our finding, that individual fish possess MWS cones with a broad range of λ_{max} values (Fig. 2F), indicates that our results are not due to a polymorphism in the RH2 opsin, as proposed for LWS opsins in guppies (*Poecilia reticulata*, Peters) by Archer and colleagues (Archer et al., 1987), but, rather, to simultaneous expression of multiple RH2 opsin subtypes as reported for zebrafish (*Danio rerio*, Hamilton) (Takechi and Kawamura, 2005).

We have not yet isolated, cloned and sequenced a second LWS opsin subtype in coho salmon, but multiple LWS opsins have been found in other teleosts (Chinen et al., 2003; Hoffmann et al., 2007; Matsumoto et al., 2006; Takechi and Kawamura, 2005; Weadick and Chang, 2007).

Significance of visual pigment changes

Changes in A_1/A_2 VP ratio and opsin expression appear to occur on different temporal scales in coho salmon. We have demonstrated that coho salmon at various life history stages (fresh or salt water) will shift their A_1/A_2 chromophore ratio in correlation with changes in season (Temple et al., 2006), a finding that was corroborated in this study. The proposed shift in opsin expression for MWS and LWS cones occurs sometime between alevin and ocean smolt stages. We predict that the change in opsin expression may occur prior to seaward migration as a means to prepare the visual system for a different photic environment and visual tasks. Other members of the genus *Oncorhynchus* lose a large portion of their UV cone population at the time of smoltification, a transition that can also be induced with the application of exogenous TH (Allison et al., 2006a; Allison et al., 2003; Browman and Hawryshyn, 1992; Hawryshyn et al., 1989).

Changes in opsin expression have been proposed to account for ontogenetic changes in photoreceptor λ_{max} in several other fish species [e.g. eels (*Anguilla* spp.) (Beatty, 1975; Carlisle and Denton, 1959; Wood and Partidge, 1993; Wood et al., 1992); cardinal fish (*Apogon brachygrammus*, Jenkins) (Munz and McFarland, 1973); yellowfin tuna (*Thunnus albacares*, Bonnaterre) (Loew et al., 2002); pollock (*Pollachius pollachius*, L.), goatfish (*Upeneus tragula*, Richardson); black bream (*Acanthopagrus butcheri*, Munro) (Shand, 1993; Shand et al., 2008; Shand et al., 2002; Shand et al., 1988); and cichlids (*Oreochromis niloticus*, L.) (Spady et al., 2006). If changes in MWS and LWS opsin expression are linked to smoltification in coho, then the two mechanisms of shifting spectral sensitivity examined here (seasonal A_1/A_2 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). Or, alternatively, it may be that VP systems in fishes remain highly plastic throughout life history and that both chromophore ratio and opsin expression are dynamic and can be tuned to environmental conditions (e.g. temperature, day length, spectral distribution of light, etc.) or visual tasks at anytime.

The dynamic nature of A_1/A_2 VP shifts and changes in opsin expression provide coho salmon with highly 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 variable spectral environments which salmonids inhabit (Novales-Flamarique and 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 freshwater and oceanic life history stages, matches the blue shift in photic environment when coho migrate from fresh water to the sea. The spectral distribution of light in freshwater environments is typically richer in long wavelength light than the open ocean (Jerlov,

1976; Lythgoe and Partridge, 1989; Tyler and Smith, 1970). Therefore, the proposed change in opsin expression that would shift LWS cones to a shorter λ_{max} may fit the hypothesis that, in fishes, double cones match the background photic environment (Levine and MacNichol, 1979; Loew and Lythgoe, 1978; Lythgoe, 1984).

The adaptive significance of the ontogenetic shift in MWS cone λ_{max} to longer wavelengths is less obvious. 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 in ocean smolts. 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 reaches the ocean. This might explain the shift to slightly longer wavelengths [see description of matched and offset pigments in Munz and McFarland (Munz and McFarland, 1975)].

Conclusions

The present findings support the seasonal hypothesis for explaining the timing of the A_1/A_2 shift in labile pigment pair species, as well as providing further evidence for the expression of more than one opsin subtype in MWS and LWS cones in coho salmon and possibly more than one RH1 opsin subtype in salmonids in general. Our research has demonstrated that coho possess a highly flexible VP spectral tuning mechanism that can be attributed to changes in A_1/A_2 ratio combined with changes in opsin expression. Furthermore, we suggest that this potential flexibility in VP λ_{max} is probably more common among fishes than was previously thought and that considerable effort will be required to elucidate the functional significance of this plasticity in the visual system.

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LIST OF ABBREVIATIONS

A_1 and A_2	the aldehydes of vitamin A_1 and A_2 (retinal and 3,4-dehydroretinal, respectively)
LWS	long wavelength sensitive
MSP	microspectrophotometer or microspectrophotometry
MWS	middle wavelength sensitive
RH1, RH2, SWS1, SWS2, MWS, LWS, UVS, RH2A, RH2B	short forms for opsins and cone types as described in the text
SWS	short wavelength sensitive
TH	thyroid hormone
λ_{max}	wavelength of maximum absorbance

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