The Journal of Experimental Biology 213, 493-501
© 2010. Published by The Company of Biologists Ltd
doi:10.1242/jeb.036301

Systemic thyroid hormone is necessary and sufficient to induce ultraviolet-sensitive cone loss in the juvenile rainbow trout retina

J. C. Raine[1,*], A. B. Coffin[1,†] and C. W. Hawryshyn[1,2,‡]

[1]Department of Biology and [2]Centre for Neuroscience Studies, Queen's University, Kingston, Ontario, Canada, K7L 3N6

*Present address: Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, S7N 5B3
†Present address: Virginia Merrill Bloedel Hearing Research Center, University of Washington, Seattle, WA 98195-7923, USA
‡Author for correspondence (craig.hawryshyn@queensu.ca)

Accepted 23 October 2009

SUMMARY

Rainbow trout possess ultraviolet-sensitive (UVS) cones in their retina that degenerate naturally during development. This phenomenon can be induced with exogenous thyroxine [T4, a thyroid hormone (TH)] treatment. However, the previous T4 exposure experiments employed static water immersion; a method that could introduce confounding stress effects on the fish. Because of this, it was uncertain if T4 alone was sufficient to induce retinal changes or if stress-related hormones were also necessary to initiate this process. Furthermore, it was unclear whether endogenous T4 was the factor responsible for initiating natural UVS cone loss during development. The current study examined the role of systemic T4 on the juvenile rainbow trout retina using a slow-release implant. Exogenous T4 treatment resulted in *SWS1* opsin downregulation and UVS cone loss after four weeks of exposure, signifying that T4 is sufficient to induce this process. Blocking endogenous T4 production with propylthiouracil (PTU, an anti-thyroid agent) attenuated *SWS1* downregulation and UVS cone loss in the retina of naturally developing rainbow trout, suggesting that endogenous T4 is necessary to initiate retinal remodelling during development. Quantitative real-time RT-PCR analysis demonstrated that several TH-regulating components are expressed in the trout retina, and that expression levels of the TH receptor isoform *TRβ* and the type 2 deiodinase (*D2*) change with T4 treatment. This suggests that T4 may act directly on the retina to induce UVS cone loss. Taken together, these results demonstrate that systemic TH is necessary and sufficient to induce *SWS1* opsin downregulation and UVS cone loss in the retina of juvenile rainbow trout.

Key words: thyroid hormone, retina, degeneration, opsin, photoreceptor, development, thyroid hormone receptors, deiodinase.

INTRODUCTION

The retina of salmonid fishes (trout and salmon) contains several analogous classes of cone photoreceptors [short wavelength-sensitive (SWS), medium wavelength-sensitive (MWS) and long wavelength-sensitive (LWS) cones] and comparable anatomical and functional organisation similar to that of humans (Kunz, 2006). Furthermore, wavelength discrimination studies in teleost fishes and humans suggest that colour vision in salmonid fishes is similar to that of humans (Spekreijse et al., 1991a; Spekreijse et al., 1991b; Wiersma and Spekreijse, 1991; Kitschmann and Neumeyer, 2005; Kunz, 2006; Joselevitch and Kamermans, 2009; Mora-Ferrer and Neumeyer, 2009). One additional and distinct cone type in these fish, the ultraviolet-sensitive (UVS) cone, contains the SWS1 opsin pigment that facilitates detection of polarised light and light in the ultraviolet spectrum (Hawryshyn et al., 1989; Hawryshyn and Hárosi, 1994; Hawryshyn, 2000; Parkyn and Hawryshyn, 2000; Allison et al., 2003). Interestingly, UVS cones are lost over much of the retina during smoltification, a developmental process involving profound changes in physiology, behaviour and morphology that prepare the freshwater juveniles (referred to as parr) for life in a dramatically different environment, the ocean (Hoar, 1988; Kunz et al., 1994; Parkyn and Hawryshyn, 2000). Smoltification occurs just prior to migration to the marine ecosystem and primarily occurs in anadromous salmonids, but land-locked species, such as rainbow trout (*Oncorhynchus mykiss*) undergo many similar changes, especially that of alterations to their visual system (Hawryshyn et al., 1989; Deutschlander et al., 2001; Veldhoen et al., 2006; Raine and Hawryshyn, 2009). During smoltification, *SWS1* opsin expression is downregulated, UVS cones in much of the retina undergo programmed cell death and UV sensitivity is decreased (Browman and Hawryshyn, 1992; Browman and Hawryshyn, 1994a; Kunz et al., 1994; Deutschlander et al., 2001; Allison et al., 2003; Allison et al., 2006; Hawryshyn et al., 2003; Kunz, 2006; Veldhoen et al., 2006; Raine and Hawryshyn, 2009). Smoltification is preceded by an increase in plasma thyroxine (T4) (Hoar, 1988), and exogenous T4 treatment has been demonstrated to elicit precocial loss of UVS cones and body silvering (a morphological change associated with smoltification) in rainbow trout (Browman and Hawryshyn, 1992; Browman and Hawryshyn 1994a; Allison et al., 2006). These studies suggest that systemic thyroid hormone (TH) may induce retinal remodelling in juvenile rainbow trout.

T4 and triiodothyronine (T3) are the two main forms of TH circulating in the blood of teleost fish. T4 is generally found in the greatest concentration in the blood stream and is considered to be a prohormone for the more biologically active form of TH, T3 (Leatherland, 1994; Hulbert, 2000). Deiodination enzymes are important regulators of TH concentrations in the blood and provide either an increased supply of T3 or clearance of both T4 and T3 from the circulation (Eales and Brown, 1993; Leatherland, 1994; Köhrle, 1999; Bianco et al., 2002; Brown, 2005; St Germain et al., 2005; Nunez et al., 2008). Local tissue deiodination is considered an important mechanism for supplying T3 to the tissues of the nervous system, and T3 levels for a target cell can be increased or decreased locally by deiodination *via* these deiodinases (Darras et al., 1998; Nunez et al., 2008).

TH appears to play a major role in vertebrate growth and development, although the precise nature of its role in teleost fishes is not clear (Leatherland, 1994; Power et al., 2001; Furlow and Neff, 2006; Nunez et al., 2008). In mammals, TH is critical for proper development of the central nervous system, including the retina (Bernal et al., 2003; Ng et al., 2001; Harpavat and Cepko, 2003). The TH receptors (TR), *TRα* and *TRβ*, have both been detected in the retina of mice, chicken, the African clawed frog (*Xenopus laevis*) and winter flounder (*Pleuornectes americanus*), and have been shown to be involved in opsin expression and cone development (Sjöberg et al., 1992; Cossette and Drysdale, 2004; Mader and Cameron, 2006; Nunez. et al., 2008). *TRβ* in particular, has been found to play a dominant role in cone development in mice, and *TRβ2* knockout mice lack M (green-sensitive) cones and have an altered distribution of S (UV-sensitive) cones across the retina relative to wild-type control mice (Ng et al., 2001; Nunez et al., 2008).

Furthermore, it has been demonstrated that deiodinase (D2) activity is present in the retina of killifish and rainbow trout and that this activity can be altered with T4 treatment (Orozco et al., 2000; Plate et al., 2002). Recently, exogenous T4 in rainbow trout parr was shown to upregulate retinal *TRβ* and downregulate *D2* across the whole retina within two days of treatment, prior to significant downregulation of *SWS1* expression (Raine and Hawryshyn, 2009). This study provides evidence that exogenous T4 may be directly involved in initiating *SWS1* downregulation and subsequent UVS cone loss in juvenile rainbow trout.

