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Spatio-temporal characterization of retinal opsin gene expression during thyroid hormone-induced and natural development of rainbow trout

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Abstract

The abundance and spatial distribution of retinal cone photoreceptors change during thyroid hormone (TH)-induced and natural development of rainbow trout (*Oncorhynchus mykiss*). These changes are thought to allow the fish to adapt to different photic environments throughout its life history. To date, the ontogeny of rainbow trout cone photoreceptors has been examined using physiological and morphological approaches. In this study, we extended these observations by measuring opsin gene expression in retinal quadrants during natural and TH-induced development. Gene expression during natural development was investigated in retinae from fish at both parr and smolt stages. The role of TH in modulating opsin gene expression was determined in TH-treated parr and control fish sampled after two, nine, and 22 days of treatment. Total RNA was isolated from each retinal quadrant and steady-state opsin mRNA levels were measured using reverse transcriptase real-time quantitative polymerase chain reaction (QPCR) analysis. Expression of ultraviolet-sensitive opsin (SWS1), rod opsin (RH1), middle wavelength-sensitive opsin (RH2), and long wavelength-sensitive opsin (LWS) transcripts vary spatially in the parr retina. Smolts, compared to parr, had downregulated SWS1 expression in all quadrants, lower LWS expression dorsally, higher RH1 expression nasally, and higher RH2 expression dorsally. In TH-treated parr, SWS1 opsin expression was downregulated in the nasal quadrants by two days. SWS1 displayed the greatest degree of downregulation in all quadrants after nine days of treatment, with an increase in short wavelength-sensitive (SWS2) and RH2 opsin mRNA expression in the temporal quadrants. This study reveals that opsin genes display spatially significant differences within rainbow trout retina in their level of mRNA expression, and that regulation of opsin expression is a dynamic process that is influenced by TH. This is particularly evident for SWS1 gene expression in parr following TH-induced and natural development.

Keywords: opsin, *Oncorhynchus mykiss*, cone photoreceptor, thyroid hormone, development

Introduction

Visual pigments are light-absorbing molecules located in the outer segments of photoreceptor cells in vertebrate retinae. A visual pigment is comprised of an opsin protein (seven transmembrane G-protein-coupled receptor) covalently bound to a light-sensitive chromophore (vitamin A_1 or A_2 in vertebrates). The spectral range over which a visual pigment absorbs is influenced by the unique electrical environment imparted to the chromophore by the amino acid side chains of the opsin transmembrane helices (Nathans, 1987). The type of chromophore associated with the opsin also affects the absorption spectrum, with vitamin A_2-based pigments being shifted to longer wavelengths than vitamin A_1-based pigments (Bridges, 1972).

Five retinal opsin groups exist, based on both nucleotide and amino acid sequence, that originated very early in vertebrate evolution: SWS1 opsins are violet- or ultraviolet-sensitive (UVS), SWS2 opsins are short wavelength-sensitive (SWS), RH1 opsins comprise the rod opsin group, RH2 opsins are rod opsin-like and are middle wavelength-sensitive (MWS), and finally LWS/MWS are long or middle wavelength-sensitive (Yokoyama, 2000; Collin & Tresize, 2004). In the rainbow trout, *Oncorhynchus mykiss*, opsin representatives of these five groups are expressed in individual cone photoreceptor subtypes, which are organized in a square mosaic pattern (Hawryshyn et al., 2001; Allison et al., 2003; Dann et al., 2004*a*). These include; a UVS, SWS1 opsin expressed in accessory corner cones (UVS corner cones), a SWS2 opsin expressed in central short single cones, a RH1, rod opsin expressed in rods, a RH2 opsin expressed in the accessory member of double cones, and a LWS opsin located in the long member of double cones. Rainbow trout possess paired pigments, meaning their opsins can bind both vitamin A_1 and

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A_2-based chromophores. The sensitivity of a given opsin type is dependent on the associated A_1/A_2 ratio (Bridges, 1972). It is important to note that sensitivity in a visual system is dictated in part by the types, numbers, and spatial distribution of visual pigments expressed in a retina.

The retina of the rainbow trout is a useful model to study developmental changes in opsin gene expression. During ontogeny, changes in visual sensitivity are correlated with remodeling of the photoreceptor mosaic (Hawryshyn et al., 1989; Beaudet et al., 1993; Allison et al., 2003). In particular, during transformation from the juvenile "parr" stage to the post-juvenile "smolt" stage, there is a loss of UV sensitivity coincident with the loss of UVS corner cones. At a later post-juvenile life history stage and at sexual maturity, UVS corner cones are present in the dorsal temporal (DT) quadrant (Beaudet et al., 1997; Deutschlander et al., 2001; Allison et al., 2003; Hawryshyn et al., 2003). These changes in the visual system, along with other physiological changes, occur naturally under the influence of thyroid hormone (TH), and can be induced by administration of exogenous TH (Hoar, 1988; Browman & Hawryshyn, 1992, 1994).

TH plays an essential role in the growth and development of all vertebrates, including fish (Leatherland, 1994; Oppenheimer et al., 1995). The thyroid gland produces TH in the form of thyroxine (T_4), which is subsequently converted through deiodinase activity to the more bioactive triiodothyronine (T_3) form in peripheral tissues (Köhrle, 2002). TH plays a role in regulation of specific gene expression by binding to and modulating the activity of nuclear thyroid hormone receptors (TRα and TRβ) that interact with TH-responsive gene promoters (Zhang & Lazar, 2000; Forrest et al., 2002). In this way, TH helps to coordinate the expression of many diverse gene products that are required during critical developmental stages. In the visual system of many vertebrates, TH has been found to influence retinal development, including retinal progenitor differentiation, photoreceptor development, chromophore abundance, and opsin expression (Allison et al., 2003; Harpavat & Cepko, 2003).

