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

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SUMMARY

The role of exogenous thyroid hormone on visual pigment content of rod and cone photoreceptors was investigated in coho salmon (*Oncorhynchus kisutch*). Coho vary the ratio of vitamin A_1- and A_2-based visual pigments in their eyes. This variability potentially alters spectral sensitivity and thermal stability of the visual pigments. We tested whether the direction of shift in the vitamin A_1/A_2 ratio, resulting from application of exogenous thyroid hormone, varied in fish of different ages and held under different environmental conditions. Changes in the vitamin A_1/A_2 visual pigment ratio were estimated by measuring the change in maximum absorbance (λ_{max}) of rods using microspectrophotometry (MSP). Exogenous thyroid hormone resulted in a long-wavelength shift in rod, middle-wavelength-sensitive (MWS) and long-wavelength-sensitive (LWS) cone photoreceptors. Rod and LWS cone λ_{max} values increased, consistent with an increase in vitamin A_2. MWS cone λ_{max} values increased more than predicted for a change in the vitamin A_1/A_2 ratio. To account for this shift, we tested for the expression of multiple RH2 opsin subtypes. We isolated and sequenced a novel RH2 opsin subtype, which had 48 amino acid differences from the previously sequenced coho RH2 opsin. A substitution of glutamate for glutamine at position 122 could partially account for the greater than predicted shift in MWS cone λ_{max} values. Our findings fit the hypothesis that a variable vitamin A_1/A_2 ratio provides seasonality in spectral tuning and/or improved thermal stability of visual pigments in the face of seasonal environmental changes, and that multiple RH2 opsin subtypes can provide flexibility in spectral tuning associated with migration–metamorphic events.

Key words: rhodopsin, porphyropsin, thyroxine, fish, vision, opsin gene sequence, expression, PCR, MSP.

INTRODUCTION

The spectral quality of light in aquatic environments is spatially and temporally more variable than that in terrestrial environments. Aquatic organisms that move between different habitats, or those that inhabit seasonally variable habitats, are faced with the challenge of tuning their spectral sensitivity to maximize detection and identification of targets (predators, prey and conspecifics). In fishes, spectral sensitivity can be adjusted by the addition or loss of a photoreceptor class or by changes to the visual pigments within the photoreceptors themselves (Beaudet and Hawryshyn, 1999; Bowmaker, 1995).

Visual pigments (VPs) comprise two components: an opsin protein and a chromophore. Specific amino acid sites throughout the opsin protein play key roles in spectral tuning of the resultant VP (Yokoyama, 2000; Yokoyama et al., 2007). There are five classes of vertebrate opsins that are categorized based on amino acid sequence and on spectral absorbance (reviewed in Bowmaker, 1995; Yokoyama, 2000). In addition to expressing a representative of one or more of each of these opsin classes, some fishes have recently been found to express different subtypes of the various opsin classes (Chinen et al., 2003; Matsumoto et al., 2006; Wood and Partridge, 1993). Changes in expression levels of opsin subtypes have been associated with ontogenetic changes and metamorphic transitions, some of which have been induced artificially with hormones or the light environment (Beatty, 1975; Carlisle and Denton, 1959; Fuller et al., 2005; Hope et al., 1998; Mader and Cameron, 2004; Shand et al., 2008; Shand et al., 2002; Takechi and Kawamura, 2005).

The other component of the VP, the chromophore, can also be varied in some species. Many freshwater and euryhaline fishes have the ability to change which chromophore is incorporated into their VPs, shifting between retinal (aldehyde of vitamin A_1) and 3,4-dehydroretinal (aldehyde of vitamin A_2), or using mixtures of both (Beatty, 1984). The wavelength of maximum absorbance (λ_{max}) of a VP based on vitamin A_2 is long-wavelength shifted relative to the same opsin combined with vitamin A_1. The long-wavelength shifted vitamin A_2-based VPs are also less thermally stable than vitamin A_1-based VPs, which could have implications for species that inhabit temperate waters where ambient temperatures vary seasonally.

Pacific salmonids are anadromous fishes restricted to temperate climates that are equipped with a remarkably dynamic visual system that varies temporally at different time scales throughout life history (Allison et al., 2006a; Allison et al., 2003; Beatty, 1966; Browman and Hawryshyn, 1994; Hawryshyn et al., 1989; Temple et al., 2006), making this group ideal for investigating adaptive changes in visual pigment composition.

Pacific salmon start life in freshwater as alevin. They become parr once their yolk sac is absorbed. They may spend anywhere from a few days to a few years in fresh water (depending on species)

before migrating to sea. A metamorphic event called smoltification precedes seaward migration, after which they are referred to as smolts. Following a period of rapid growth at sea, the length of which differs among species, they return to their natal streams to spawn and die (Groot and Margolis, 1991). During smoltification, they lose most of their ultraviolet-sensitive (UVS) cones through programmed cell death (Allison et al., 2006a). On their return migration back to fresh water, some of these UVS cones are regenerated (Allison et al., 2006a; Beaudet et al., 1997). It has been proposed that the VP vitamin A_1/A_2 ratio in salmon might follow a similar pattern, changing at the time of the metamorphic–migration event, with vitamin A_2 dominating in fresh water and vitamin A_1 dominating at sea (Alexander et al., 1994; Novales Flamarique, 2005). However, recent observations show a seasonal pattern in vitamin A_1/A_2 VP ratio in all ages of coho salmon (*Oncorhynchus kisutch*) (Walbaum 1792), evidence that this ratio is correlated with seasonal changes in environmental variables and not with the pattern of migration (Temple et al., 2006). With regard to the timing of changes in vitamin A_1/A_2 ratio, we refer to these two models as the migration–metamorphosis and the seasonal hypotheses.

In salmonids, thyroid hormones (THs) are responsible for many of the structural, physiological and behavioral changes associated with smoltification, e.g. silvering, changes in body shape, increased saltwater tolerance, change in rheotaxis, cell proliferation in olfactory epithelium, loss of UVS cones and changes in visual pigment gene expression (Allison et al., 2003; Folmar and Dickhoff, 1980; Grau et al., 1982; Higgs et al., 1982; Hoar, 1988; Lema and Nevitt, 2004; McBride et al., 1982; Specker et al., 2000; Staley and Ewing, 1992; Veldhoen et al., 2006). Earlier research established that TH treatment increased the proportion of vitamin A_2 in the VPs of coho and other salmonids (Beatty, 1972). However, a recent study (Alexander et al., 1998) suggested that the direction of shift in vitamin A_1/A_2 ratio may vary with temperature. Alexander et al. (Alexander et al., 1998) found that coho held in warm water increased the proportion of vitamin A_2 in their VPs when treated with exogenous TH, but that coho held in cold water decreased the proportion of vitamin A_2 in their VPs when treated with exogenous TH.

In the present study, we investigated the effect of TH treatment on VP compositions in coho salmon to determine whether there is variability in the direction of vitamin A_1/A_2 ratio shifts at different times of year and under different environmental conditions, including warm and cold temperatures. We report that TH treatment increased the proportion of vitamin A_2 under all conditions tested. We also provide evidence for a change in opsin expression in middle-wavelength-sensitive (MWS) cones.

MATERIALS AND METHODS

Animal care and experimental design

A series of five experiments was used to compare the effects of TH on vitamin A_1/A_2 VP ratios in coho salmon of various ages, tested at different times of year and held under different environmental conditions (for details, see Table 1).

Coho were provided by two local hatcheries (Robertson Creek hatchery, Department of Fisheries and Oceans, Canada, Port Alberni, British Columbia and Target Marine, commercial hatchery, Sechelt, British Columbia). Both facilities rear coho from eggs to smolts, under natural environmental conditions, in outdoor ponds and tanks. Fish for use in these experiments were transported to the University of Victoria aquatic facilities within a few days of commencing each experiment. Care and treatment of fish was in accordance with University of Victoria's Animal Care Committee, under the auspices of Canadian Council for Animal Care.

Two different TH delivery systems were used for TH treatment: TH-treated food and an exogenous TH bath. Both techniques are effective in stimulating smoltification-like transitions in coho (Alexander, 1998; Alexander et al., 1998; Munz and Beatty, 1965) and vitamin A_1/A_2 ratio shifts in this and other species (Allen, 1977; Allison et al., 2004; Beatty, 1969a; Beatty, 1972; Cristy, 1974; Jacquest and Beatty, 1972). The TH-food treatment was used in Experiments I and II to deliver TH to 150 coho housed in a 750 l cylindrical fiberglass tank with flow-through water replacement. However, it was not possible to control the dose of TH delivered to each fish using TH-treated food. High variation in vitamin A_1/A_2 ratios among the food-treated fish and incomplete transition to vitamin A_2 VP dominance (see Results) led us to use a bath treatment for the remaining experiments in which fewer fish were used. Although an investigation into the differences between these two treatments might be fruitful, it was beyond the scope of this study. Our goal was to determine the direction of shift in vitamin A_1/A_2 ratio and both TH treatments were consistent in this regard.