However, while several lines of evidence implicate T4 in UVS cone loss, the precise role and mechanism of T4 in eliciting this developmental phenomenon is unresolved. It is still unclear if T4 alone is sufficient to induce UVS cone loss, as previous T4 exposure methods may have introduced confounding variables such as stress effects from daily handling. Cortisol and other stress-related factors can modulate circulating T3 levels in vertebrates (Kühn et al., 1998), which in this case could act in concert with T4 to trigger retinal remodelling. Furthermore, the role of endogenous T4 in natural UVS cone loss has not been examined. The present study utilises a slow-release implant technique to examine the role of exogenous and endogenous TH in retinal remodelling in juvenile rainbow trout. We demonstrate that exogenous T4 treatment is sufficient to induce *SWS1* downregulation and UVS cone loss, and that blocking endogenous T4 with the anti-thyroid agent PTU delayed retinal remodelling during development, suggesting that endogenous T4 is necessary for this process.

MATERIALS AND METHODS

Experimental animals

Rainbow trout (*Oncorhynchus mykiss* Walbaum) parr were obtained from Rainbow Springs Trout Hatchery (Thamesford, ON, Canada). For all of the experiments, fish were held at a mean temperature of 15°C in constantly flowing aerated water. The 12 h:12 h light:dark cycle fluorescent lighting used in these experiments was spectrally comparable with Veldhoen et al. (Veldhoen et al., 2006). Fish were fed a low ration (1% g body mass day^{-1}) of a commercial trout food (Classic Sinking Feed, Martin Mills, Elmira, ON, Canada) to slow growth and prevent potential premature smoltification. Fish care and treatment were performed in accordance with the Canadian Council for Animal Care regulations and the Queen's University Animal Care Committee.

For the purposes of this study, the terms 'parr' and 'smolt', which are commonly used to represent pre- and post-migratory stages, respectively, of anadromous salmonid species, were used here to represent the comparable developmental stages in rainbow trout. A 'true' smolt undergoes all of the behavioural, physiological and morphological changes necessary for survival in the ocean whereas a land-locked species like rainbow trout undergoes a 'pseudo'-smoltification process (see Hoar, 1988). However, because anadromous rainbow trout (steelhead trout) were not available for study, and because rainbow trout are a popular and easily available model organism that exhibit similar changes in their visual system to 'true' smolts (Allison et al., 2006; Veldhoen et al., 2006; Raine and Hawryshyn, 2009), the latter were used in this study.

Exogenous T4 or PTU exposure

A coconut oil implant was employed to expose rainbow trout parr to exogenous T4 and PTU. Use of the implant allows the fish to be held under optimal flow-through conditions in an effort to remove the potential effects of stress resulting from the previously used immersion method, where fish were held in static water and handled daily during water changes. The coconut oil implant method used in the current study was based on a method used previously for altering other blood hormone levels in salmonids (Flett and Leatherland, 1989; Vijayan and Leatherland, 1989; Holloway and Leatherland, 1997). This is the first time exogenous T4 and PTU have been delivered *via* a coconut oil implant. For this reason, a pilot study was employed to determine the optimal amount of coconut oil and T4 required to elevate plasma T4 levels to appropriate levels for the duration of the experiment (data not shown).

At the beginning of the current experiment, the rainbow trout parr were 8.7±0.7 g wet mass. The coconut oil implant consisted of 20 µl of coconut oil per gram of mean body mass of the fish. Forty fish were allocated to each of four aquaria. Each of these four tanks corresponded to one of the four experimental treatments: a control treatment with only the vehicle [dimethyl sulfoxide (DMSO)] in the implant; a T4 treatment (L-thyroxine sodium salt pentahydrate; Sigma-Aldrich, St Louis, MO, USA) utilising 3 µg T4 µl^{-1} coconut oil; a PTU treatment (6-propyl-2-thiouracil; Sigma-Aldrich) of 3 µg PTU µl^{-1} coconut oil; and a T4/PTU treatment implanted with 3 µg of T4 and 3 µg of PTU µl^{-1} coconut oil. PTU prevents endogenous production of T4 and would therefore decrease systemic levels of T4 in the trout and serve as a contrary treatment to T4. The T4 implant was designed to produce blood plasma T4 levels similar to those obtained using the typical immersion treatment method utilising 300 µmol l^{-1} T4 in solution. It was predicted that T4 treatment would induce premature smoltification and associated retinal changes whereas PTU treatment would prevent natural smoltification. The combined T4/PTU treatment was predicted to induce changes similar to those seen with T4 alone, as exogenous T4 delivery should not be affected by PTU.

Sampling protocol

Complete sampling from each group occurred after four weeks of treatment, at which point experiments with T4 and T4/PTU implants were terminated. Only the control and PTU-implanted fish were retained past the four-week sampling point in order to examine the effects of PTU exposure on natural smoltification. The ration level was increased to 4% g body mass day^{-1} after the four-week sampling point to allow rapid growth and development of the fish in the two remaining treatments. Fish were visually monitored for changes in size, body colouring and rounding of the rostrum indicative of natural pseudo-smoltification, which occurred by the ninth week of treatment. The experiment was terminated at this point.

Fish were dark adapted prior to euthanasia with 150 mg l^{-1} tricaine methanesulfonate (MS-222; Crescent Research Chemical, Phoenix, AZ, USA). Following euthanasia, wet mass and fork length

(tip of snout to fork in tail) measurements were immediately recorded. Blood was then quickly collected after transverse severance of the tail, slightly caudal to the anal fin. Heparinised microcapillary tubes (Fisher Scientific, Ottawa, ON, Canada) were used to collect blood from the caudal blood capillaries. Following cervical transection, the eyes were quickly removed and the neural retina was subsequently dissected free of the retinal pigment epithelium from both eyes under deep red illumination. The isolated retina from the right eye (N=5 fish per treatment) was placed in 4% paraformaldehyde to preserve the tissue for assessment of UVS cone presence using immunohistochemistry on the whole retina. The left isolated retina (N=5 fish per treatment) was immediately placed in RNAlater (Ambion Inc., Austin, TX, USA) for gene expression analysis. The remaining fish in each group were used for blood collection and length and mass measurements.

Plasma T4 analysis

The blood-containing hematocrit tubes were removed from ice and centrifuged at 1500× gravity at 4°C to separate the blood plasma from the cellular components. 100 µl of plasma was pooled from 2–3 fish to generate five replicates for each treatment. This was the minimum volume of plasma required for T4 analysis performed by a diagnostic lab using a standard radioimmunoassay procedure validated with spiked trout plasma (Texas Vet Med Diagnostic Laboratory, College Station, TX, USA). Plasma samples were stored at –20°C until T4 analysis.

SWS1 opsin immunohistochemistry

Fixed whole retinas were immunostained with a mouse polyclonal antibody to the N-terminal portion of the rainbow trout SWS1 opsin (Allison et al., 2006). All solutions were prepared in phosphate buffer (PB, pH 7.4), and all steps were performed at room temperature unless otherwise noted. Retinas were incubated with PB containing 5% normal goat serum and 0.3% Triton-X (both from Sigma-Aldrich) to reduce non-specific binding. Retinas were incubated overnight at 4°C with the primary SWS1 opsin antibody diluted 1:5000 in PB containing 1% goat serum and 0.3% Triton-X. The tissue was rinsed in fresh PB and incubated for one hour in the secondary antibody (Alexa Fluor 594 goat anti-mouse, Invitrogen, Carlsbad, CA, USA) diluted 1:750 in 1% goat serum with 0.3% Triton-X. The tissue was then rinsed with fresh PB and counter-stained with Alexa Fluor 488 peanut lectin-agglutinin (PNA; Invitrogen). PNA has been found to non-specifically label cones in many vertebrate species, including rainbow trout, and was used as a positive control (Blanks and Johnson, 1984; Allison et al., 2006). The immunostained whole retinas were mounted photoreceptor-side up with Prolong Gold mounting medium (Invitrogen) on FisherFrost Plus histology slides with a cover slip.