To date, morphological approaches such as *in situ* hybridization, immunocytochemistry, and topographical mapping have been employed to study the ontogeny of cone photoreceptors in the rainbow trout (Beaudet et al. 1997; Allison et al., 2003; Hawryshyn et al., 2003). In the present study, we extend these investigations and quantify opsin gene expression profiles during natural and TH-induced development. As the loss of UVS corner cones has previously been shown to be asymmetric across the retina (Beaudet et al., 1997; Allison et al., 2003; Hawryshyn et al., 2003), we included a spatial analysis by examining retinal quadrants. Examination of the steady-state levels of five opsin gene mRNA transcripts was performed using a reverse transcriptase real-time quantitative polymerase chain reaction (QPCR) method. Changes in mRNA abundance were observed for all opsins in TH-treated parr. In particular, SWS1 mRNA displayed strong TH-dependent downregulation in the retina of hormone-treated parr and was also downregulated during the transition from parr to smolt.

Materials and methods

Animals

Animal care and treatment was in accordance with and approved by the University of Victoria Animal Care Committee, under the auspices of the Canadian Council of Animal Care. Wild stock rainbow trout (*Oncorhynchus mykiss*) were obtained from the Vancouver Island Trout Hatchery (Duncan, BC, Canada). Fish were maintained indoors in 350 L flow-through freshwater (15 ± 1°C) holding tanks. The University of Victoria Aquatic Holding Facility uses a continuous flow recirculation system, which controls temperature, biological waste, and appropriate gas levels. Illumination was provided by fluorescent lights (Stanpro FU32T8/65K/8 6500K) on a 12 h light and 12 h dark cycle. The spectral distribution (Fig. 1) was measured using an Ocean Optics USB 2000 spectrophotometer (Ocean Optics, Dunedin, FL). Fish were fed Trout AB feed (Moore-Clark, Vancouver, BC, Canada) three times per week. The parr and smolts used in this study were from the same source; however, the smolts were from a different brood stock. The smolts were maintained under facility conditions for 16 months and the parr for six weeks prior to experimentation.

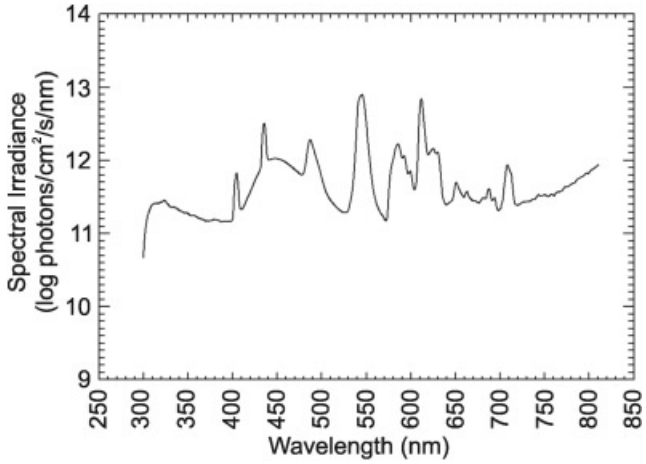

Fig. 1. Spectral irradiance at the water surface of tanks in the fish holding facility, as measured by the Ocean Optics Inc USB2000 spectrophotometer.

Technically, the terms "parr" and "smolt" are used to describe pre- and post-migratory stages of anadromous salmonids, respectively. Anadromous fish migrate from fresh to saltwater when they become smolts, and then back to freshwater as reproductive adults. Rainbow trout are a non-anadromous population of the facultative anadromous species, remaining in fresh water throughout their life history. However, changes observed in the rainbow trout visual system during development are similar to the changes that occur in their migratory counterparts.

TH-induced and natural development

For the TH-induction experiment, 30 parr were split between three 15 L tanks (10 fish per tank). Thirty additional parr were placed in three separate 15 L control tanks (10 fish per tank). L-thyroxine sodium salt pentahydrate (T_4; Sigma, St. Louis, MO) dissolved in 0.1 M NaOH at a concentration of 3 mg/ml was added to the water at a dilution of 1:10,000 to a final nominal concentration of 300 μg/L. Static renewal of water and chemicals was performed daily. Control animals were treated identically with the exception that only 0.1 M NaOH vehicle was added to the water. Fish were fed a maintenance diet during T_4 exposures to minimize growth. Ten fish were sampled from each control and TH treatment tank at two, nine, and 22 days following the initiation of the experiment. Previous experiments have indicated that changes in visual sensitivity of TH-treated parr occur by six weeks of hormone treat-

ment (Browman & Hawryshyn, 1992, 1994; Allison et al., 2003). We therefore chose three earlier time points to detect changes in opsin gene expression that are involved in this process. Average standard lengths (±1 S.D.) were as follows: control day two = 53 ± 6 mm, treatment day two = 56 ± 6 mm, control day nine = 53 ± 4 mm, treatment day nine = 53 ± 7 mm, control day 22 = 58 ± 5 mm, and treatment day 22 = 56 ± 5 mm. For the natural development experiment, 10 parr and 10 smolts were sampled from their holding tanks. Average standard lengths (±1 S.D.) were 53 ± 5 mm for parr and 264 ± 18 mm for smolts.

Retinal isolation

Fish were dark-adapted and then killed by immersion in 300 mg/L tricaine methanesulfonate (Crescent Research Chemical, Phoenix, AZ) for a period of 10 min. Dark-adaptation commenced at 09:30 h and retinal collection was conducted between 11:00 h and 13:00 h during the light phase of the photoperiod. Neural retina was dissected free of pigmented epithelium from the left eye of each animal under deep red light illumination and in ice-cold phosphate-buffered saline. Immediately after dissection, each isolated retina was preserved in 1 ml RNA*later* (Ambion, Inc., Austin, TX) and stored at 4°C. Each retina was subsequently cut into DT, dorsal nasal (DN), ventral nasal (VN), and ventral temporal (VT) quadrants using the embryonic fissure as a standardized landmark. Individual quadrants were placed in fresh RNA*later* (0.5 ml for parr quadrants and 1 ml for smolt quadrants) and stored at 4°C.

Preparation of retinal total RNA and cDNA

Total RNA was isolated from retinal tissue using TRIzol reagent (Invitrogen Canada Inc., Burlington, Ontario, Canada) as per the manufacturer's recommended protocol. Each retinal quadrant was placed in a 1.5 ml microcentrifuge tube containing TRIzol reagent (200 μl for parr quadrants and 700 μl for smolt quadrants) and a 3 mm tungsten carbide bead, and was homogenized using a Retsch MM301 Mixer mill (Retsch GmBH & Co. Ltd. KG, Hann, Germany) for 6 min at 20 Hz. Homogenization racks were rotated 180° halfway through the procedure. 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 parr retinal quadrants. Isolated RNA was re-suspended in RNase-free water (20 μl for parr quadrant samples and 50 μl for smolt quadrant samples). RNA concentration was determined for smolt retinal samples by measuring absorbance using spectrophotometry at a standard wavelength of 260 nm.