For Experiments I and II, fish were sampled weekly to track temporal changes in vitamin A_1/A_2 ratio. TH-treated fish were fed commercial salmon pellets sprayed with ethanol containing dissolved L-thyroxine and 3,5,3′-triiodo-L-thyronine (Sigma, St Louis, MO, USA). A mixture of 120 p.p.m. by weight L-thyroxine (T_4) and 12 p.p.m. 3,5,3′-triiodo-L-thyonine (T_3) was used to approximate plasma TH levels found in salmonids prior to smoltification. This dose stimulated smoltification-like transitions in other *Oncorhynchus* spp. (Ebbesson et al., 2000; Plate, 2001). TH-treated fish were fed the T_3–T_4 diet for 1 month, and then given the control diet for an additional 2 weeks. The control group was fed the same commercial salmon pellets, sprayed only with ethanol,

Table 1. Details of environmental conditions, treatments and dates of experiments I–V

Experiment	Dates	Light source	Water temperature	Photoperiod (L:D)	Treatment type	Age (months)	Length (cm)	Weight (g)
I	29.01.2002–25.03.2002	6500°K fluorescent	15±1°C	12 h:12 h	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	12 h:12 h	T_3/T_4 on food	7	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	7 h:19 h	T_4 in water	13	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	4	5.4±0.3	1.8±0.4

for the entire experiment. Both control and treatment fish were fed to satiation every other day. Weekly, three to ten fish were sampled from both control and treatment groups (for details, see sample sizes in results).

For Experiments III–V, T_4 was dissolved in 1.5 ml of 0.1 moles l^{-1} NaOH and added to tank water for a final concentration of 300 μg l^{-1} T_4. Control fish received the vehicle only (1.5 ml of 0.1 moles l^{-1} NaOH). Water was changed three times per week. Both control and treatment groups were fed to satiation every other day with commercial salmon pellets. Five to 10 fish were sampled from both control and treatment groups after 4–6 weeks of treatment.

Microspectrophotometry

Fish were dark adapted for at least 1 hour prior to sacrifice by 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 dorsalmost section of the dorsal hemisphere. This retinal sample was teased apart on a glass coverslip and a drop of minimum essential medium (Sigma, Oakville, ON, Canada; 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.

A CCD–microspectrophotometer (MSP), which 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, Connecticut, USA)] to the photoreceptor outer segment. Beam size was ~2×3 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 1340×400 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) to 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, Germany) was used to position of the photoreceptor outer segment (OS) relative to the measurement beam. The path of the motorized stage was recorded to prevent repeated measurements of photoreceptor OSs. 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: (1) presence of a baseline on the long-wavelength limb (Harosi and MacNichol, 1974); (2) λ_{max} near the expected wavelength for known *Oncorhynchus* spp. photoreceptors [UVS≈350–380 nm; short-wavelength-sensitive (SWS)≈420–450 nm; MWS≈490–550 nm; and long-wavelength-sensitive (LWS)≈540–630 nm; rod≈500–530 nm (Hawryshyn et al., 2001; Hawryshyn and Harosi, 1994)]; (3) minimal absorbance by photoproduct; and (4) signal-to-noise ratio of the main absorption band (α-band) greater than 5:1. Determinations of λ_{max}, and percent vitamin 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 de-trended 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 center of the α-band. The fit of the normalized curve was compared with a nonlinear least-squares routine to the upper 20% of the weighted vitamin A_1/A_2 ratio averaged Govardovskii et al. (Govardovskii et al., 2000) template (based on the center 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 (Munz and Beatty, 1965) for coho rod pigments. Rod absorbance curves were compared (minimum variance fit) to the Munz and Beatty (Munz and Beatty, 1965) template, which extends from the λ_{max} to a point at 20% of the maximum on the long-wavelength arm. This template (Munz and Beatty, 1965) assumes that λ_{max} values of coho rods vary from 503–527 nm. Their model is in close agreement with Harosi's (Harosi, 1994), which predicts that a vitamin A_1-based VP with a λ_{max} of 503 nm shifts to 529 nm if the A_1-based chromophore is replaced with a vitamin A_2-based chromophore in the same opsin (see Results). However, many of the rods we measured had λ_{max} values that exceeded 527 nm and therefore would not fit the Munz and Beatty (Munz and Beatty, 1965) template. In these cases, we used the estimate obtained by the fit to the Govardovskii et al. (Govardovskii et al., 2000) template.

Gene discovery

Coho salmon parr, obtained from Robertson Creek hatchery in 2004 and maintained in outdoor aquatic facilities, were dark adapted for 1 hour and then killed by immersion in 300 mg l^{-1} tricaine methanesulfonate (Crescent Research Chemical, Phoenix, AZ, USA). Neural retina was dissected free of pigmented epithelium under infrared illumination. Immediately after dissection, total RNA was isolated from the tissue using TRIzol reagent (Invitrogen Canada, Burlington, ON, Canada) as per the manufacturer's recommended protocol. Retinal tissue was placed in a 1.5 ml microcentrifuge tube containing 700 μl TRIzol and was homogenized using a disposable Kontes Pellet Pestle with cordless motor tissue grinder (Kimble Kontes, NJ, USA). Isolated RNA was re-suspended in 50 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 of total RNA. Each RNA sample was annealed with 500 ng random hexamer oligonucleotide (Amersham Biosciences, Baie d'Urfe, Québec, Canada) and cDNA was prepared using Superscript II RNase H reverse transcriptase (Invitrogen) as described by the manufacturer's protocol.

A degenerate forward primer (5′-GCTATTGAGAGGTA-CATNGT-3′) was designed based on an alignment of consensus RH2 opsin open reading frame (ORF) sequences and was synthesized by Operon Biotechnologies (Huntsville, AL, USA). A degenerate reverse primer (Johnson et al., 1993) was also used (5′-RAANATNACNGGRTTRAA-3′). All primer pairs used in this

study were diluted and combined in an equimolar ratio to a final concentration of 10 μM. Primers were used to amplify cDNA synthesized from 1 μg parr retinal total RNA. The 20 μl reaction contained 20 mmol l^{-1} Tris-HCl, 50 mmol l^{-1} KCl, 1.5 mmol l^{-1} $MgCl_2$, 200 μmol l^{-1} dNTPs, 1 μmol l^{-1} of each primer, 2 μl cDNA diluted 1:20 and 1.0 U Platinum Taq DNA polymerase (Invitrogen). The thermocycle program was 94°C for 9 min, followed by 30 cycles of 94°C for 30 s, 50°C for 1 min and 72°C for 1.5 min, and a final extension at 72°C for 10 min. Amplicons were separated in a 1.5% 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, Mississauga, ON, 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 coding sequence of the alternate RH2 was isolated from control fish used in Experiment III using the BD SMART RACE cDNA Amplification Kit (BD Biosciences Clontech, Mississauga, ON, 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 Invitrogen and Operon Biotechnologies, respectively, as follows: 3′-RACE primer (5′CTATGCCAGCTTTGCTGCCTGGATT-3′) and 5′-RACE primer (5′-GGCAGCACAGGCCATTGCCATGAC-3′). The 5′-RACE reaction used the following thermal profile: initial denaturation at 94°C for 9 min 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 min, 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 obtained from 5′ and 3′ RACE were assembled and compared with sequences in GenBank using Blastn (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence alignments were performed using 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., 2004; Jokela et al., 2003). This approach is not typical of MSP studies, which classically report the mean λ_{max} for each class of receptor from all fish sampled (e.g. Cummings and Partridge, 2001; Harosi and Kleinschmidt, 1993; Hawryshyn et al., 2001; Nawrocki, 1985; Novales Flamarique, 2005). However, it is statistically correct to treat the fish as the sample unit, rather than the individual photoreceptors, because photoreceptors from the same fish lack independence (pseudoreplication). When comparisons were made between fish for a particular receptor class, we used the mean λ_{max} of all receptors collected from each fish (fish mean λ_{max}). When comparisons were made between control and treatment groups for a particular receptor class, we 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}).

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 (α=0.05).

RESULTS

Exogenous TH treatments (both TH diet and TH bath) resulted in typical parr–smolt-like transitions in all five experiments. TH-treated fish exhibited physical characteristics associated with post smoltification: loss of parr marks, increased silvering, blue–green dorsal coloration, and decreased condition index (body weight to length ratio). None of these changes was observed in control fish.