Immunostained retinas were visualised with a Zeiss Axioskop 2 compound microscope (Carl Zeiss Canada Ltd, Toronto, ON, Canada) equipped for epifluorescence and photographed with a Retiga EXi 12-bit CCD cooled monochromatic digital camera (QImaging, Surrey, BC, Canada). Images were captured using Northern Eclipse 7.0 (Empix Imaging, Mississauga, ON, Canada) software. Monochromatic images were pseudocoloured using ImageJ 1.40g (W.S. Rasband, National Institutes of Health, Bethesda, MD, USA) according to the fluorophore used (for example, tissue labelled with Alexa 594 secondary antibody was pseudocoloured red). Images were cropped and adjusted for brightness and contrast using Adobe PhotoShop 7.0 (Adobe Systems, Mountain View, CA, USA) but image content was not altered.

qRT-PCR analysis of gene expression

Each retina was homogenised individually with a Retsch MM301 mixer mill (Retsch GmBH & Co. Ltd KG, Hann, Germany). Total RNA was isolated from RNAlater-protected retinas using an Absolutely RNA miniprep kit (Stratagene, La Jolla, CA, USA). Quantification of total RNA isolations was performed using RiboGreen (Invitrogen Canada Inc., Burlington, ON, Canada) following the manufacturer's protocol and using an MX3000P real-time quantitative PCR thermocycler (Stratagene). Briefly, master mixes were generated containing TE buffer, Ribogreen and known concentrations of RNA to generate a standard curve using duplicate 25 µl volumes. A mastermix for each unknown was generated from 1 µl of each unknown, Ribogreen and TE buffer to run 25 µl per well in triplicate. The fluorescence signal generated from the RNA standard curve was used to assess the concentrations of the unknown samples. 1 µg total RNA of each of the retinal RNA isolates was then reverse-transcribed to cDNA using a Superscript III First Strand Synthesis SuperMix kit for qRT-PCR (Invitrogen).

Quantitative RT-PCR (qRT-PCR) analysis of individual total RNA samples from each retina was performed for each of the *O. mykiss* gene specific primers designed for *SWS1*, *SWS2*, *TRα*, *TRβ*, *D2* and a housekeeping gene, ribosomal protein L8 (*L8*) (Table 1). All primer pairs were validated in previous studies (Raine et al., 2004; Veldhoen et al., 2006; Raine and Hawryshyn, 2009). *L8* has been demonstrated to be effective as a house-keeping gene, as it does not vary significantly during TH treatment and development in rainbow trout or anurans (Shi and Liang, 1994; Callery and Elinson, 2000; Crump et al., 2002; Veldhoen et al., 2006; Raine and Hawryshyn, 2009). Primer optimisation reactions were run to determine the optimal concentration of primers to use in the qRT-PCR reactions; the resulting optimal equimolar amounts of primer varied between 100 and 300 µmol l^{-1} (Raine and Hawryshyn, 2009). Standard curves were run for each primer pair using serial dilutions of rainbow trout parr retinal cDNA. The magnesium chloride concentration in the standard curve reactions was altered between 3 and 5 mmol l^{-1} as needed, to generate repeatable R^2 values greater than 0.98 and reaction efficiencies between 90 and 100% (Raine and Hawryshyn, 2009).

Standard curves were run in triplicate and unknowns were run in duplicate. Master mixes were used whenever possible to reduce the possibility of pipetting error. Each 10 µl reaction contained Supermix-UDG (Invitrogen), ROX and 2 µl of 20-fold diluted retinal

Table 1. Primer sequences and amplicon sizes for the gene-specific primers used in this study

Gene	Forward primer	Reverse primer	Amplicon size (bp)	Reference
SWS1	GGCTTTCTACCTACAGACTGC	CCTGCTATTGAACCCATGC	258	Veldhoen et al., 2006
SWS2	GGCACTGCTATCAACGTCCT	CCACTACTGAGAGAGACCATAA	244	Veldhoen et al., 2006
TRα	GCACAACATTCCCCACTTCT	AGTTCGTTGGGACACTCCAC	117	Raine et al., 2004
TRβ	TCACCTGTGAAGGATGCAAG	GACAGCGATGCACTTCTTGA	152	Raine et al., 2004
D2	ATTTTGTATGCCGATGCACA	TACGGCGCTAACCTCTGTTT	207	Raine and Hawryshyn, 2009
L8	GGTGTGGCTATGAATCCTGT	ACGACGAGCAGCAATAAGAC	113	Veldhoen et al., 2006

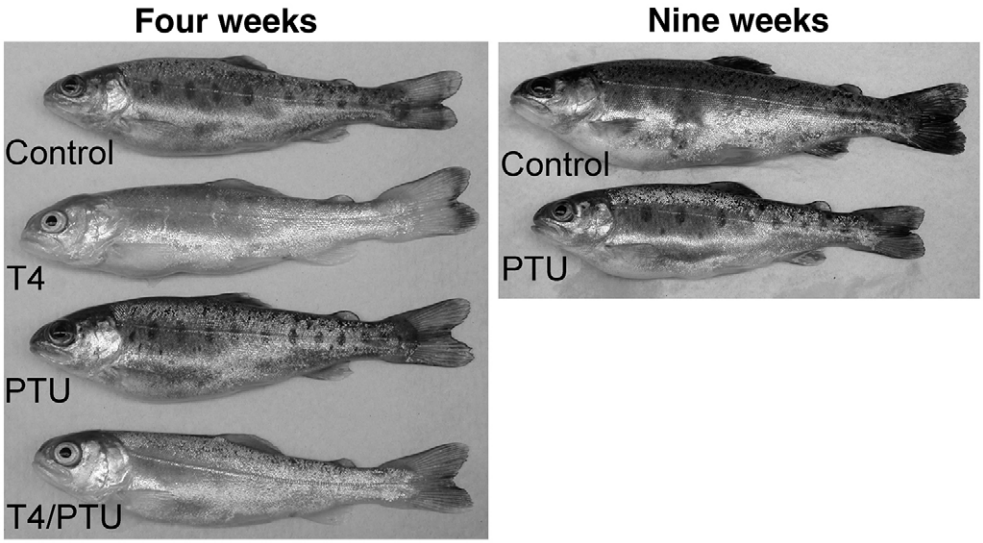

Fig. 1. External morphology of treated rainbow trout parr four weeks (left) and nine weeks (right) post-treatment. Left: After four weeks, control and PTU-treated fish have conspicuous parr marks that are absent in T4- and T4/PTU-treated fish. Right: After nine weeks, control fish show morphological signs of smoltification (e.g. silvering) whereas PTU-treated fish are smaller and still have parr marks. PTU, propylthiouracil.

cDNA. The thermocycling program was 50°C for 2 min, 95°C for 2 min followed by 40 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C. A dissociation curve was generated at the conclusion of each qRT-PCR run to ensure that all products generated during the thermocycling program produced only one distinct DNA peak that dissociated at a consistent temperature. DNA amplification was performed using a MX3000P real-time quantitative PCR thermocycler (Stratagene), and the resultant amplification curves and data analysis were performed by the accompanying MXPro v. 4.0 software using the efficiency-corrected comparative quantitation method (Pfaffl, 2001). A relative method of qRT-PCR quantification was determined to be most relevant for analysis of the experimental data, because changes relative to the control fish were the target of the investigation and absolute copy numbers of RNA were not required for this purpose.