Total cDNA was synthesized using 10 μl of the RNA sample (determined to represent approximately 1 μg total RNA) isolated from parr quadrants and 1 μg of RNA isolated from smolt quadrants. 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. mykiss* SWS1, SWS2, RH1, RH2, LWS, and ribosomal protein L8 (L8) open reading frame (ORF) sequences (GenBank accession numbers AF425074, AF425075, AF425072, AF425076, AF425073, AY957563, respectively) using Primer Premier V4.1 software (Premier Biosoft International, Palo Alto, CA), and synthesized by Alpha DNA (Montreal, Québec, Canada) (Table 1). Primer pairs were diluted and combined in an equimolar ratio to a final concentration of 10 μM. We chose L8 as our normalization reference for gene across samples as, in this study, its expression did not vary significantly either spatially within the retina or following TH treatment (data not shown). In addition, L8 has been shown to remain invariant following TH treatment and during natural post-embryonic development in anuran models (Shi & Liang, 1994; Callery & Elinson, 2000; Crump et al., 2002). We utilized primers designed for amphibian L8 (forward primer 5′-CAGGGGACAGAGAAAAGGTG-3′, reverse primer 5′-GTCTT ATTGCTGCTCGTCGT-3′) to PCR-amplify and clone a partial L8 ORF sequence from rainbow trout retinal cDNA (GenBank accession number AY957563).

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 on a ChemiImager 4000 (Alpha Innotech Corp., San Leandro, CA) (Fig. 2a). 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 Inc., Mississauga, Ontario, Canada) and sequenced (Centre for Biomedical Research DNA Sequencing Facility, University of Victoria). Positive identification of cloned DNA amplicons served to confirm that each gene-specific primer pair was

Table 1. *Gene-specific primer sequences used in QPCR. Linear R^2 value is a measure of PCR efficiency, calculated from the slope of the standard plots generated for each target sequence*

Gene	Forward Primer	Reverse Primer	Amplicon Size (bp); Linear R^2 Value
SWS1	GGCTTTCTACCTACAGACTGC	CCTGCTATTGAACCCATGC	258; 0.956
SWS2	GGCACTGCTATCAACGTCCT	CCACTACTGAGAGAGACCATAA	244; 0.987
RH1	CGTCCCTATGTCCAATGCT	AGTGGTGAAGCCTCCGATT	250; 0.952
RH2	GAACGGCACAGAAGGAAGCA	AAAGAGGACCATTATCATTC	265; 0.984
LWS	ATGCAGCCAGGCGACAAA	ATTCCGCAAGTGGAGACA	376; 0.892
L8	GGTGTGGCTATGAATCCTGT	ACGACGAGCAGCAATAAGAC	126; 0.998