Experiments I and II: timeline of shift in vitamin A_1/A_2 ratio during TH treatment

In Experiments I and II, performed in winter and summer, respectively (Table 1), we made weekly comparisons of the mean λ_{max} of rods from both control and TH-treated groups. In both experiments, rod λ_{max} gradually increased in TH-treated groups relative to controls, and after 4–5 weeks TH-treated groups had shifted to significantly (P<0.05) longer wavelengths than controls (Table 2; Fig. 1A,B). At the end of the treatment period, when TH-treated fish were put on the control diet, there was a rapid decrease in λ_{max}, and after 2 weeks mean λ_{max} values of TH-treated and control groups were not significantly (P>0.4) different (Fig. 1A,B).

Experiments III–V

In Experiments III–V, exogenous TH significantly (P<0.001) increased group mean λ_{max} of rods, MWS and LWS cones. For rods, the long-wavelength shift in group mean λ_{max} matched that anticipated for a shift from vitamin A_1- to A_2-based VP dominance (Table 2, Fig. 2). The distribution of λ_{max} values from all rods from all fish sampled in Experiments III–V extended from ~500 to 540 nm (Fig. 3A).

Experiments III–V: MWS and LWS cone photoreceptors

For LWS cones, like rods, the mean λ_{max} values of TH-treated groups were shifted to significantly (P<0.001) longer wavelengths than controls in all three experiments (Fig. 2). Among the three experiments, the control groups did not differ significantly (ANOVA; F_{16}=0.861, P=0.444) nor did the TH-treated groups (ANOVA; F_{17}=0.209, P=0.746). The range of λ_{max} values for LWS cones extended from 563 nm to 633 nm (Fig. 3B).

For MWS cones, like the rods and LWS cones, the λ_{max} values of TH-treated groups were shifted to longer wavelengths than the controls in all three experiments. However, we did not perform ANOVA tests because we had evidence that more than one variable

Table 2. Comparisons of rod λ_{max} between control and treatment groups for experiments I–V

Group	Expt I (week 5)	Expt II (week 4)	Expt III	Expt IV	Expt V
Control	510.4±6.3 (3)	509.5±1.8 (8)	513.0±3.8 (8)	510.8±1.9 (5)	509.6±0.7 (6)
Treatment	521.0±4.8 (3)	515.2±4.9 (8)	532.3±1.1 (7)	531.5±1.4 (5)	533.0±1.0 (5)
t-test	t_4=–3.561, P=0.024	t_{14}=–3.042, P=0.009	t_{14}=–13.916, P<0.001	t_7=–18.760, P<0.001	t_8=–43.853, P<0.001

Rod group mean λ_{max} (nm) values are given ±s.d. *N* values are in parentheses.

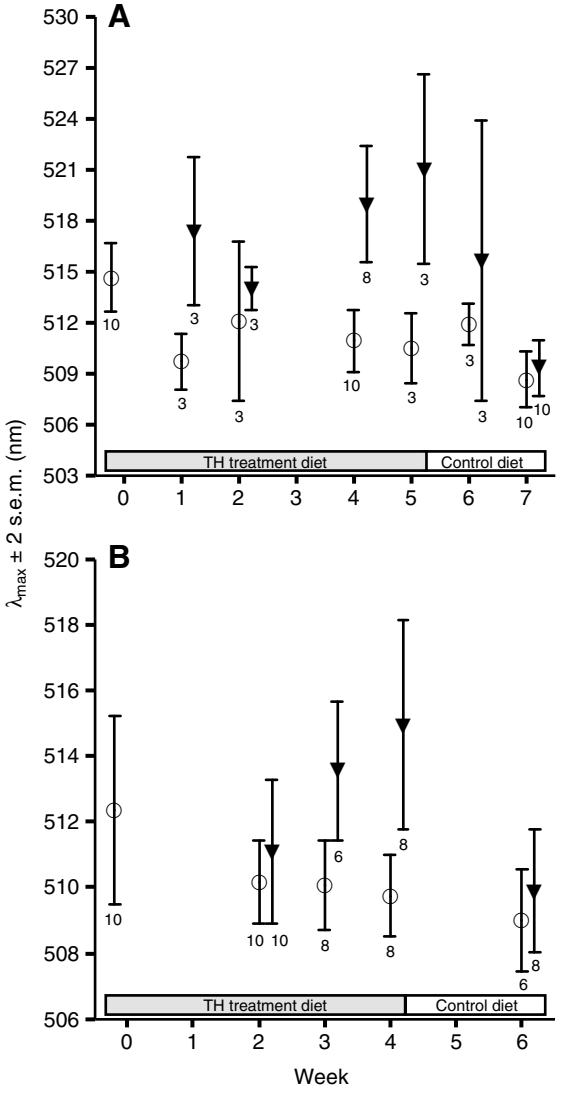

Fig. 1. Group mean λ_{max} ± 2 s.e.m. of rods for the control group (circles) and TH treatment group (triangles) from Experiment I performed January–March (A) and Experiment II performed in July and August (B). Each point is the mean rod λ_{max} of all coho salmon (*Oncorhynchus kisutch*, Walbaum) sampled 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 ~20 rods measured using MSP. Treated fish received commercial salmon pellets sprayed with 12 p.p.m. T_3 and 120 p.p.m. T_4 by weight dissolved in ethanol. The horizontal bar shows the timing of the transition from the treatment (gray) to the control (white) diet for the treatment group. The control group remained on a diet of commercial salmon pellets sprayed with ethanol only for the duration of the experiments.

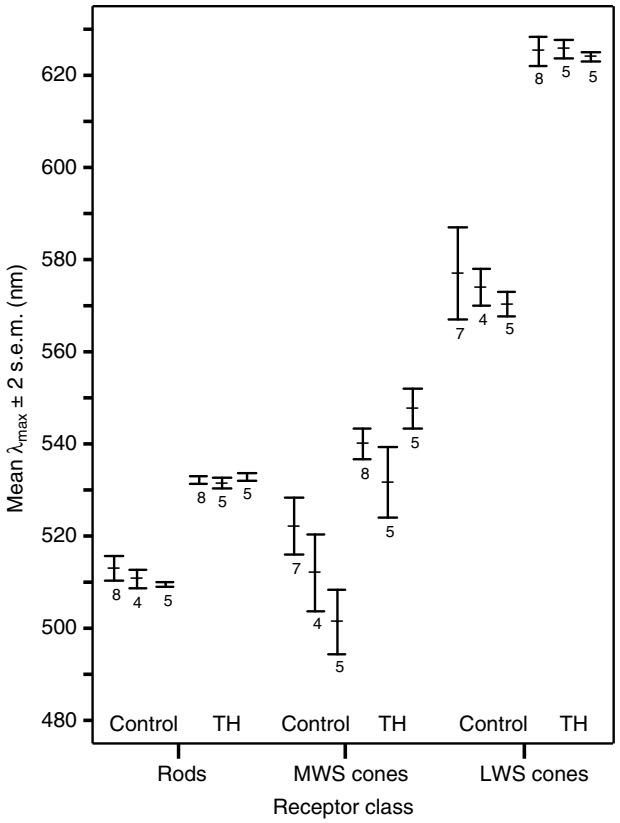

Fig. 2. Group mean λ_{max} ± 2 s.e.m. of rods, middle-wavelength-sensitive (MWS) and long-wavelength-sensitive (LWS) cones from control and treatment groups for Experiments III–V (plotted in numerical order from left to right). Absorbance spectra of individual photoreceptors from the dorsal retina of 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 ~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 (P<0.001) in all three experiments.

was changing (see below). The group mean λ_{max} values for MWS cones ranged from 501.5 to 547.7 nm (Fig. 2). The range of λ_{max} values for all individual MWS cones (from all fish, from Experiments III–V) spanned a spectral range from below 490 nm to above 550 nm (Fig. 3C). Both measures of change in λ_{max} suggest a shift that was greater than predicted for a shift from vitamin A_1- to A_2-based chromophores in a single opsin. There are six empirical models that predict the change in λ_{max} ($\Delta\lambda_{max}=A_2\lambda_{max}-A_1\lambda_{max}$) that occurs when vitamin A_1 and A_2 chromophores are exchanged in a single VP opsin (Bridges, 1965; Dartnall and Lythgoe, 1965; Harosi, 1994; Parry and Bowmaker, 2000; Tsin et al., 1981; Whitmore and Bowmaker, 1989). For vitamin A_1-based VPs with λ_{max} values between 495 nm and 512 nm (Fig. 4), $\Delta\lambda_{max}$ is predicted to be between 16.6 nm (Parry and Bowmaker, 2000) and 36.0 nm (Whitmore and Bowmaker, 1989). The $\Delta\lambda_{max}$ value we observed for MWS cones was between 46.2 nm and 60 nm (13.8 nm range for $\Delta\lambda_{max}$ is the difference between using the group mean λ_{max} value or the mean of all individual cones, respectively). Regardless of how it was calculated, the magnitude of $\Delta\lambda_{max}$ was greater than predicted by any of the existing models. We therefore hypothesized that more than one subtype of the RH2 opsin was being expressed in coho MWS cones. Given this proposed explanation, it was not appropriate to treat all MWS cones as if originating from a single normally distributed population. Therefore, MWS cone λ_{max} values are hereafter described by the range of λ_{max} values recorded from all individual MWS cones from each group of fish.