Statistical analysis

Statistical analysis was performed on all data using SigmaPlot 11 software (Systat Software Inc., San Jose, CA, USA). One-way analysis of variance (ANOVA) was used to compare data from all treatment groups for each method of analysis. The Holm–Sidak method was subsequently employed to detect differences between the treatment groups. A probability level of 0.05 was used in all cases to indicate significance. All data were expressed as means ± standard error. All qRT-PCR data were expressed as means ± standard error relative to control retina.

RESULTS

Morphological changes

After four weeks of treatment, control fish retained parr bars and a sloped rostrum, while T4-treated fish exhibited body silvering and a rounded rostrum indicative of smoltification (Fig. 1). As predicted, PTU-treated fish were similar in external morphology to the control fish. T4/PTU-treated fish displayed morphological changes comparable with those of the T4-treated group, complete with body silvering and rounded snouts (Fig. 1). No significant differences in wet mass or fork length were observed between groups after four weeks of treatment (Table 2).

While the control and PTU-treated fish appeared similar after four weeks of treatment, the two groups were morphologically distinct by week nine of the experiment (Fig. 1). After nine weeks the control fish appeared to have gone through the metamorphic process of smoltification, displaying the external morphology of a juvenile rainbow trout, including rounded rostrum, faded parr bars and multi-coloured, iridescent scales similar to that of adult fish. The PTU-treated fish, which retained their parr bars, exhibited more morphological similarity to that of the control fish at the beginning of the study (Fig. 1). The fork length and wet mass of PTU-treated fish after nine weeks was also significantly less than that of the controls (Table 2).

Plasma T4 levels

Plasma T4 levels were significantly elevated in fish treated for four weeks with T4 or T4/PTU implants as compared with control and PTU-treated fish (Fig. 2). Control and PTU-treated fish exhibited low plasma T4 levels at 0.46±0.28 ng ml^{-1} and 0.23±0.23 ng ml^{-1}, respectively, while T4- and T4/PTU-treated fish had significantly higher plasma T4 values at 269.33±46.61 ng ml^{-1} and 60.80±29.35 ng ml^{-1}, respectively. Interestingly, the plasma T4 levels from fish in the T4/PTU-treatment group were dramatically lower than those that received only T4 treatment (P<0.05).

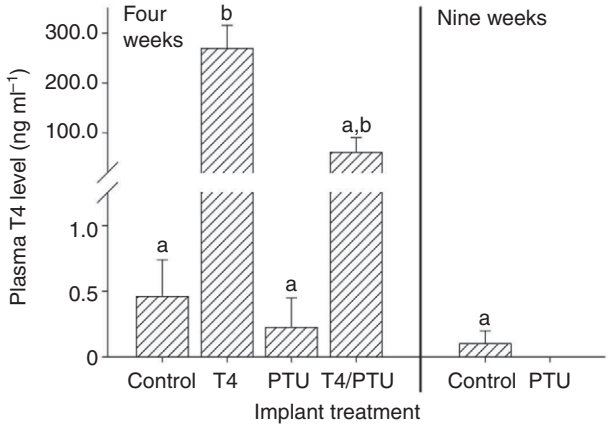

Fig. 2. Plasma T4 levels for fish treated with coconut oil implants containing vehicle alone (control), T4, propylthiouracil (PTU) or both T4 and PTU for either four (left) or nine (right) weeks, with the latter time point considered equivalent to the smolt developmental stage. Groups that are not significantly different from one another (P<0.05) are labelled with the same letter (N=6; bars represent means ± s.e.m.).

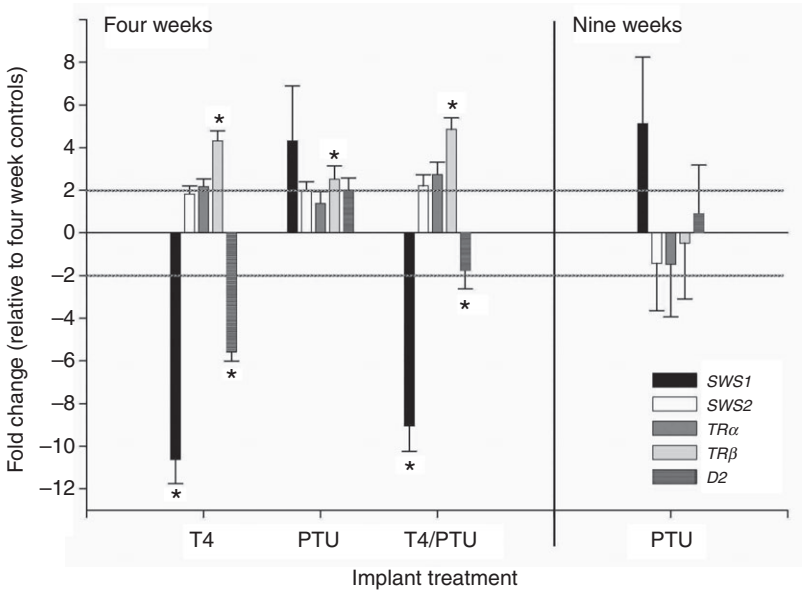

Fig. 3. Relative gene expression levels assessed by qRT-PCR for *SWS1* and *SWS2* opsins, thyroid hormone receptors (*TRα*, *TRβ*) and deiodinase 2 (*D2*) in rainbow trout four weeks post-implant (left) and at the smolt developmental stage (nine weeks, right). *N*=5; data are presented as means ± s.e.m. * indicates fold-change differences at $P<0.05$ relative to controls, and the lines at ±2 indicate the boundaries at which fold-change differences are considered significant.

After nine weeks of treatment, control fish had low plasma T4 levels at 0.1 ng ml^{-1}, and in the PTU-treatment group plasma T4 levels were below the detection threshold (Fig. 2).

qRT-PCR analysis

After four weeks of treatment, *SWS1* expression significantly decreased in both the T4- and T4/PTU-implanted fish relative to implant controls (Fig. 3, $P<0.05$). PTU-treated fish showed modest upregulation of *SWS1* at both the 4-week and 9-week time points when compared with the control fish but this trend was not significant at either time point (Fig. 3, $P=0.103$ at four weeks and $P=0.208$ at nine weeks). When the individual qRT-PCR data for each fish were compared with the corresponding fish size and UVS cone immunostaining results, a pattern emerged, suggesting that *SWS1* upregulation in PTU-treated fish may be dependent on fish size; smaller fish had higher *SWS1* levels (Table 3).

As some previous studies have suggested that *SWS1* downregulation coincides with *SWS2* upregulation in the rainbow trout retina (Cheng et al., 2006; Cheng and Flamarique, 2007), relative transcript levels for *SWS2* (blue) opsin were also examined. *SWS2* did not exhibit any significant changes in transcript level over the course of the experiment in any treatment group or time point examined (Fig. 3, $P=0.224$).

Table 2. Wet masses and fork lengths for rainbow trout parr treated with vehicle (control), T4, PTU or a combination of T4 and PTU for four weeks or nine weeks (to smoltification in controls)

		Wet mass (g)		Fork length (cm)	
Treatment	Day 0	Four weeks	Nine weeks	Four weeks	Nine weeks
Control	10.19±0.43	11.15±0.88	53.51±5.90[a]	9.84±0.27	15.39±0.50[a]
T4	10.98±0.67	12.07±2.48	–	10.13±0.54	–
PTU	10.62±0.48	10.34±0.70	30.79±6.38[b]	9.60±0.24	12.34±0.97[b]
T4/PTU	11.50±0.61	12.57±2.00	–	10.53±0.38	–

Values with different letters are significantly different from one another; all data are expressed as means ± s.e.m.; $P<0.05$. PTU, propylthiouracil.