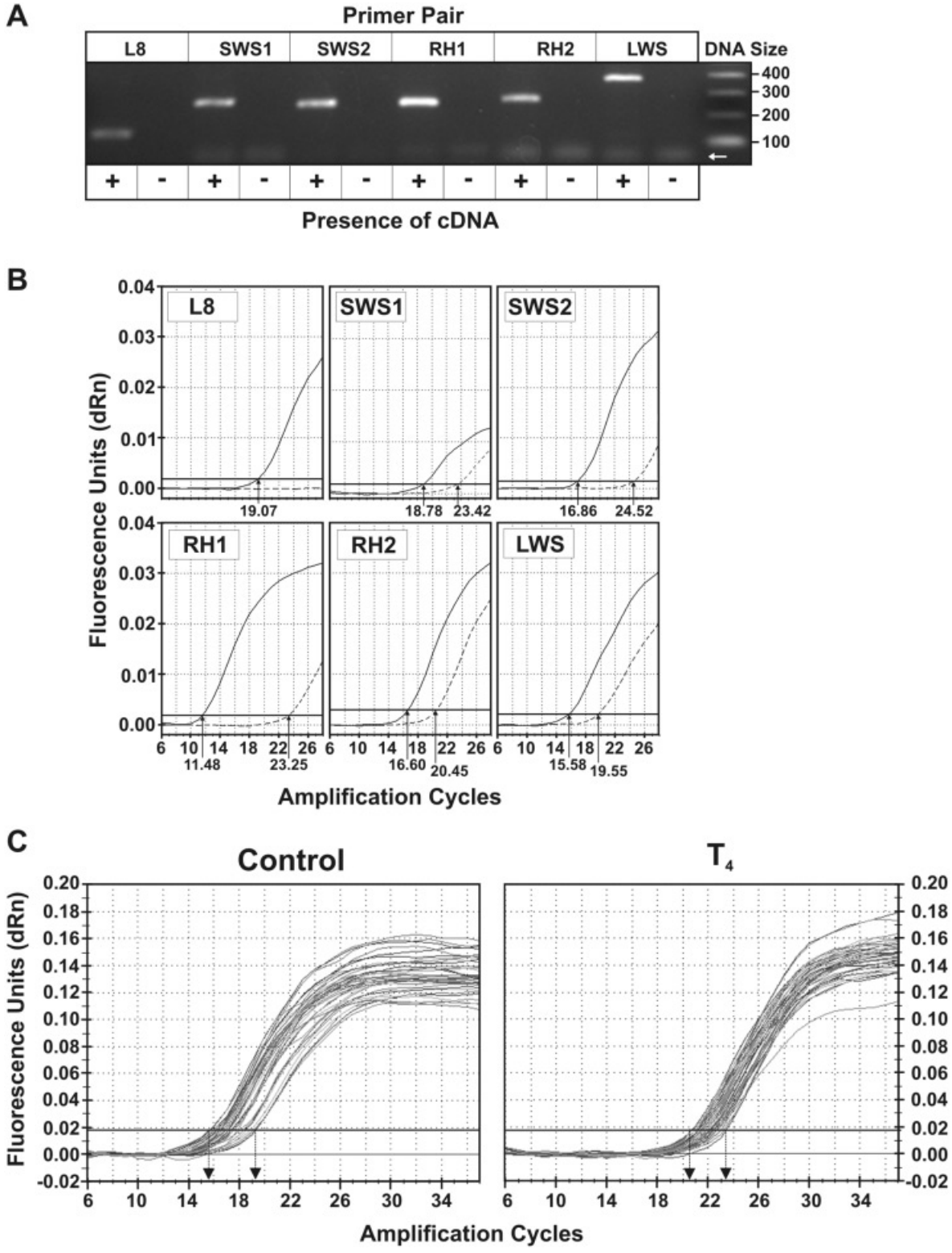

Fig. 2. Quality control assessment of real-time quantitative polymerase chain reaction (QPCR) analysis performed on opsin genes expressed in the rainbow trout retina. A. Gene-specific DNA amplicons produced during the QPCR run of a cDNA sample prepared from parr whole retina were separated on a 1.5% agarose gel. Reactions without cDNA added were also performed to assess non-specific background amplification. Primer dimers are denoted by a white arrow. B. Amplification curves derived for six gene targets analyzed for expression in rainbow trout parr whole retina. Shown are the cDNA-associated amplification signal(solid line) and the no DNA template control (dashed line) fluorescent signals with associated Ct values identified by arrows. C. A representitive example of a shift in the QPCR-derived amplification curves following changes in SWS1 gene expression in the DT region of parr retina following exposure to TH for nine days. The arrows show the hormone-dependent shift in Ct values that reflect a change in steady-state mRNA abundance.

amplifying the correct cDNA target sequence from rainbow trout retinal samples.

Real-time quantitative polymerase chain reaction

QPCR analysis of individual retinal cDNA samples was carried out using L8, SWS1, SWS2, RH1, RH2, and LWS primer sets. All primer pairs were tested for good quality signal-to-noise detection of target gene sequences expressed in a cDNA sample prepared from parr retinal tissue (Fig. 2a and Fig. 2b). Each 15 μl reaction contained 10 mM Tris HCl, 50 mM KCl, 3 mM $MgCl_2$, 0.01% Tween 20, 0.8% glycerol, 40,000-fold dilution of SYBR Green I (Molecular Probes Inc., Eugene, OR), 200 μM dNTPs, 83 nM ROX reference dye (Stratagene, La Jolla, CA), 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 a 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, 55°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 eight randomly chosen cDNA samples and genomic DNA isolated from parr retina using the SWS2 primer set (DNA amplicons of 244 bp and approximately 550 bp, respectively). No genomic DNA contamination was evident in the cDNA samples used for the QPCR (data not shown). Opsin gene expression for each retinal sample was analyzed in quadruplicate, averaged, and normalized to expression of the L8 control. DNA-associated fluorescent signal collected from each sample reaction was smoothed using a three-point moving average method and corrected for volumetric variation using the ROX passive reference dye (that is, dRn selection). Cycle threshold (Ct) values, defined as the cycle at which the SYBR Green I-detected amplification of specific DNA sequence crosses a threshold fluorescent value 10 standard deviations above the baseline signal that is determined between cycles 5 and 9 of the thermocycle program, were subsequently collected. An example of amplification curves obtained for SWS1 in the DT retinal region of parr treated with TH for nine days and matched time point controls ($n = 10$ for each) is shown (Fig. 2c). A delay in amplification reflecting decreased SWS1 mRNA abundance following hormone treatment is evident. Collected Ct data 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.

Statistical analyses

The TH treatment study measured five opsin genes in four quadrants for a total of 20 response variables. These variables cannot be analyzed individually because they are correlated with each other. Analyzing each variable separately can easily lead to false rejection of the null hypothesis of no treatment effect because of the repeated tests. When working with multiple, inter-correlated variables, an alternative to analyzing each measurement separately is to extract principal components of the correlation matrix and analyze the principal components. Principal components are interpreted by the contribution of individual variables to the component scores. Sequential components are, by definition, uncorrelated with each other. This method thus summarizes many variables into fewer uncorrelated variables while retaining most of the variance in the original data (Quinn & Keough, 2002). Principal component analysis (PCA) was performed on the correlation matrix using prcomp() in program R v1.7.1 (http://www.r-project.org/). We analyzed the first two principal components using general linear models with Type III sums of squares.

Results

Opsin gene expression in parr retina

Opsin gene expression was measured in each retinal quadrant of 10 parr (Fig. 3). The DT quadrant contains the highest levels of SWS1, RH1, and LWS gene transcripts. The highest levels of RH2 opsin gene expression are in the VT quadrant. SWS2 opsin gene expression does not vary spatially.

Opsin gene expression in TH-induced parr retina

We measured the effects of TH exposure on opsin gene expression in parr at two, nine, and 22 days of treatment (Fig. 4). Results are presented as fold-change relative to matched controls. PCA of the TH treatment data set resulted in three components, which cumulatively accounted for 54.9% of the total variance in the original data set of 20 variables. The derived variables PC1, PC2, and PC3 accounted for 31.3%, 15.2%, and 8.4% of the total variance, respectively. Analysis of variance of each principal component (Table 2) revealed that PC3 did not vary significantly with treatment or time (data not shown). We therefore present and discuss the results of PC1 and PC2.

The PC1 score was high for TH treatment and differed significantly from control at day 9 (Fig. 6). A significant interaction term between treatment and time reflects the temporally limited window of TH action (Table 2). SWS1 gene expression loaded negatively on PC1, while SWS2, RH2, and LWS loaded positively, and RH1 had neutral values (Fig. 5). Therefore, high positive PC1 scores (Fig. 6) indicate individuals with relatively low SWS1 gene expression, high SWS2 and RH2 gene expression, moderate LWS gene expression and no change in RH1 expression.

Fish treated with TH had higher PC2 scores than control fish at two, nine, and 22 days (Fig. 7). SWS1 gene expression loaded negatively on PC2, as did RH1 and RH2 gene expression in the nasal quadrants, and LWS gene expression in all quadrants. SWS2 gene expression loaded positively on PC2 in the temporal quadrants. Therefore, high positive PC2 scores (Fig. 7) represent individuals that, for a given value of PC1, have relatively low SWS1 and LWS gene expression in all quadrants, and low SWS2, RH1 and RH2 gene expression in the nasal quadrants.