Modeling multiple opsins in MWS cones

Estimating the λ_{max} of the hypothesized opsin subtypes was complicated by the variation in λ_{max} that resulted from the variable chromophore ratio. To address this, we plotted λ_{max} values from measurements made on ~100 MWS–LWS double cones from

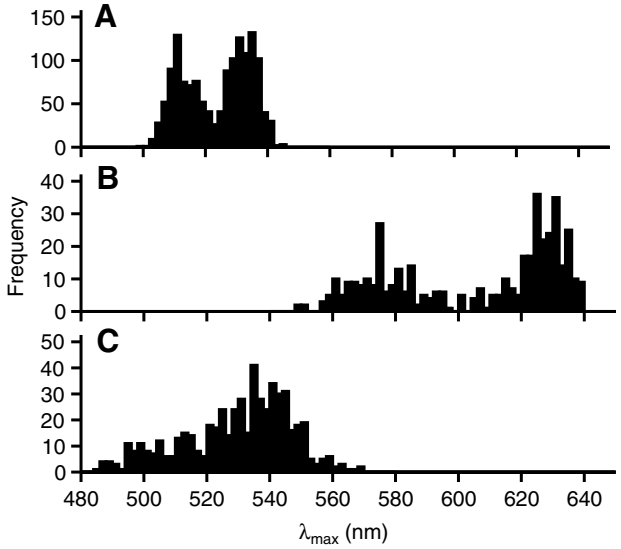

Fig. 3. Frequency histograms of λ_{max} values for rods (A), long-wavelength-sensitive (LWS) cones (B) and middle-wavelength-sensitive (MWS) cones (C) recorded from all control and TH-treated coho salmon (*Oncorhynchus kisutch*, Walbaum) used in Experiments III–V.

which both OSs had been recorded (Fig. 5). The expectation for such a plot, for a species with only one opsin in each of its double cone OSs and using only one chromophore type (i.e. not coho salmon), is 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 vitamins A_1 and A_2 (as was thought to be the case for coho), the prediction is a distribution of points tightly grouped along a straight line that extends diagonally away from the origin (Loew and Dartnall, 1976). Our observation did not match either of these predictions and, instead, looked like a diagonal line with a positive slope stretched out along the horizontal axis (Fig. 5). This distribution indicated the presence of more than one opsin being expressed in MWS cone OSs.

To estimate λ_{max} values of the two hypothesized opsin subtypes, we defined the limits of the distribution with lines that took into account a measurement error of ±3 nm on all sides (Fig. 5), except the lower side (see below). The distribution of λ_{max} values for LWS cones extended from 563 to 633 nm. The distribution for MWS cones extended from 495 to 548 nm. To calculate the λ_{max} values of the corresponding vitamin A_1- or A_2-based VP pairs, we assigned the lower limit of the λ_{max} values as the λ_{max} for the vitamin A_1-based VP and the upper limit as the vitamin A_2-based VP. These values were then used in equations provided in the six published models predicting $\Delta\lambda_{max}$ (Bridges, 1965; Dartnall and Lythgoe, 1965; Harosi, 1994; Parry and Bowmaker, 2000; Tsin et al., 1981; Whitmore and Bowmaker, 1989) to estimate the λ_{max} of predicted

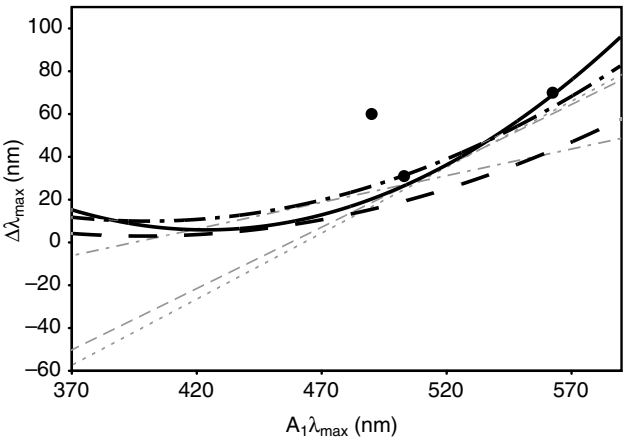

Fig. 4. Six models that predict the expected shift in visual pigment λ_{max} ($\Delta\lambda_{max}=A_2\lambda_{max}-A_1\lambda_{max}$) when one opsin is combined with vitamins A_1 and A_2 chromophores as a function of the λ_{max} of the vitamin A_1 member. These models are compared with the observed values for rods, middle-wavelength-sensitive (MWS) and long-wavelength-sensitive (LWS) cones in coho salmon (*Oncorhynchus kisutch*, Walbaum). Lines representing each model are from Bridges (Bridges, 1965) (gray dotted), from Dartnall and Lythgoe (Dartnall and Lythgoe, 1965) (gray dashed), from Tsin et al. (Tsin et al., 1981) (gray dotted and dashed), from Whitmore and Bowmaker (Whitmore and Bowmaker, 1989) (black dotted and dashed), from Harosi (Harosi, 1994) (solid black), and from Parry and Bowmaker (Parry and Bowmaker, 2000) (black dashed). The observed range for rods, MWS and LWS cones based on the mean per fish are plotted as filled circles. The spectral shift observed in rods and LWS cones falls within the predicted range of the models, indicating that the observed variance in λ_{max} values can be explained by a change in chromophore ratio. The spectral shift of MWS cones lies outside the range predicted by all six models and therefore the variance in λ_{max} of MWS cones cannot be explained by a shift in chromophore ratio alone: a second RH2 opsin subtype is implicated in this shift.

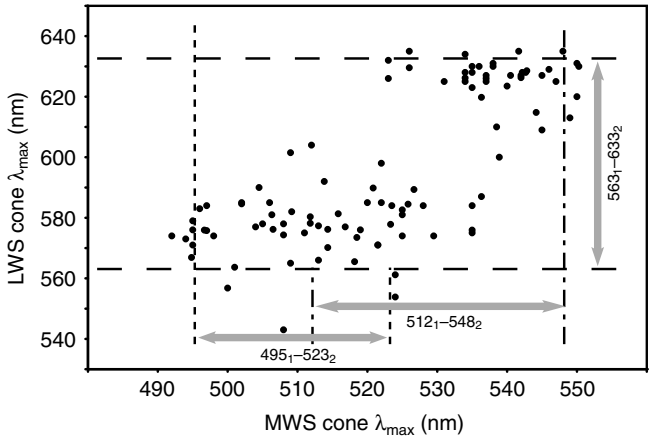

Fig. 5. Scatter plot showing λ_{max} values for middle-wavelength-sensitive (MWS) and long-wavelength-sensitive (LWS) outer segments from individual double cones where both outer segments were measured. The horizontal dashed lines indicate the minimum and maximum λ_{max} values predicted for a single LWS opsin with a λ_{max} range of 563_1–633_2 nm using Harosi's (Harosi, 1994) model (for model selection criteria, see text). Vertical dotted line predicts 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 495_1 nm as the vitamin A_1 observed value, we used Whitmore and Bowmaker's (Whitmore and Bowmaker, 1989) model to calculate that the same opsin would have a λ_{max} of 523_2 nm if combined with a vitamin A_2 chromophore. Using the inverse of Whitmore and Bowmaker's (Whitmore and Bowmaker, 1989) model, we estimated that the vitamin A_1-based visual pigment that corresponded to the 548_2 nm MWS cone value at the long-wavelength end of the range would have a λ_{max} of 512_1 nm.

VP pair. 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 $\Delta\lambda_{max}$ for the given vitamin A_1–A_2 VP pair [Harosi's (Harosi, 1994) model for the long-wavelength range and Whitmore and Bowmaker's (Whitmore and Bowmaker, 1989) for the middle-wavelength range].

The LWS cone λ_{max} distribution was explained by a single LWS opsin combining with vitamins A_1 and A_2 to give a range of 563_1–633_2 nm (subscripts denote vitamins A_1 and A_2 chromophore types, respectively). The most parsimonious model to account for the observed range in MWS cones was to assign the longest value (548 nm) as the vitamin A_2 state of one VP pair and the shortest value (495 nm) as the vitamin A_1 state of a second VP pair. We proposed that one VP pair had a range of 495_1–523_2 nm and the other VP pair had a range of 512_1–548_2 nm.

Molecular evidence for two RH2 opsin subtypes

To test the hypothesis that coho express a second RH2 opsin subtype, retinal material was collected from fish that showed a large range in MWS cone λ_{max} values. Two subtypes of the RH2 opsin were isolated, cloned and sequenced. One subtype had been previously identified in coho [RH2 (Dann et al., 2004)], here renamed RH2A, and the second was a novel RH2 sequence that we have named RH2B (GenBank Accession Number DQ309027) in accordance with nomenclature recently used for other fish opsin subtypes (Carleton and Kocher, 2001; Collin et al., 2003; Matsumoto et al., 2006).