Table 3. A comparison of measurements corresponding to individual fish within the PTU-treatment group at four weeks post-injection and the smolt developmental stage (nine weeks post-treatment)

Treatment	Fish number	Wet mass (g)	Fork length (cm)	Region containing UVS cones (stained)	*SWS1* expression (fold change)
Four week PTU	1	9.64	9.2	–	3.38
	2	12.06	10.3	–	0.07
	3	13.67	10.6	D	–2.16
	4	8.77	9.4	D	9.42
	5	8.69	8.8	D, V	10.92
	6	9.67	9.4	D	–
	7	9.91	9.5	D, V	–
Nine week PTU	1	17.41	9.8	D, V	10.86
	2	11.9	9.8	D, V	4.36
	3	33.44	13.9	D	0.19

Not all analyses were performed on the same fish, thus some of the data in the table is not available for the same individual. D, dorsal retina; V, ventral retina; UVS, ultraviolet sensitive; PTU, propylthiouracil; all data are expressed as means ± s.e.m.; $P<0.05$. – represents analysis not done.

The relative expression levels of the *TRα*, *TRβ* and *D2* transcripts were quantified and compared with time-matched control fish (Fig. 3). There were no significant differences in *TRα* expression levels across treatment groups or time points. However, fish in all three groups (T4, PTU, T4/PTU) treated for four weeks demonstrated a significant elevation in *TRβ* transcripts relative to time-matched control fish.

There were no changes in *TRβ* mRNA levels in the PTU-treated fish after nine weeks of treatment when compared with time-matched controls. The level of *D2* mRNA was significantly lower in both the T4 and T4/PTU treatments relative to the control fish at four weeks post-implant. Although there did appear to be an increasing trend in the level of transcript for *D2* in the PTU-treated group after four weeks when compared with the control fish, this trend was not statistically significant. Both control and PTU-treated fish displayed similar *D2* expression levels after nine weeks of treatment.

Immunohistochemical detection of UVS cones

Immunostaining of whole retina with anti-SWS1 opsin demonstrated that UVS cones were present in the dorsal retina of both the control and PTU-treated fish, while both the T4- and T4/PTU-treated fish demonstrated UVS cone loss in the dorsal retina at the conclusion of the 4-week treatment period (Fig. 4). UVS cones were also missing from the ventral retina of T4 and T4/PTU-treated fish by four weeks (data not shown). The ventral retina of all five of the control fish was also devoid of UVS cones by week four of the experiment (Fig. 5). This suggests that naturally occurring UVS cone loss prior to smoltification begins in the ventral hemisphere. By contrast, the UVS cone population was retained in the ventral retina of two out of the five fish treated for four weeks with PTU (Fig. 5). A comparison of individual fish sizes, *SWS1* opsin gene expression and UVS cone location in the retina demonstrated that smaller PTU-treated fish (under 10 g wet mass) exhibited higher *SWS1* expression levels and more ventral UVS cone retention than larger PTU-treated fish (Table 3).

Control and PTU-treated fish exhibited greater differences in UVS cone distribution across the retina after nine weeks of treatment, when control fish resembled smolts. The control fish possessed immunolabelled UVS cones only in the dorsal-temporal quadrant of the retina, while the PTU-treated fish displayed additional ventral and dorso-nasal labelling (Fig. 6).

DISCUSSION

The current study demonstrates that systemic T4 is necessary and sufficient to induce UVS cone loss in the juvenile rainbow trout retina. Exogenous T4, administered *via* slow-release implant, triggered precocious smoltification, including *SWS1* downregulation and UVS cone loss. The necessity of endogenous T4 in naturally occurring UVS cone loss was revealed using exogenous treatment with the anti-thyroid agent PTU. Decreased T4 production following PTU treatment attenuated natural *SWS1* opsin downregulation and UVS cone loss in the retina of rainbow trout smolt. This is the first study to show that T4 is involved in both induced and natural *SWS1* downregulation and UVS cone loss in rainbow trout. Finally, qRT-PCR analysis demonstrates that several components of TH regulation are present in the rainbow trout retina and that expression of these components changes with T4 treatment, suggesting that TH may act directly on the retina to induce UVS cone loss during smoltification.

The T4-induced downregulation of *SWS1* and corresponding UVS cone loss seen in the present study is in keeping with several prior studies in juvenile rainbow trout (Browman and Hawryshyn, 1992; Browman and Hawryshyn, 1994a; Deutschlander et al., 2001; Allison et al., 2006; Veldhoen et al., 2006; Raine and Hawryshyn, 2009). However, previous studies involved the immersion of fish in static solutions containing exogenous T4. This method requires daily handling of the fish in order to ensure the removal of accumulated biological waste products and to provide a relatively

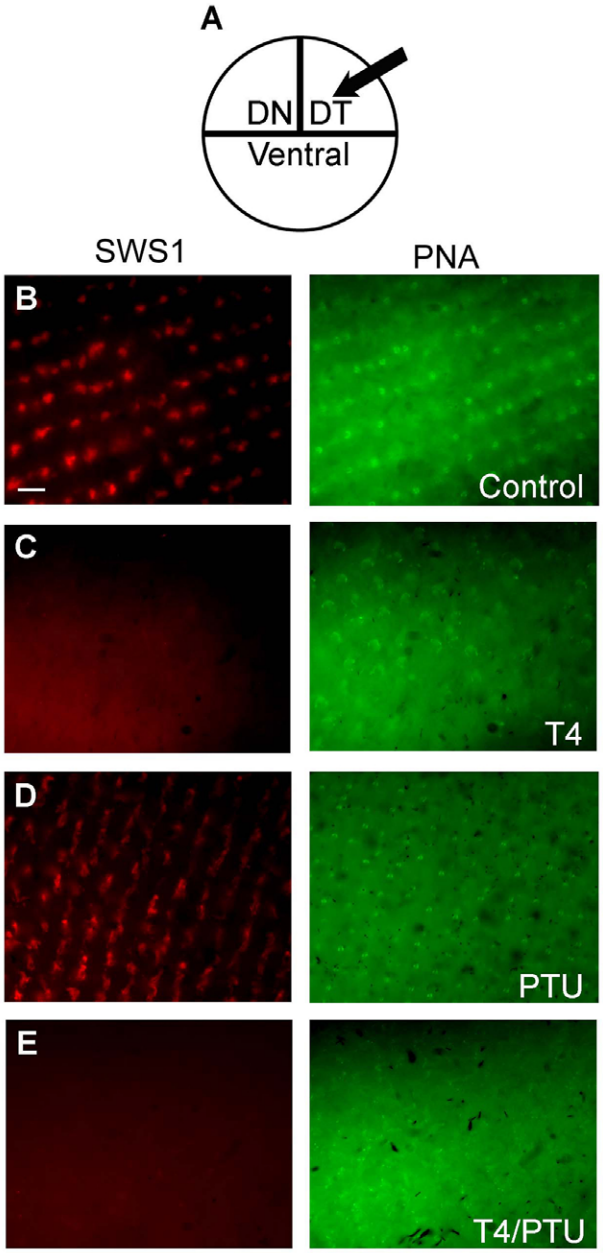

Fig. 4. Immunolabelling of ultraviolet-sensitive (UVS) cones in the dorsal temporal (DT) retina four weeks after the initiation of treatment with control, T4, PTU or T4 and propylthiouracil (PTU) coconut oil implants. (A) A diagram of a retina illustrates the quadrant compared in the subsequent images. (B–E) SWS1 immunolabelling is shown in the left (red) image of each panel, while peanut lectin-agglutinin (PNA) counterstaining (green) of the same region is shown on the right. (B, D) UVS cones are present in the DT retina of control and PTU-treated parr after four weeks of treatment. (C, E) The DT quadrant of the retina lacks UVS cones in fish treated for four weeks with T4 or T4/PTU. PNA counter-labelling demonstrates the presence of other cones types. Scale bar=12 μm and applies to panels B–E. DN, dorsal nasal.