Opsin expression in smolt retina

Opsin gene expression was measured in each retinal quadrant of 10 smolts, and presented as fold-change relative to the expression levels observed in parr retina (Fig. 8). SWS1 gene expression decreased between 11- and 100-fold in all quadrants. LWS gene expression also decreased two-fold in the dorsal quadrants. A two-fold or greater increase in gene transcript levels was observed for RH1 nasally and RH2 dorsally.

Discussion

The visual pigments and corresponding visual sensitivities of many fish have been correlated with their photic environment

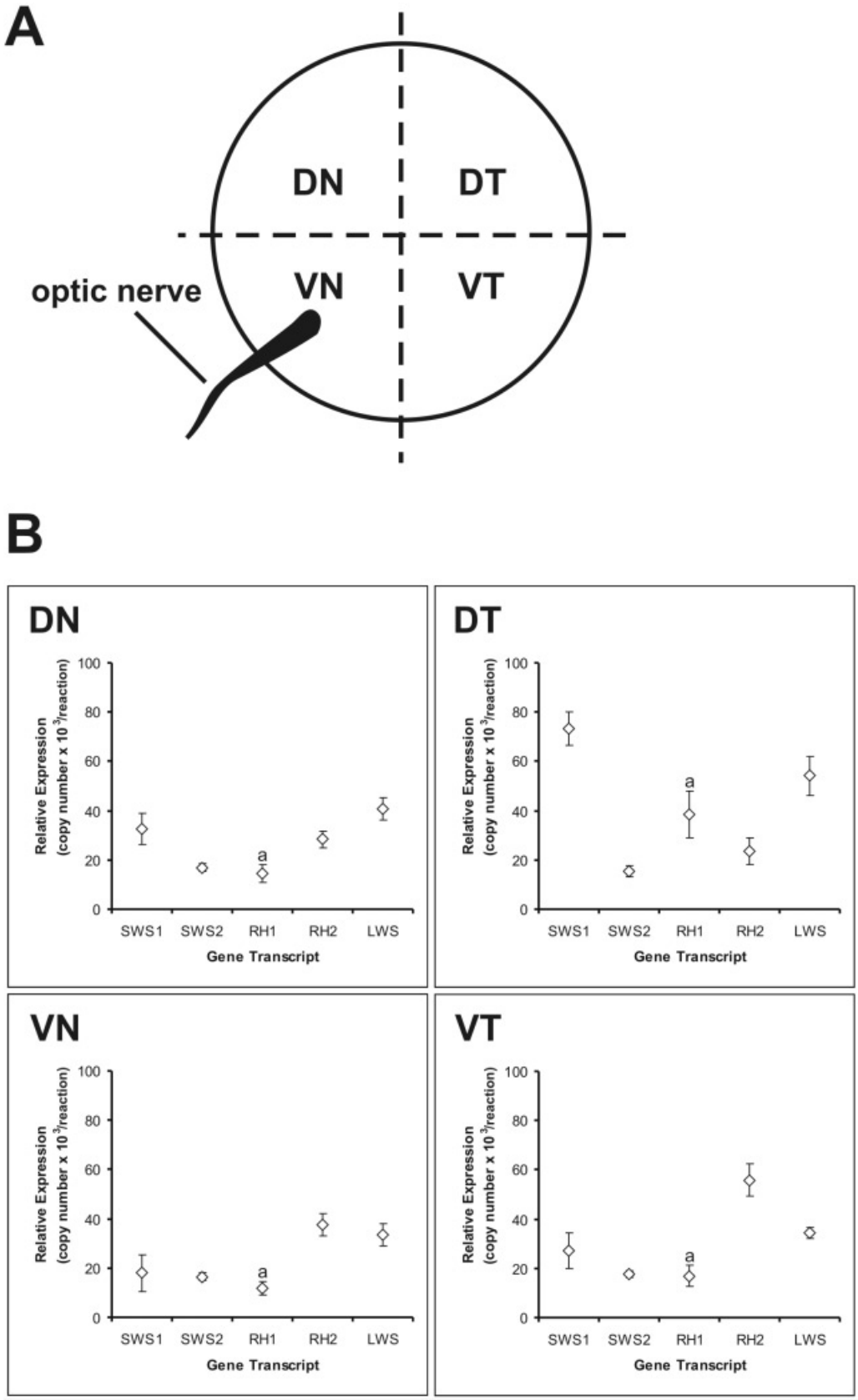

Fig. 3. Opsin gene expression in the retina of parr rainbow trout measured using real-time quantitative polymerase chain reaction (QPCR). A. Schematic representation of the retina depicting the location of sampled quadrants relative to the optic nerve. B. Relative opsin expression in retinal quadrants of parr ($n = 10$) normalized to the invariant ribosomal protein L8 gene. Error bars are ± S.E.M. [a]Expression levels of RH1 were out of scale, therefore, values were reduced by a factor of 100 for comparison.