The possession of several amino acids that are conserved among vertebrate opsins suggests that RH2B is a functional opsin. The predicted RH2B amino acid sequence has a lysine at position 296 that forms a Schiff's base linkage with the chromophore (Wang et al., 1980). RH2B has glutamic acid at position 113, which is the counter ion of the protonated Schiff's base (Sakmar et al., 1989; Zhukovsky and Oprian, 1989), and cysteine residues at positions 110 and 187, which form a disulphide bond within the opsin (Karnik et al., 1988). RH2B also has three amino acids, S240, T243 and V250, which are located in cytoplasmic loop 3 and are conserved in all opsins (Archer, 1995).

Alignment of RH2A and RH2B sequences (Fig. 6) reveals 48 amino acid differences (86.1% amino acid sequence identity). The substitution of glutamate (E) in RH2A for glutamine (Q) in RH2B at position 123 (analogous to position 122 in bovine rod opsin) could play an important role in tuning the λ_{max} of these pigments (see Discussion).

Summary of results

The main findings of these experiments were: (1) exogenous TH induced smoltification-like transitions in pre-smolt coho under all environmental conditions tested and throughout the year; (2) exogenous TH resulted in a long-wavelength shift in rod λ_{max} in all five experiments; (3) the observed range of group mean λ_{max} values for rods (503–533 nm) was consistent with a shift in vitamin A_1/A_2 ratio; (4) 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 vitamin A_1/A_2 ratio within a single opsin; and (5) a second RH2 opsin subtype was isolated, cloned and sequenced.

DISCUSSION

Effect of TH on rod photoreceptors

Exogenous TH increased the λ_{max} of all photoreceptors regardless of differences in age, time of year, rearing conditions or TH delivery method. In rods, the 30 nm increase in group mean λ_{max} was consistent with a change in vitamin A_1/A_2 VP ratio based on predicted $\Delta\lambda_{max}$ values (Bridges, 1965; Dartnall and Lythgoe, 1965; Harosi, 1994; Parry and Bowmaker, 2000; Tsin et al., 1981; Whitmore and Bowmaker, 1989). This TH-induced increase in vitamin A_2 is consistent with nearly all previous studies with teleosts (Alexander et al., 1998; Allen, 1971; Allen, 1977; Allison et al., 2004; Beatty, 1969a; Cristy, 1974; Jacquest and Beatty, 1972; McFarland and Allen, 1977; Munz and Swanson, 1965; Tsin and Beatty, 1979), except one trial in a study by Alexander et al. (Alexander et al., 1998), which showed a decrease in vitamin A_2 in coho reared at cold temperatures (5°C). Further investigation is required to understand the factors that account

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RH2A MQNGTEGSNF YIPMSNRTGL VRSPFEYPQY YLAPPWQYYC LAVYTFFLIC 50
RH2B MQNGTEGNNF YIPMSNRTGL VRSPFLYQQY YLADPWQFYL LAVYMFFLIC 50
           *                    * *      *   * *     *

RH2A FGFPINGLTL YVTATNKKLR QPLNFILVNL AAAGMIMVLF GFTITITSAV 100
RH2B FGFPINGLTL YVTATNKKLQ QPLNFILANL AAAGMIMVMF GFTITITSAV 100
                          *        *           *

RH2A NGYFIWGPLG CAIEGFMATL GGEVALWSLV VLAVERYIVV CKPMGSFTFT 150
RH2B NGYFVFGPMG CAIEGFMATL GGQVALWSLV VLAIERYIVV CKPMGSFTFT 150
         **  *               *           *

RH2A STHAGAGVAF TWIAAMACAA PPLLGWSRYI PEGMQCSCGP DYYTLAEGFN 200
RH2B TTHAGAGCAF TWVMAMACAA PPLVGWSRYI PEGMQCSCGP DYYTLAEGFN 200
     *      *     **          *

RH2A NESYVIYMFS CHFIIPVCLI AYTYGSLVLT VKAAAASQQD SASTQKAEKE 250
RH2B NESYVIYMFT CHFCVPVVTI FFTYGSLVLT VKAAAASQQD SASTQKAEKE 250
              *    **  **  **

RH2A VTRMCILMVC GFMVAWTPYA TLAAYIFFNK GIAFSAQSMA IPAFFSKSSA 300
RH2B VTRMCFLMVC GFLIAWTPYA SFAAWIFFNK GAAFTATAMA IPAFFSKSSA 300
          *       **       **  *       *  * **

RH2A LFNPIIYVLM NKQFRGCMLA AVGMKA-EEG ETSVSTSKTE VSSAGPA    346
RH2B IFNPVIYVLM NKQFRSCMLA AVGISSGAED ETSVSASKTE VSSVGPA    347
     *   *           *        ***** *      *        *
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Fig. 6. Alignment between the amino acid sequences of the coho (*Oncorhynchus kisutch*, Walbaum) RH2A (Dann et al., 2004) and RH2B protein deduced from coho middle-wavelength-sensitive (MWS) opsin cDNA show 86.1 percent amino acid sequence identity. Conserved residues in positions analogous to bovine I113, C187 and K296 are in bold. The 48 amino acid differences between the RH2A and RH2B are marked by asterisks below the sequence. The E123Q substitution (analogous to position 122 in bovine rod opsin), which may be responsible for the short-wavelength shift of the RH2B relative to RH2A, is in bold with an asterisk below.

for the difference between our results in cold water (3–5°C) and those of Alexander et al. (Alexander et al., 1998).

The λ_{max} values recorded from individual rods, rather than group mean values, had a range that was greater than the $\Delta\lambda_{max}$ models predicted, and the mean λ_{max} of rods from the TH-bath treated fish was 532.7 nm, which is long-wavelength shifted by nearly 5 nm relative to previous reports for coho salmon (Alexander, 1998; Alexander et al., 1994; Beatty, 1972; Munz and Beatty, 1965; Temple et al., 2006). One explanation is that our TH-bath treatment may have resulted in a complete transition to vitamin A_2-based VPs, whereas previous studies used fish that may have had intermediary vitamin A_1/A_2 ratios owing to the source of fish or treatments given [e.g. wild *versus* untreated fish (Alexander et al., 1994; Temple et al., 2006), TH in diet (Alexander et al., 1998) (present study), or intraperitoneal injections of TH for less than 14 days (Beatty, 1972)]. The large shifts in λ_{max}, resulting from TH treatment may rarely occur naturally, but they demonstrate the extent of adaptability of this species.

Alternatively, it is possible that a second opsin subtype is expressed in coho rods. European eels (*Anguilla anguilla* L.) express two RH1 opsin subtypes. Under natural conditions, the European eel alters expression of its RH1 opsin subtypes upon migrating from fresh water to the sea (Carlisle and Denton, 1959), and they have been induced to shift between the two subtypes using exogenous hormone treatment (Hope et al., 1998; Wood and Partridge, 1993). Recent evidence suggests that rainbow trout (*O. mykiss*) could also have two RH1 opsin genes (Allison et al., 2006b). The expression of these two genes was shown to vary between TH-treated and control fish, as proposed here for the RH2 opsin subtypes. It is possible that the wide range of values observed for rods was the result of a TH-induced shift to a second RH1 opsin subtype in coho.

LWS cone λ_{max} range

In our plot of LWS verses MWS cone λ_{max} values, we observed three outliers below the lower horizontal line at 563 nm, which delineated the vitamin A_1 state of the proposed LWS opsin (Fig. 5). The data points came from three separate control fish. We are collecting measurements from additional fish as part of a subsequent study to define more accurately the lower limit of λ_{max} values for LWS cones in coho salmon. Again, we would like to suggest this is preliminary evidence for the presence of multiple subtypes of the LWS opsin.

Two RH2 opsin subtypes in coho MWS cones

MSP and molecular approaches have provided independent evidence for the presence of two RH2 opsin subtypes in coho salmon. The λ_{max} values recorded from MWS cones ranged from 495 nm to 548 nm ($\Delta\lambda_{max}$=53 nm). This observation argues strongly for two opsin subtypes, especially in the context of several models predicting a $\Delta\lambda_{max}$ no greater than 36 nm (Bridges, 1965; Dartnall and Lythgoe, 1965; Harosi, 1994; Parry and Bowmaker, 2000; Tsin et al., 1981; Whitmore and Bowmaker, 1989). Our molecular analysis led to the isolation and sequencing of a novel subtype of the RH2 opsin gene, thus confirming our hypothesis. When compared with the existing sequence [RH2A (Dann et al., 2004)], there were 48 amino acid differences (Fig. 6). Key among these is likely to be the substitution of glutamate for glutamine at position 123, which is analogous to 122 in bovine RH1. This particular site plays an important role in spectral tuning of both RH1 and RH2 opsins (Yokoyama et al., 1999). The E122Q substitution results in a short-wavelength shift in bovine RH1 by 20–25 nm (Sakmar et al., 1989). In zebrafish, three out of the four RH2 opsin subtypes have glutamate at position 122 and all three absorb maximally at shorter wavelengths (17–38 nm) than the fourth, which has glutamine at position 122 (Chinen et al., 2003; Chinen et al., 2005). Based on our estimates from Fig. 5, the predicted values for the RH2A and RH2B opsin subtypes, when combined with vitamin A_1, were 512_1 nm and 495_1 nm, respectively. This observed shift in λ_{max} was within the range previously reported for other opsins differing by an E122Q substitution. Based on its sequence and on our MSP results, RH2B was short-wavelength shifted by ~17 nm relative to RH2A.