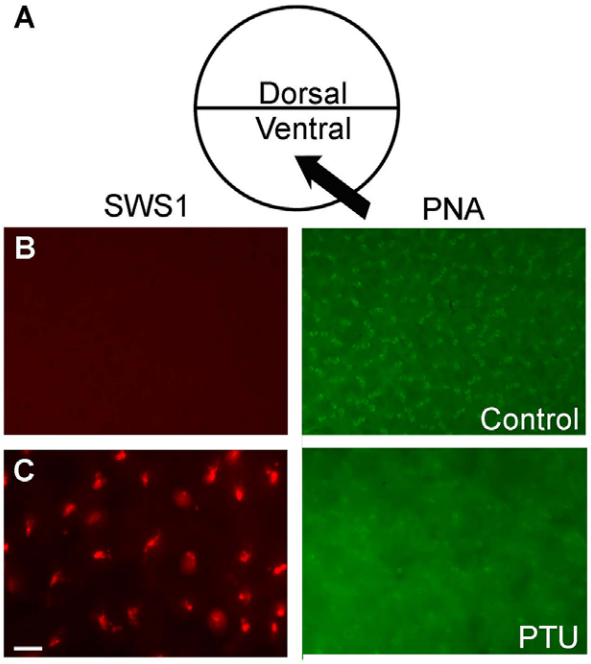

Fig. 5. Ultraviolet-sensitive (UVS) cone immunolabelling in the ventral retina (A) of rainbow trout parr four weeks after the initiation of treatment with control or PTU coconut oil implants. (B–C) SWS1 immunolabelling (left, red) and peanut lectin-agglutinin (PNA) counterstaining (right, green). (B) Control fish lack immunostained UVS cones in the ventral retina. (C) UVS cones are still present in the ventral retina of this PTU-treated fish. Scale bar=12 μm and applies to B and C. PTU, propylthiouracil.

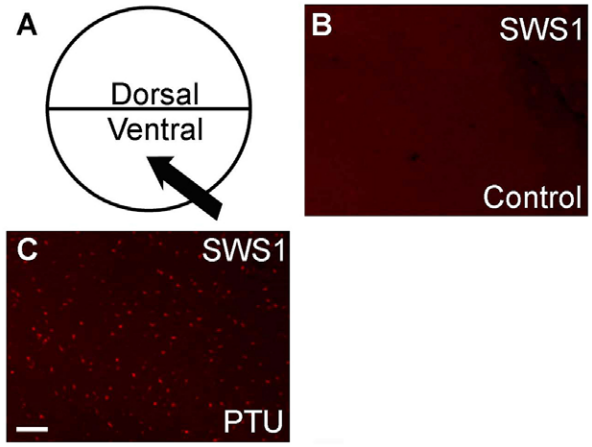

Fig. 6. Ultraviolet-sensitive (UVS) cone immunolabelling in the ventral retina (A) of control and PTU-treated fish at the smolt developmental stage (nine weeks post-treatment). (B) The ventral retina of control smolt is missing UVS cones, while (C) UVS cones are present in the ventral retina of PTU-treated fish of the same age. Scale bar=50 μm. PTU, propylthiouracil.

constant supply of hormone. Increases in circulating cortisol levels associated with stress, including handling stress, have been found to interact with TH in fishes (Kühn et al., 1998). Thus, there is the potential for cortisol to be generated using this treatment method. This raises the possibility that cortisol is necessary in combination with exogenous T4 treatment to induce precocious UVS cone loss. The use of a coconut oil implant to provide long-term exposure of rainbow trout parr to exogenous T4 in this study reduced the potentially confounding effects of stress inherent in the previously utilised T4-treatment method, allowing the fish to be held under optimal conditions with minimal researcher-induced stress. This provides strong evidence that exogenous T4 alone induces UVS cone loss in rainbow trout.

SWS1 downregulation and UVS cone loss were attenuated in PTU-treated fish after both four and nine weeks of treatment, demonstrating that endogenous TH is necessary for these events. Treatment with PTU consistently prevents TH synthesis and significantly decreases plasma TH levels in all teleost species examined (for reviews, see Leatherland, 1994; Cyr and Eales, 1996). By nine weeks, the effects of PTU treatment were demonstrated by undetectable levels of plasma T4, lack of change in body colouration and decreased growth when compared with the control fish. Differences between retinas of control and PTU-treated fish were most evident at the nine-week time point. By this time, the PTU treatment had most likely prevented the endogenous production of TH associated with natural smoltification; these fish were smaller and retained their parr marks, while control fish were larger, with colouration resembling adult fish. In other studies, treatment of salmonids with 25 mg l^{-1} (Sullivan et al., 1987) or 30 mg l^{-1} (Ebbesson et al., 1998) PTU has been shown to reduce plasma total T4, body growth, food conversion efficiency, pigment changes and the ability to osmoregulate in seawater. Ebbesson et al. found a decrease in plasma total T3 levels and a corresponding decrease in plasma growth hormone (GH) with PTU treatment (Ebbesson et al., 1998). The results of these studies provide support for the lack of both body growth and changes in body pigment observed in the nine-week PTU-treated trout in the current study. This also provides supporting evidence that a sufficient systemic dose of PTU was released from the implant in the PTU-treated trout to inhibit T4 production.

While there was a trend toward a higher level of *SWS1* expression in the PTU-treated fish relative to the control fish at both sampling times, this trend was not statistically significant. In an effort to illuminate results that are slightly obscured by the high variation in *SWS1* opsin gene expression in the four- and nine-week PTU-treated groups, results for each individual fish were collated based on body size, *SWS1* opsin expression relative to the control parr and UVS cone distribution (Table 3). These results demonstrate that smaller fish were most likely to exhibit higher expression levels of *SWS1* and a greater distribution of UVS cones across their retina. At the start of the experiment, some ventral UVS cone loss was evident in some of the same population of parr (data not shown), and the onset of UVS cone loss has been suggested to occur early in the parr stage (Cheng et al., 2007). PTU treatment was not expected to trigger regeneration of UVS cones already lost *via* natural processes but merely to arrest additional endogenous T4-induced loss after the initiation of PTU treatment. Therefore, early *SWS1* downregulation and UVS cone loss in the experimental fish may have partially masked effects of the PTU treatment. Long-term PTU treatment begun with much younger and smaller parr (<5 g) would be necessary to substantiate this hypothesis.

Fish treated with both T4 and PTU resembled T4-treated fish in all measurements examined, including body morphology, *SWS1* expression levels and UVS cone loss. These results were expected, as PTU acts directly on the thyroid tissue to inhibit endogenous T4 synthesis but PTU was not expected to affect the high circulating T4 levels resulting from exogenous administration. Interestingly, plasma T4 levels were noticeably lower in T4/PTU-treated fish than

in the T4-only fish, although the reason for this is not clear. It is tempting to interpret the lower plasma T4 level in the T4/PTU fish as a result of the PTU treatment but there is no direct evidence to support this. Nevertheless, the exogenous T4 level administered in these T4/PTU fish was sufficient to induce precocious smoltification and retinal remodelling.

The present study demonstrates that manipulation of systemic T4 levels in rainbow trout leads to changes in retinal gene expression of the TH-regulating components examined. Specifically, expression of the nuclear receptor *TRβ* was significantly upregulated in fish treated with T4, PTU and T4/PTU implants for four weeks, prior to complete natural developmental loss of UVS cones from most of the central retina. *D2* expression was downregulated following both T4 and T4/PTU treatments, while expression levels in PTU-treated fish were not different from controls. Alternately, *TRα* expression levels did not differ between any of the treatment groups, and there were no differences in gene expression of the *TR*s or *D2* between PTU-treated and control fish after nine-weeks of treatment, when complete natural developmental loss of UVS cones from most of the central retina was completed.