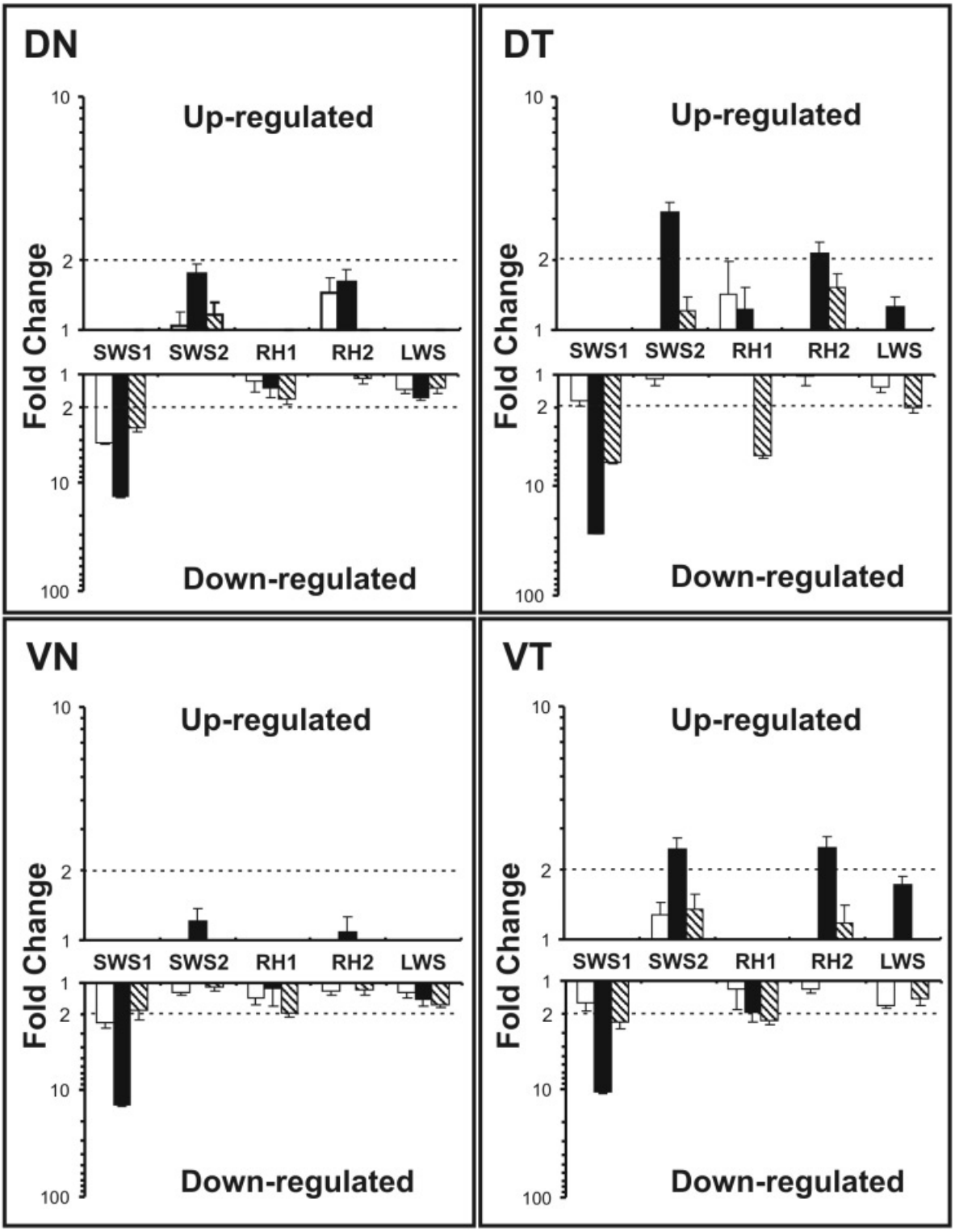

Fig. 4. Opsin gene expression in retinal quadrants of TH treated parr presented as fold-change relative to matched time point controls after 2 (white bars), 9 (black bars), and 22 (hatched bars) days of exposure. Ten fish were sampled from each control and TH treatment tank at each time point. Error bars are ± S.E.M.

(Loew & Lythgoe, 1978; Bowmaker, 1995). In addition, fish that migrate horizontally or vertically between different photic environments at different life history stages change their photoreceptor mosaic and opsin complement (Beaudet & Hawryshyn, 1999; Helvik et al., 2001). Parr rainbow trout live and feed in shallow freshwater streams, where the light is spectrally broad, and includes UV light (Novales-Flamarique et al., 1992). After the transformation from parr to smolt, fish migrate to lake waters

Table 2. *Two-way analysis of variance of PC1 and PC2 values*

	Degrees of Freedom	Sum of Squares	F value	Probability (F)
PC1				
Treatment	1	56.931	21.552	<0.0001
Time	2	110.037	20.828	<0.0001
Treatment by time	2	55.826	10.567	0.0001
Residuals	53	140.004		
PC2				
Treatment	1	83.594	69.832	<0.0001
Time	2	25.487	10.646	0.0001
Treatment by time	2	4.097	1.711	0.1904
Residuals	53	63.444		

where they spend their adult life until returning to spawn in their native stream. In the lake environment, they inhabit waters at greater depths, where the light is spectrally enriched for blue/green (that is, more short/middle-wavelength light) (Novales-Flamarique et al., 1992; Beaudet & Hawryshyn, 1999). Variation in the photic environment may require changes in opsin gene expression in order to modulate visual sensitivity, allowing subsequent adaptation to different light environments. Such a dynamic system has been demonstrated in cichlid fish (Carleton & Kocher, 2001) and in ayu (Minamoto & Shimizu, 2005).

This study represents the first quantitative evaluation of retinal opsin gene expression in the context of both TH-induced and natural development in rainbow trout. We demonstrate that treatment with TH affected transcript levels of opsin genes, both spatially and temporally, throughout the retinal hemisphere (see Fig. 4). Most notably, SWS1 gene expression was strongly down-regulated in all quadrants after nine days of treatment. This observation is consistent with results of previous studies of TH-treated rainbow trout where it has been shown that UV sensitivity and UVS corner cones, which express the SWS1 opsin gene, disappear in parr under the influence of TH (Browman & Hawryshyn, 1992, 1994; Allison et al., 2003). Loss of UVS corner cones is a gradual process, with complete loss occurring by six weeks of TH treatment (Browman & Hawryshyn, 1992, 1994; Allison et al., 2003). Importantly, the TH-induced change in SWS1 mRNA abundance precedes the point at which complete loss of UVS corner cones is known to occur, providing evidence that TH is modulating opsin gene expression during parr development.