The magnitude of shift in λ_{max} between control and TH-treated groups suggested that TH played a role in regulating expression levels of RH2A and RH2B opsin subtypes. This is consistent with the function of TH as a signaling mechanism in vertebrate metamorphosis (Power et al., 2001), and in retinal development and opsin expression (Allison et al., 2003; Browman and Hawryshyn, 1992; Harpavat and Cepko, 2003; Roberts et al., 2006). Current efforts in our laboratory are aimed at determining the spatiotemporal expression patterns of RH2A and B opsins in coho and other Pacific salmonids.

The ecological significance of the second RH2 opsin subtype was not immediately evident. The λ_{max} of MWS cones in TH-treated fish were long-wavelength shifted relative to control fish, suggesting that the natural state for post smoltification coho was to express the long-wavelength shifted RH2A opsin. This would seem counterintuitive as after smoltification, coho migrate to sea where the spectral distribution is short-wavelength shifted relative to most freshwater habitats (Tyler and Smith, 1970).

A similar shift in λ_{max} of MWS cones was reported to occur naturally in coho by Novales Flamarique (Novales Flamarique, 2005). He described the change in MWS cone λ_{max} as being a ‘compensatory’ shift in response to the simultaneous loss of UVS cones from the retina. We suggest that MWS cones are shifted to longer wavelengths so that they are offset from the main spectral distribution of light along the sidewelling line of sight. Several marine fish have been shown to employ this tactic to enhance the detection of bright reflective targets against the nearly monochromatic blue–green background (McFarland and Munz, 1975). When coho move from fresh water to sea they shift their diet from predominantly terrestrial and aquatic insects to small fish and crustaceans. Crypsis in freshwater streams is accomplished by earthy and dark coloration patterns that match the substrate, therefore prey detection would be optimized by having VPs matched to the spectral background. In the open ocean, crypsis is accomplished by being transparent, or silvery, to reflect the monochromatic background light (Denton and Nicol, 1965; Johnsen, 2002; McFall-Ngai, 1990). The long-wavelength shift in MWS cone λ_{max} would offset the VP improving the detection of brightly colored prey. The change in MWS cone λ_{max} might be equally important for conspecific recognition. Coho are territorial while in freshwater streams but join schools when they enter the estuaries, and they alter their appearance at smoltification by changing their dark reddish-brown parr marks and dorsal pigmentation to silvery sides and blue–green dorsal pigmentation (Groot and Margolis, 1991).

Migration–metamorphosis or seasonal vitamin A_1/A_2 ratio shift

Our observation, of an increase in vitamin A_2 with the application of TH lends support to the seasonal hypothesis for explaining the timing of shifts in A_1/A_2 ratio. Given that metamorphic transitions are driven by TH signaling mechanisms in vertebrates (Mader and

Cameron, 2004; Power et al., 2001), and that TH induces smoltification-like transitions in salmon (see Introduction), then under the migration–metamorphosis hypothesis, it would be expected that TH induce a decrease in A_2 as naturally occurs when salmon undergo smoltification prior to seaward migration. In fact the opposite was observed. The λ_{max} of rods increased in a manner consistent with an increase in vitamin A_2 under all conditions tested, leading us to conclude that the vitamin A_1/A_2 ratio is independent of smoltification in coho salmon. These findings support our previous observations (Temple et al., 2006) that the A_1/A_2 ratio in coho salmon is linked to seasonal environmental changes, a pattern also observed in other teleosts (Allen et al., 1982; Beatty, 1969b; Dartnall et al., 1961; Ueno et al., 2005), amphibians (Makino et al., 1983) and invertebrates (Suzuki et al., 1984).

The selective advantage of shifting from vitamin A_2 in winter to vitamin A_1 in summer could be that it improves the signal-to-noise ratio in the face of seasonal variation in temperature and light conditions. Vitamin A_1-based VPs are more thermally stable than are vitamin A_2-based VPs (Ala-Laurila et al., 2003; Ala-Laurila et al., 2004; Barlow, 1957). And because photoreceptors are unable to distinguish between photo- and thermal-isomerization events (Barlow, 1957) vitamin A_1-based VPs will provide a higher signal-to-noise ratio (Aho et al., 1988; Barlow, 1988). The eyes of exothermic organisms such as fish, amphibians and invertebrates are subject to environmental changes and as temperature increases, the signal-to-noise ratio worsens (Baylor et al., 1980). The possible advantages of long-wavelength-shifted vitamin A_2-based VPs must be countered by the poorer signal-to-noise ratio at higher temperatures (Allen and McFarland, 1973). We interpret the variable vitamin A_1/A_2 ratio in coho as a trade-off between increasing spectral breadth of sensitivity and minimizing noise when temperatures rise (Temple et al., 2006).

In summary, exogenous TH produced long-wavelength shifts in photoreceptor λ_{max} that were explicable by changes in vitamin A_1/A_2 VP chromophore ratio combined with changes in opsin subtype expression. In addition to expressing representatives of all five vertebrate opsin classes, coho were found to express at least two subtypes of RH2 opsin. Our MSP evidence also indicates the possibility of two subtypes of the RH1 and LWS opsin genes. The potential to vary spectral sensitivity, through variable opsin expression together with alterations in the vitamin A_1/A_2 VP ratio, provides coho with a dynamic visual system that operates over spatially and temporally diverse spectral environments encountered throughout their complex life history.

LIST OF SYMBOLS AND ABBREVIATIONS

λ_{max}	wavelength of maximum absorbance
CCD	charge couple device
LED	light emitting diode
MSP	microspectrophotometer or microspectrophotometry
OS	outer segment
PCR	polymerase chain reaction
RPE	retinal pigmented epithelium
TH	thyroid hormone
VP	visual pigment

RH1, RH2, SWS1, SWS2, MWS, LWS, UVS, RH2A, RH2B are all short forms for opsins and cone types as described in the text

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REFERENCES

Aho, A. C., Donner, K., Hyden, C., Larsen, L. O. and Reuter, T. (1988). Low retinal noise in animals with low body temperature allows high visual sensitivity. *Nature* **334**, 348-350.

Ala-Laurila, P., Albert, R. J., Saarinen, P., Koskelainen, A. and Donner, K. (2003). The thermal contribution to photoactivation in A2 visual pigments studied by temperature effects on spectral properties. *Vis. Neurosci.* **20**, 411-419.

Ala-Laurila, P., Pahlberg, J., Koskelainen, A. and Donner, K. (2004). On the relation between the photoactivation energy and the absorbance spectrum of visual pigments. *Vision Res.* **44**, 2153-2158.

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

Alexander, G., Sweeting, R. and McKeown, B. (1994). The shift in visual pigment dominance in the retinae of juvenile coho salmon (*Oncorhynchus kisutch*): an indicator of smolt status. *J. Exp. Biol.* **195**, 185-197.

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

Allen, D. M. (1971). Photic control of the proportions of two visual pigments in a fish. *Vision Res.* **11**, 1077-1112.

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

Allen, D. M. and McFarland, W. N. (1973). The effect of temperature on rhodopsin-porphyropsin ratios in a fish. *Vision Res.* **13**, 1303-1309.

Allen, D. M., Loew, E. R. and McFarland, W. N. (1982). Seasonal change in the amount of visual pigment in the retinae of fish. *Can. J. Zool.* **60**, 281-287.

Allison, W. T., Dann, S. G., Veldhoen, K. M. and Hawryshyn, C. W. (2006a). Degeneration and regeneration of ultraviolet cone photoreceptors during development in rainbow trout. *J. Comp. Neurol.* **499**, 702-715.

Allison, W. T., Veldhoen, K. M. and Hawryshyn, C. W. (2006b). Proteomic analysis of opsins and thyroid hormone-induced retinal development using isotope-coded affinity tags (ICAT) and mass spectrometry. *Mol. Vision* **12**, 655-672.