Several previous studies have demonstrated *TR* expression in the vertebrate retina (Sjöberg et al., 1992; Harpavat and Cepko, 2003; Mader and Cameron, 2006; Applebury et al., 2007; Ng et al., 2009; Raine and Hawryshyn, 2009). Both *TRα* and *TRβ* are expressed in the retina of another fish, the winter flounder (*Pleuronectes americanus*), in addition to rainbow trout (Mader and Cameron, 2006; Raine and Hawryshyn, 2009). Upregulation of retinal *TRα* gene expression has been shown to accompany TU treatment (an anti-thyroid agent similar to PTU) using the whole fish immersion method in post-metamorphic flounder (Mader and Cameron, 2006). Conversely, changes in *TRα* were not apparent in the present study. However, this may be a result of several differences between the two studies, including treatment delivery method, PTU/TU concentration, species and developmental stage examined. In winter flounder, the increase in *TRα* gene expression did not correspond to an increase in levels of the corresponding protein, suggesting that functional TRα may be regulated by post-transcriptional mechanisms and not involved in retinal changes accompanying flounder metamorphosis (Mader and Cameron, 2006). Interestingly, Mader and Cameron did note an increase in *TRβ* expression in the retina of post-metamorphic flounder compared with the pre-metamorphic condition (Mader and Cameron, 2006). As flounder metamorphosis is a TH-driven process, this latter finding suggests a role for TRβ in the developing flounder retina.

TH receptors are expressed in the developing mouse retina and multiple studies in mouse models of TH disruption provide compelling evidence that *TRβ2* (a mammalian splice variant of *TRβ*) regulates opsin expression during mammalian retinal development (Ng et al., 2001; Roberts et al., 2006; Applebury et al., 2007; Pessôa et al., 2008; Lu et al., 2009). Mouse cone photoreceptors express one of two opsins, a short-wavelength-sensitive S-opsin and a middle-wavelength-sensitive M-opsin (Wikler et al., 1996). Endogenous T3 binding to *TRβ2* is necessary to inhibit S-opsin expression and turn on M-opsin expression in developing mouse retina *in vivo* (Roberts et al., 2006; Pessôa et al., 2008). Mouse S-opsin belongs to the SWS1 opsin subfamily (Yokoyama and Yokoyama, 1996) and suggests that TH regulation of SWS1 *via* TRβ may be an evolutionarily conserved vertebrate feature.

Previous studies postulated a correlation between *SWS1* downregulation and *SWS2* opsin upregulation in the retina of juvenile salmonids, and suggested that UVS cones do not degenerate but instead transform into blue cones (Cheng et al., 2006; Cheng et al., 2009; Cheng and Flamarique, 2007). Although this phenomenon was not specifically investigated in this study, the expression of *SWS2* opsin at the four- and nine-week sampling periods did not negatively co-vary with *SWS1* expression. In the present study, *SWS2* was always slightly but not significantly upregulated after four weeks of treatment, regardless of whether relative *SWS1* expression levels were reduced (T4, T4/PTU treatment) or increased (PTU treatment). Therefore, the current data do not support the hypothesis of negative co-regulation between *SWS1* and *SWS2*. The present study, however, examined transcript levels across the whole retina and this approach could mask any potential regional variation. Thus, although this study does not provide any evidence to support the claim that *SWS1* and *SWS2* opsins are co-expressed, neither do the results provide any evidence to unequivocally dispute the claim.

The present study demonstrates that exogenous T4 alone is sufficient to induce UVS cone loss in the juvenile rainbow trout retina, and that endogenous T4 is necessary to trigger natural *SWS1* downregulation and corresponding UVS cone loss. The TH-regulating TRs and D2 are expressed in the trout retina and their expression changes with systemic T4 manipulation. This is the first study to provide evidence for a role of endogenous T4 in natural UVS cone loss in salmonid fishes.

LIST OF ABBREVIATIONS

D1–D3	deiodinase 1–3
DN	dorsal nasal
DT	dorsal ventral
PB	phosphate buffer
PNA	peanut lectin-agglutinin
PTU	propylthiouracil
T3	triiodothyronine
T4	thyroxine
TH	thyroid hormone
TR	thyroid hormone receptor
TRα	thyroid hormone receptor isoform alpha
TRβ	thyroid hormone receptor isoform beta
UVS	ultraviolet-sensitive

ACKNOWLEDGEMENTS

The authors would like to thank Dr Duncan McKenzie for facilitating access to the Texas Vet Med Diagnostic Laboratory, College Station, Texas. Grant Sponsors: Canada Research Chair program and NSERC Discovery grant (C.W.H.).

REFERENCES

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

Applebury, M. L., Farhangfar, F., Glösmann, M., Hashimoto, K., Kage, K., Robbins, J. T., Shibusawa, N., Wondisford, F. E. and Zhang, H. (2007). Transient expression of thyroid hormone nuclear receptor TRß2 sets S opsin patterning during cone photoreceptor genesis. *Dev. Dyn.* **236**, 1203-1212.

Bernal, J., Guadaño-Ferraz, A. and Morte, B. (2003). Perspectives in the study of thyroid hormone action on brain development and function. *Thyroid* **13**, 1005-1012.

Bianco, A. C., Salvatore, D., Gereben, B., Berry, M. J. and Larsen, P. R. (2002). Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. *Endocr. Rev.* **23**, 38-89.

Blanks, J. C. and Johnson, L. V. (1984). Specific binding of peanut lectin to a class of retinal photoreceptor cells. A species comparison. *Invest. Ophthalmol. Vis. Sci.* **25**, 546-557.

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

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

Browman, H. and Hawryshyn, C. (1994b). Retinoic acid modulates retinal development in the juveniles of a teleost fish. *J. Exp. Biol.* **193**, 191-207.

Brown, D. D. (2005). The role of deiodinases in amphibian metamorphosis. *Thyroid* **15**, 815-821.