Both the molecular mechanisms underlying UVS cone loss and the role of TH in the process are currently under investigation. There is evidence in rainbow trout (Allison et al., 2006) and Atlantic salmon (*Salmo salar*) (Kunz et al., 1994) that UVS corner cones undergo apoptosis during development. It has been postulated that UVS corner cone loss involves repression of opsin transcription within the SWS1 proximal promoter leading

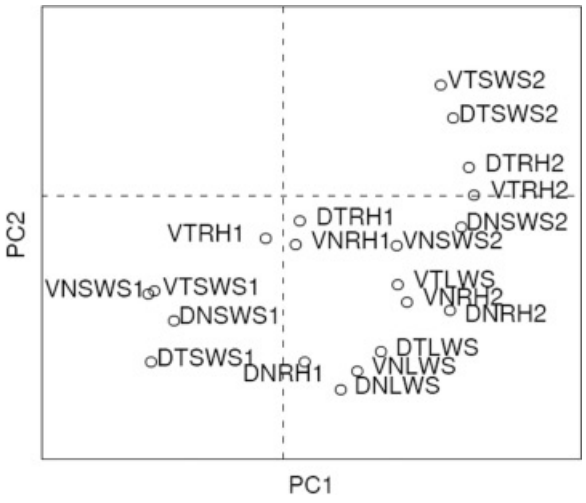

Fig. 5. Loadings of the individual opsin measurements on the first two principal components. The intersection of the dashed lines is (PC1,PC2) = (0,0). Fish with high values for variables to the right of the figure and low values to the left of the figure will have high PC1 scores. Variables near the origin have little influence on PC1 scores. Similarly, fish with high values of variables near the top of the figure and low values near the bottom of the figure will have high PC2 scores.

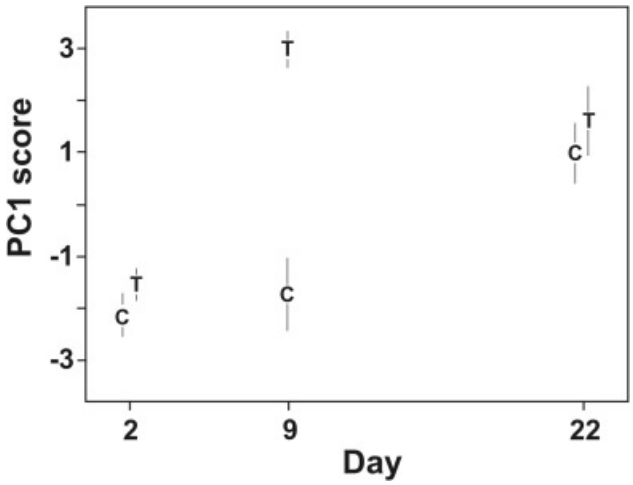

Fig. 6. First principal component scores ± S.E.M. of control and TH-treated parr at each experimental time point (2, 9, and 22 days).

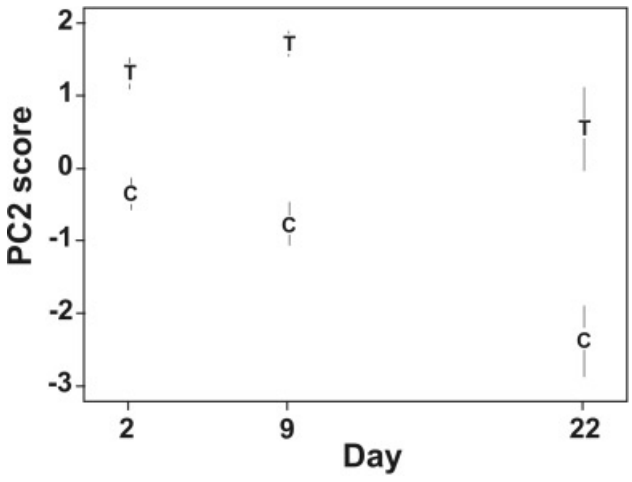

Fig. 7. Second principal component scores ± S.E.M. of control and TH-treated parr at each experimental time point (2, 9, and 22 days).

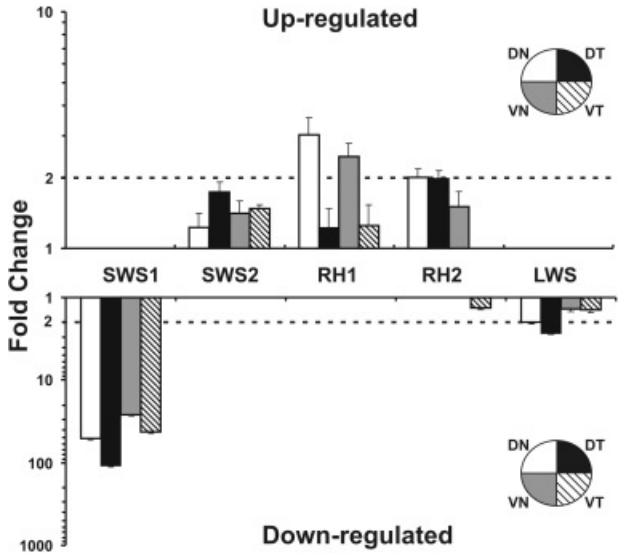

Fig. 8. Developmental change in opsin gene expression in retinal quadrants of smolts (n = 10) presented as fold-change relative to parr. Error bars are ± S.E.M.

to changes in phototransduction and metabolism (Dann et al., 2004*b*). Chromatin immunoprecipitation assays have revealed that the transcription factors c-jun and NF-κB bind the SWS1 proximal promoter in the parr rainbow trout retina, and not to other opsin promoters (Dann et al., 2004*b*). Both c-jun, as a component of the AP-1 complex, and NF-κB have been implicated in photoreceptor apoptosis due to photo-oxidative stress induced by light damage (Hafezi et al., 1999; Krishnamoorthy et al., 1999; Grimm et al., 2001). In addition, the activity of both transcription factors is repressed by molecules involved in TH signaling, such as the TR and silencing mediator of retinoic acid and thyroid hormone receptors (SMRT) (Schmidt et al., 1993; Lee et al., 2000). More research needs to be conducted on the activity of regulatory sequences found within the SWS1 promoter, in order to clarify the role, if any; these associated nuclear factors play in SWS1-specific opsin expression. While the current study assesses factors that affect mRNA abundance, it is important to note that regulation of functional opsin within the photoreceptor cell may also include translational and post-translational mechanisms of action.