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

Allison, W. T., Haimberger, T. J., Hawryshyn, C. W. and Temple, S. E. (2004). Visual pigment composition in zebrafish: evidence for a rhodopsin-porphyropsin interchange system. *Vis. Neurosci.* **21**, 945-952.

Archer, S. (1995). Molecular biology of visual pigments. In *Neurobiology and Clinical Aspects of the Outer Retina* (ed. M. B. A. Djamgoz, S. Archer and S. Vallerga), pp. 79-104. London: Chapman & Hall.

Barlow, H. B. (1957). Purkinje shift and retinal noise. *Nature* **179**, 255-256.

Barlow, H. B. (1988). The thermal limit to seeing. *Nature* **334**, 296-297.

Baylor, D. A., Matthews, G. and Yau, K. W. (1980). Two components of electrical dark noise in toad retinal rod outer segments. *J. Physiol.* **309**, 591-621.

Beatty, D. D. (1966). A study of the succession of visual pigments in Pacific salmon (*Oncorhynchus*). *Can. J. Zool.* **44**, 429-455.

Beatty, D. D. (1969a). Visual pigment changes in juvenile kokanee salmon in response to thyroid hormones. *Vision Res.* **9**, 855-864.

Beatty, D. D. (1969b). Visual pigments of the burbot, *Lota lota*, and seasonal changes in their relative proportions. *Vision Res.* **9**, 1173-1183.

Beatty, D. D. (1972). Visual pigment changes in salmonid fishes in response to exogenous L-thyroxine, bovine TSH and 3-dehydroretinol. *Vision Res.* **12**, 1947-1960.

Beatty, D. D. (1975). Visual pigments of the American eel *Anguilla rostrata*. *Vision Res.* **15**, 771-776.

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

Beaudet, L. and Hawryshyn, C. W. (1999). Ecological aspects of vertebrate visual ontogeny. In *Adaptive Mechanisms in the Ecology of Vision* (ed. S. N. Archer, M. B. A. Djamgoz, E. R. Loew, J. C. Partridge and S. Vallerga), pp. 413-437. London: Kluwer Academic.

Beaudet, L., Novales Flamarique, I. and Hawryshyn, C. W. (1997). Cone photoreceptor topography in the retina of sexually mature Pacific salmonid fishes. *J. Comp. Neurol.* **383**, 49-59.

Bowmaker, J. K. (1995). The visual pigments of fish. *Prog. Retin. Eye Res.* **15**, 1-27.

Bridges, C. D. B. (1965). The grouping of fish visual pigments about preferred positions in the spectrum. *Vision Res.* **5**, 223-238.

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

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

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

Carlisle, D. B. and Denton, E. J. (1959). On the metamorphosis of the visual pigments of *Anguilla anguilla* (L.). *J. Mar. Biol. Assoc. U.K.* **38**, 97-102.

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G. and Thompson, J. D. (2003). Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res.* **31**, 3497-3500.

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

Chinen, A., Matsumoto, Y. and Kawamura, S. (2005). Reconstitution of ancestral green visual pigments of zebrafish and molecular mechanism of their spectral differentiation. *Mol. Biol. Evol.* **22**, 1001-1010.
Collin, S. P., Hart, N. S., Shand, J. and Potter, I. C. (2003). Morphology and spectral absorption characteristics of retinal photoreceptors in the southern hemisphere lamprey (*Geotria australis*). *Vis. Neurosci.* **20**, 119-130.
Cristy, M. (1974). Effects of prolactin and thyroxine on the visual pigments of trout, *Salmo gairdneri*. *Gen. Comp. Endocrinol.* **23**, 58-62.
Cummings, M. E. and Partridge, J. C. (2001). Visual pigments and optical habitats of surfperch (Embiotocidae) in the California kelp forest. *J. Comp. Physiol. A* **187**, 875-889.
Dann, S. G., Allison, W. T., Levin, D. B., Taylor, J. S. and Hawryshyn, C. W. (2004). Salmonid opsin sequences undergo positive selection and indicate an alternative evolutionary relationship in *Oncorhynchus*. *J. Mol. Evol.* **58**, 400-412.
Dartnall, H. J. A. and Lythgoe, J. N. (1965). The spectral clustering of visual pigments. *Vision Res.* **5**, 81-100.
Dartnall, H. J. A., Lander, M. R. and Munz, F. W. (1961). Periodic changes in the visual pigment of a fish. In *Progress in Photobiology* (ed. B. C. Christensen and B. Buchman), pp. 203-213. Amsterdam: Elsevier.
Denton, E. J. and Nicol, J. A. (1965). Studies on reflexion of light from silvery surfaces of fishes, with special reference to the bleak *Alburnus alburnus*. *J. Mar. Biol. Assoc. U. K.* **45**, 683-703.
Ebbesson, L. O. E., Björnsson, B. T., Stefansson, S. O. and Ekström, P. (2000). Free plasma thyroxine levels in coho salmon, *Oncorhynchus kisutch*, during parr-smolt transformation: comparison with total thyroxine, total triiodothyronine, and growth hormone levels. *Fish Physiol. Biochem.* **22**, 45-50.
Folmar, L. C. and Dickhoff, W. W. (1980). The parr-smolt transformation (smoltification) and seawater adaptation in salmonids – a review of selected literature. *Aquaculture* **21**, 1-37.
Fuller, R. C., Carleton, K. L., Fadool, J. M., Spady, T. C. and Travis, J. (2005). Genetic and environmental variation in the visual properties of bluefin killifish, *Lucania goodei*. *J. Evol. Biol.* **18**, 516-523.
Govardovskii, V. I., Fyhrquist, N., Reuter, T., Kuzmin, D. G. and Donner, K. (2000). In search of the visual pigment template. *Vis. Neurosci.* **17**, 509-528.
Grau, E. G., Specker, J. L., Nishioka, R. S. and Bern, H. A. (1982). Factors determining the occurrence of the surge in thyroid activity in salmon during smoltification. *Aquaculture* **28**, 49-57.
Groot, C. and Margolis, L. (1991). *Pacific Salmon Life Histories*. Vancouver: University of British Columbia Press.
Harosi, F. I. (1987). Cynomolgus and rhesus monkey visual pigments. Application of Fourier transform smoothing and statistical techniques to the determination of spectral parameters. *J. Gen. Physiol.* **89**, 717-743.
Harosi, F. I. (1994). An analysis of two spectral properties of vertebrate visual pigments. *Vision Res.* **34**, 1359-1367.
Harosi, F. I. and Kleinschmidt, J. (1993). Visual pigments in the sea lamprey, *Petromyzon marinus*. *Vis. Neurosci.* **10**, 711-715.
Harosi, F. I. and MacNichol, E. F., Jr (1974). Visual pigments of goldfish cones. Spectral properties and dichroism. *J. Gen. Physiol.* **63**, 279-304.
Harpavat, S. and Cepko, C. L. (2003). Thyroid hormone and retinal development: an emerging field. *Thyroid* **13**, 1013-1019.
Hawryshyn, C. W. and Harosi, F. I. (1994). Spectral characteristics of visual pigments in rainbow trout (*Oncorhynchus mykiss*). *Vision Res.* **34**, 1385-1392.
Hawryshyn, C. W., Arnold, M. G., Chaisson, D. J. and Martin, P. C. (1989). The ontogeny of ultraviolet photosensitivity in rainbow trout (*Salmo gairdneri*). *Vis. Neurosci.* **2**, 247-254.
Hawryshyn, C. W., Haimberger, T. J. and Deutschlander, M. E. (2001). Microspectrophotometric measurements of vertebrate photoreceptors using CCD-based detection technology. *J. Exp. Biol.* **204**, 2431-2438.
Higgs, D. A., Fagerlund, U. H. M., Eales, J. G. and McBride, R. E. (1982). Application of thyroid and steroid hormones as anabolic agents in fish culture. *Comp. Biochem. Physiol.* **73B**, 143-176.
Hoar, W. S. (1988). The physiology of smolting salmonids. In *Fish Physiology*. Vol. XI (ed. W. S. Hoar and D. J. Randall), pp. 275-343. Toronto: Academic Press.
Hope, A. J., Partridge, J. C. and Hayes, P. K. (1998). Switch in rod opsin gene expression in the European eel, *Anguilla anguilla* (L.). *Proc. R. Soc. Lond. B Biol. Sci.* **265**, 869-874.
Jacquest, W. L. and Beatty, D. D. (1972). Visual pigment changes in the rainbow trout, *Salmo gairdneri*. *Can. J. Zool.* **50**, 1117-1126.
Johnsen, S. (2002). Cryptic and conspicuous coloration in the pelagic environment. *Proc. Biol. Sci.* **269**, 243-256.
Johnson, R. L., Grant, K. B., Zankel, T. C., Boehm, M. F., Merbs, S. L., Nathans, J. and Nakanishi, K. (1993). Cloning and expression of goldfish opsin sequences. *Biochemistry* **32**, 208-214.
Jokela, M., Vartio, A., Paulin, L., Fyhrquist-Vanni, N. and Donner, K. (2003). Polymorphism of the rod visual pigment between allopatric populations of the sand goby (*Pomatoschistus minutus*): a microspectrophotometric study. *J. Exp. Biol.* **206**, 2611-2617.
Karnik, S. S., Sakmar, T. P., Chen, H. B. and Khorana, H. G. (1988). Cysteine residues 110 and 187 are essential for the formation of correct structure in bovine rhodopsin. *Proc. Natl. Acad. Sci. USA* **85**, 8459-8463.
Lema, S. C. and Nevitt, G. A. (2004). Evidence that thyroid hormone induces olfactory cellular proliferation in salmon during a sensitive period for imprinting. *J. Exp. Biol.* **207**, 3317-3327.
Loew, E. R. and Dartnall, H. J. A. (1976). Vitamin A1/A2-based visual pigment mixtures in cones of the rudd. *Vision Res.* **16**, 891-896.
Mader, M. M. and Cameron, D. A. (2004). Photoreceptor differentiation during retinal development, growth, and regeneration in a metamorphic vertebrate. *J. Neurosci.* **24**, 11463-11472.
Makino, M., Nagai, K. and Suzuki, T. (1983). Seasonal variation of the vitamin A2-based visual pigment in the retina of adult bullfrog, *Rana catesbeiana*. *Vision Res.* **23**, 199-204.
Matsumoto, Y., Fukamachi, S., Mitani, H. and Kawamura, S. (2006). Functional characterization of visual opsin repertoire in Medaka (*Oryzias latipes*). *Gene* **371**, 268-278.
McBride, J. R., Higgs, D. A., Fagerlund, U. H. M. and Buckley, J. T. (1982). Thyroid hormones and steroid hormones: potential for control of growth and smoltification of salmonids. *Aquaculture* **28**, 201-210.
McFall-Ngai, M. (1990). Crypsis in the pelagic environment. *Am. Zool.* **30**, 175-188.
McFarland, W. N. and Allen, D. M. (1977). The effect of extrinsic factors on two distinctive rhodopsin-porphyropsin systems. *Can. J. Zool.* **55**, 1000-1009.
McFarland, W. N. and Munz, F. W. (1975). Part III. The evolution of photopic visual pigments in fishes. *Vision Res.* **15**, 1071-1080.
Munz, F. W. and Beatty, D. D. (1965). A critical analysis of the visual pigments of salmon and trout. *Vision Res.* **5**, 1-17.
Munz, F. W. and Swanson, R. T. (1965). Thyroxine-induced changes in the proportions of visual pigments. *Am. Zool.* **5**, 583.
Nawrocki, L. (1985). Development of the neural retina in the zebrafish, *Danio rerio*. In *Biology*, 146 pp. University of Oregon.
Novales Flamarique, I. (2005). Temporal shifts in visual pigment absorbance in the retina of Pacific salmon. *J. Comp. Physiol. A* **191**, 37-49.
Parry, J. W. and Bowmaker, J. K. (2000). Visual pigment reconstitution in intact goldfish retina using synthetic retinaldehyde isomers. *Vision Res.* **40**, 2241-2247.
Plate, E. M. (2001). Olfactory imprinting in sockeye salmon (*Oncorhynchus nerka*). In *Biology*, 161 pp. Victoria: University of Victoria.
Power, D. M., Llewellyn, L., Faustino, M., Nowell, M. A., Bjornsson, B. T., Einarsdottir, I. E., Canario, A. V. and Sweeney, G. E. (2001). Thyroid hormones in growth and development of fish. *Comp. Biochem. Physiol.* **130C**, 447-459.
Roberts, M. R., Srinivas, M., Forrest, D., Morreale de Escobar, G. and Reh, T. A. (2006). Making the gradient: thyroid hormone regulates cone opsin expression in the developing mouse retina. *Proc. Natl. Acad. Sci. USA* **103**, 6218-6223.
Sakmar, T. P., Franke, R. R. and Khorana, H. G. (1989). Glutamic acid-113 serves as the retinylidene Schiff base counterion in bovine rhodopsin. *Proc. Natl. Acad. Sci. USA* **86**, 8309-8313.
Shand, J., Hart, N. S., Thomas, N. and Partridge, J. C. (2002). Developmental changes in the cone visual pigments of black bream *Acanthopagrus butcheri*. *J. Exp. Biol.* **205**, 3661-3667.
Shand, J., Davies, W. L., Thomas, N., Balmer, L., Cowing, J. A., Pointer, M., Carvalho, L. S., Trezise, A. E. O., Collin, S. P., Beazley, L. D. et al. (2008). The influence of ontogeny and light environment on the expression of visual pigment opsins in the retina of the balck bream, *Acanthopagus butcheri*. *J. Exp. Biol.* **211**, 1495-1503.
Smith, H. O. (1980). Recovery of DNA from gels. *Meth. Enzymol.* **65**, 371-380.
Specker, J. L., Eales, J. G., Tagawa, M. and Tyler, W. A. (2000). Parr-smolt transformation in Atlantic salmon: thyroid hormone deiodination in liver and brain and endocrine correlates of change in rheotactic behavior. *Can. J. Zool.* **78**, 696-705.
Staley, K. B. and Ewing, R. D. (1992). Purine levels in the skin of juvenile coho salmon (*Oncorhynchus kisutch*) during parr-smolt transformation and adaptation to seawater. *Comp. Biochem. Physiol.* **101B**, 447-452.
Suzuki, T., Makino-Tasaka, M. and Eguchi, E. (1984). 3-Dehydroretinal (vitamin A_2 aldehyde) in crayfish eye. *Vision Res.* **24**, 783-787.
Takechi, M. and Kawamura, S. (2005). Temporal and spatial changes in the expression pattern of multiple red and green subtype opsin genes during zebrafish development. *J. Exp. Biol.* **208**, 1337-1345.
Temple, S. E., Plate, E. M., Ramsden, S., Haimberger, T. J., Roth, W. M. and Hawryshyn, C. W. (2006). Seasonal cycle in vitamin A_1/A_2-based visual pigment composition during the life history of coho salmon (*Oncorhynchus kisutch*). *J. Comp. Physiol. A* **192**, 301-313.
Tsin, A. T. C. and Beatty, D. D. (1979). Scotopic visual pigment composition in the retina and vitamins A in the pigment epithelium of the goldfish. *Exp. Eye Res.* **29**, 15-26.
Tsin, A. T., Liebman, P. A., Beatty, D. D. and Drzymala, R. (1981). Rod and cone visual pigments in the goldfish. *Vision Res.* **21**, 943-946.
Tyler, J. E. and Smith, R. C. (1970). *Measurements of Spectral Irradiance Underwater*. London: Gordon and Breach, Science Publishers.
Ueno, Y., Ohba, H., Yamazaki, Y., Tokunaga, F. and Hariyama, T. (2005). Seasonal variation of chromophore composition in the eye of the Japanese dace, *Tribolodon hakonensis*. *J. Comp. Physiol. A* **191**, 1137-1142.
Veldhoen, K., Allison, W. T., Veldhoen, N., Anholt, B. R., Helbing, C. C. and Hawryshyn, C. W. (2006). Spatio-temporal characterization of retinal opsin gene expression during thyroid hormone-induced and natural development of rainbow trout. *Vis. Neurosci.* **23**, 169-179.
Wang, J. K., McDowell, J. H. and Hargrave, P. A. (1980). Site of attachment of 11-cis-retinal in bovine rhodopsin. *Biochemistry* **19**, 5111-5117.
Whitmore, A. V. and Bowmaker, J. K. (1989). Seasonal variation in cone sensitivity and short-wave absorbing visual pigments in the rudd *Scardinius erythrophthalmus*. *J. Comp. Physiol. A* **166**, 103-116.
Wood, P. and Partridge, J. C. (1993). Opsin substitution induced in retinal rods of the eel (*Anguilla anguilla* (L.)): a model for G-protein-linked receptors. *Proc. R. Soc. Lond. B Biol. Sci.* **254**, 227-232.
Yokoyama, S. (2000). Molecular evolution of vertebrate visual pigments. *Prog. Retin. Eye Res.* **19**, 385-419.
Yokoyama, S., Zhang, H., Radlwimmer, F. B. and Blow, N. S. (1999). Adaptive evolution of color vision of the Comoran coelacanth (*Latimeria chalumnae*). *Proc. Natl. Acad. Sci. USA* **96**, 6279-6284.
Yokoyama, S., Tada, T. and Yamato, T. (2007). Modulation of the absorption maximum of rhodopsin by amino acids in the C-terminus. *Photochem. Photobiol.* **83**, 236-241.
Zhukovsky, E. A. and Oprian, D. D. (1989). Effect of carboxylic acid side chains on the absorption maximum of visual pigments. *Science* **246**, 928-930.