Co-incident with the observed TH-induced decrease in SWS1 mRNA expression was an increase in both SWS2 and RH2 transcript abundance in the temporal half of the parr retina (see Fig. 4). TH treatment of rainbow trout smolts also results in a higher density of cones expressing SWS2 and RH2 opsins in the temporal region of the retina (Hawryshyn et al., 2003). The increase in SWS2 gene expression could be responsible for the transient shift in the UV sensitivity peak to longer wavelengths previously observed in both TH-treated parr and during the transition from parr to smolt stages of natural development (Hawryshyn et al., 1989; Browman & Hawryshyn 1992). In this case, we have suggested that another opsin may be expressed in UVS cones. Opsin co-expression in cone photoreceptors has been observed in a variety of vertebrates (Shand et al., 1988; Röhlich et al., 1994; Makino & Dodd, 1996; Applebury et al., 2000; Xiao & Hendrickson, 2000; Parry & Bowmaker, 2002; Cheng & Novales Flamarique, 2004). A relative increase in middle wavelength sensitivity compared with short wavelength sensitivity in rainbow trout has been observed with TH treatment and during natural development (Deutschlander et al., 2001) and it is possible that the increase in RH2 mRNA expression is responsible for this change in sensitivity. We are currently exploring the possibility that there maybe multiple copies of the SWS1 and other classes of opsin genes and their ontogenetic pattern of expression. Assessing the functional state of colour vision throughout ontogeny will permit us to evaluate the timing of expression and the relative importance of possessing multiple suites of opsin genes. Recent research (Dann et al., 2004*b*) has reported on the regulatory potential of TH, specifically on the SWS1 opsin gene promoter. We are now focusing our efforts on identifying transcription factor binding sites on opsin gene promoters that are responsible for differential patterns of expression.

Repression of SWS1 with concomitant induction of a MWS opsin (RH2) is a pattern of expression that suggests the involvement of TRβ activity. TRβ regulates mouse cone opsin expression in a TH-dependent manner, specifically suppressing SWS1 and inducing MWS opsin expression (Yanagi et al., 2002). TRβ-deficient mice have revealed that the receptor is necessary for the development of green cone photoreceptors (which express a MWS pigment of the LWS/MWS opsin group) and in the maintenance of the normal dorsal-ventral organization of cones expressing LWS/MWS and SWS1 opsins (Ng et al., 2001). This regulatory control by TRβ is dependent on the presence of DNA-binding activity (Shibusawa et al., 2003). TH-associated spatial and temporal modulation of opsin expression may also involve the activities of deiodinases. This suggestion comes from work in *Xenopus laevis*, where dorsal-ventral asymmetry exists in type 3 deiodinase expression that serves to inactivate TH, and establishes a dorsal-ventral asymmetry of retinal cell proliferation at metamorphosis (Marsh-Armstrong et al., 1999). Deiodinases are active in the retina of rainbow trout and their activity is increased with TH treatment, although no spatial information is available at present (Plate et al., 2002).

Similar to TH-treated parr, naturally developed smolts display a decrease in SWS1 opsin gene expression in all quadrants (compare Fig. 4 and Fig. 8). In part, this result was unexpected, as previous studies have shown that both smolts and sexually mature fish possess UV sensitivity, UVS opsin transcripts, and UVS corner cones in the DT quadrant (Allison et al., 2003; Beaudet et al., 1997; Deutschlander et al., 2001; Hawryshyn et al., 2003). It has yet to be resolved whether the DT population of UVS corner cones is maintained during development or whether they undergo a gradual and complete loss and are then regenerated in the DT quadrant. There is evidence that UVS corner cones regenerate from progenitor cells within the rainbow trout retina (Allison et al., 2006). The low levels of SWS1 expression in smolts may reflect an absence of UVS corner cones, rather than an immediate TH-induced change in gene expression as observed in the TH-induction of parr; it is possible that the smolts in this study were at a point during development where UVS corner cones have yet to regenerate.

However, if UVS corner cones do not undergo apoptosis and are maintained in the DT retina, then smolts may continue to express very low levels of SWS1 opsin. Since the DT region is exposed to lower intensities of light, UVS corner cones presumably would not be subject to a photic load comparable to other retinal quadrants, resulting in a lower demand for SWS1 opsin synthesis. Trout, like many other fishes, have a rostral–caudal axis of accommodation resulting in an elliptical pupil (e.g., Andison & Sivak, 1996). The pupil has an aphakic space, normally open at the

rostral end that permits light to enter the eye exposing the rostral half of the retinal hemisphere to non-refracted light (Bunt, 1991). The degree of illumination of the retina in the VN and DN retina can be considerable, especially in shallow waters, which have a reflective substrate. In conflict with the suggestion that a low photic load results in decreased opsin expression, is the observation that the DT quadrant in parr contains the higher levels of SWS1, RH1 and LWS opsin gene expression (see Fig. 3). Furthermore, downregulation in rod opsin synthesis and rod outer segment disk renewal has been shown in rats following UVA exposure (Rapp & Ghalayini, 1999). The link, if any, between UV exposure and transcriptional control of the SWS1 opsin promoter would necessarily be dependent on intermediary regulatory factors (e.g., c-jun, NF-κB, SMRT, TRs) in order to maintain spatially restricted expression in the DT retina. Both c-jun and NF-κB are activated by UVA light, and are implicated in a variety of UV-induced signal transduction pathways (Vile et al., 1995; Bender et al., 1997; Silvers & Bowden, 2002).

In summary, the TH-associated changes in opsin mRNA expression observed within the rainbow trout retina are in accordance with previous examinations of both TH-induced and natural developmental changes in spectral sensitivity and photoreceptor topography (Beaudet et al., 1993, 1997; Browman & Hawryshyn, 1994; Deutschlander et al., 2001; Allison et al., 2003, Hawryshyn et al., 2003). These results, in particular, highlight the important role TH plays in the regulation of SWS1 expression and form the basis of the first topographic map of opsin gene expression in the rainbow trout. Spatial asymmetry of opsin expression and photoreceptor cell type is not an unusual phenomenon, with examples of different types of asymmetries (central-peripheral, hemispheric, and quadrant) observed in the retina of a variety of vertebrates (Wikler & Rakic, 1990; Beaudet et al., 1997; Applebury et al., 2000; Hart, 2001; Hawryshyn et al., 2003; Takechi & Kawamura, 2005). The functional relevance of topographic variation in opsin expression is presumably to maximize photon capture and resolution within a particular photic habitat. Further research is required in order to understand the molecular basis of opsin expression as it relates to visual ecology and behavior in vertebrates.

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