Callery, E. M. and Elinson, R. P. (2000). Thyroid hormone-dependent metamorphosis in a direct developing frog. *Proc. Natl. Acad. Sci. USA* **97**, 2615-2620.
Cheng, C. L. and Flamarique, I. N. (2007). Chromatic organization of cone photoreceptors in the retina of rainbow trout: single cones irreversibly switch from UV (SWS1) to blue (SWS2) light sensitive opsin during natural development. *J. Exp. Biol.* **210**, 4123-4135.
Cheng, C. L., Flamarique, I. N., Hárosi, F. I., Richers-Haunerland, J. and Haunerland, N. H. (2006). Photoreceptor layer of salmonid fishes: transformation and loss of single cones in juvenile fish. *J. Comp. Neurol.* **495**, 213-235.
Cheng, C. L., Gan, K. J. and Flamarique, I. N. (2009). Thyroid hormone induces a time-dependent opsin switch in the retina of salmonid fishes. *Invest. Ophthalmol. Vis. Sci.* **50**, 3024-3032.
Cossette, S. M. and Drysdale, T. A. (2004). Early expression of thyroid hormone receptor beta and retinoid X receptor gamma in the *Xenopus* embryo. *Differentiation* **72**, 239-249.
Crump, D., Werry, K., Veldhoen, N., Van Aggelen, G. and Helbing, C. C. (2002). Exposure to the herbicide acetochlor alters thyroid hormone-dependent gene expression and metamorphosis in *Xenopus laevis*. *Environ. Health Perspect.* **110**, 1199-1205.
Darras, V. M., Mol, K. M., van der Geyten, S. and Kühn, E. R. (1998). Control of peripheral thyroid hormone levels by activating and inactivating deiodinases. *Ann. NY Acad. Sci.* **839**, 80-86.
Deutschlander, M. E., Greave, D. K., Haimberger, T. J. and Hawryshyn, C. W. (2001). Functional mapping of ultraviolet photosensitivity during metamorphic transitions in a salmonid fish, *Oncorhynchus mykiss*. *J. Exp. Biol.* **204**, 2401-2413.
Eales, J. G. and Brown, S. B. (1993). Measurement and regulation of thyroidal status in teleost fish. *Rev. Fish Biol. Fish* **3**, 299-347.
Ebbesson, L. O. E., Björnsson, B. Th., Stefansson, S. O. and Ekström, P. (1998). 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.
Flett, P. A. and Leatherland, J. F. (1989). Dose-related effects of 17ß-oestradiol (E2)o n liver weight, plasma E2, protein, calcium and thyroid hormone levels, and measurement of the binding of thyroid hormones to vitellogenin in rainbow trout, *Salmo gairdneri* Richardson. *J. Fish Biol.* **34**, 515-521.
Furlow, J. D. and Neff, E. S. (2006). A developmental switch induced by thyroid hormone: *Xenopus laevis* metamorphosis. *Trends Endocrinol. Metab.* **17**, 40-47.
Harpavat, S. and Cepko, C. L. (2003). Thyroid hormone and retinal development: an emerging field. *Thyroid* **13**, 1013-1019.
Hawryshyn, C. W. (2000). Ultraviolet polarization vision in fishes: possible mechanisms for coding e-vector. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **355**, 1187-1190.
Hawryshyn, C. W. and Hárosi, 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., Martens, G., Allison, W. T. and Anholt, B. R. (2003). Regeneration of ultraviolet-sensitive cones in the retinal cone mosaic of thyroxin-challenged post-juvenile rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **206**, 2665-2673.
Hoar, W. (1988). The physiology of smolting salmonids. In *Fish Physiology* (eds. W. Hoar and D. Randall), pp. 275-343. San Diego: Academic Press.
Holloway, A. C. and Leatherland, J. F. (1997). Effect of gonadal steroid hormones on plasma growth hormone concentrations in sexually immature rainbow trout, *Oncorhynchus mykiss*. *Gen. Comp. Endocrinol.* **105**, 246-254.
Hulbert, A. J. (2000). Thyroid hormones and their effects: a new perspective. *Biol. Rev.* **75**, 591-631.
Joselevitch, C. and Kamermans, M. (2009). Retinal parallel pathways: seeing with our inner fish. From retinal and cortical circuitry to clinical application. In memory of Henk Spekreijse – a life dedicated to Vision Research. *Vision Res.* **49**, 943-959.
Kitschmann, M. and Neumeyer, C. (2005). Generalization and categorization of spectral colors in goldfish I. Experiments with one training wavelength. *J. Comp. Physiol. A Neuroethol. Sens. Neural. Behav. Physiol.* **191**, 1025-1036.
Köhrle, J. (1999). Local activation and inactivation of thyroid hormones: the deiodinase family. *Mol. Cell. Endocrinol.* **151**, 103-119.
Kühn, E. R., Geris, K. L., van der Geyten, S., Mol, K. A. and Darras, V. M. (1998). Inhibition and activation of the thyroidal axis by the adrenal axis in vertebrates. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **120**, 169-174.
Kunz, Y. W. (2006). Review of development and aging in the eye of teleost fish. *Neuroembryol. Aging* **4**, 31-60.
Kunz, Y. W., Wildenburg, G., Goodrich, L. and Callaghan, E. (1994). The fate of ultraviolet receptors in the retina of the Atlantic salmon (*Salmo salar*). *Vision Res.* **34**, 1375-1383.
Leatherland, J. F. (1994). Reflections on the thyroidology of fishes: from molecules to humankind. *Guelph Ichthyol. Rev.* **2**, 1-67.
Lu, A., Ng, L., Kefas, B., Davies, T. F., Hernandez, A., Chan, C. C. and Forrest, D. (2009). Retarded development expression and patterning of retinal cone opsins in hypothyroid mice. *Endocrinology* **150**, 1536-1544.
Mader, M. M. and Cameron, D. A. (2006). Effects of induced systemic hypothyroidism upon the retina: regulation of thyroid hormone receptor alpha and photoreceptor production. *Mol. Vis.* **12**, 915-930.
Mora-Ferrer, C. and Neumeyer, C. (2009). Neuropharmacology of vision in goldfish: a review. *Vis. Res.* **49**, 960-969.
Ng, L., Hurley, J. B., Dierks, B., Srinivas, M., Saltó, C., Vennström, B., Reh, T. A. and Forrest, D. (2001). A thyroid hormone receptor that is required for the development of green cone photoreceptors. *Nat. Genet.* **27**, 94-98.
Ng, L., Ma, M., Curran, T. and Forrest, D. (2009). Developmental expression of thyroid hormone receptor β2 protein in cone photoreceptors in the mouse. *NeuroReport* **20**, 627-631.
Nunez, J., Celi, F. S., Ng, L. and Forrest, D. (2008). Multigenic control of thyroid hormone functions in the nervous system. *Mol. Cell. Endocrinol.* **287**, 1-12.
Orozco, A., Linser, P. and Valverde-R, C. (2000). Kinetic characterization of outer-ring deiodinase activity (ORD) in the liver, gill and retina of the killifish *Fundulus heteroclitus*. *Comp. Biochem. Physiol. B* **126**, 283-290.
Parkyn, D. C. and Hawryshyn, C. W. (2000). Spectral and ultraviolet-polarization sensitivity in juvenile salmonids: a comparative analysis using electrophysiology. *J. Exp. Biol.* **203**, 1173-1191.
Pessôa, C. N., Santiago, L. A., Santiago, D. A., Machado, D. S., Rocha, F. A., Ventura, D. F., Hokoç, J. N., Pazos-Moura, C. C., Wondisford, F. E., Gardino, P. F. and Ortiga-Carvalho, T. M. (2008). Thyroid hormone action is required for normal cone opsin expression during mouse retinal development. *Invest. Ophthalmol. Vis. Sci.* **49**, 2039-2045.
Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45.
Plate, E. M., Adams, B. A., Allison, W. T., Martens, G., Hawryshyn, C. W. and Eales, J. G. (2002). The effects of thyroxine or a GnRH analogue on thyroid hormone deiodination in the olfactory epithelium and retina of rainbow trout, *Oncorhynchus mykiss*, and sockeye salmon, *Oncorhynchus nerka*. *Gen. Comp. Endocrinol.* **127**, 59-65.
Power, D. M., Llewellyn, L., Faustino, M., Nowell, M. A., Björnsson, B. T., Einarsdottir, I. E., Canario, A. V. and Sweeney, G. E. (2001). Thyroid hormones in growth and development of fish. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **130**, 447-459.
Raine, J. C. and Hawryshyn, C. W. (2009). Changes in thyroid hormone reception precede SWS1 opsin down-regulation in trout retina. *J. Exp. Biol.* **212**, 2781-2788.
Raine, J. C., Cameron, C., Vijayan, M. M., Lamarre, J. and Leatherland, J. F. (2004). The effect of elevated oocyte triiodothyronine content on development of rainbow trout embryos and expression of mRNA encoding for thyroid hormone receptors. *J. Fish Biol.* **65**, 206-226.
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.
Shi, Y. B. and Liang, V. C. (1994). Cloning and characterization of the ribosomal protein L8 gene from *Xenopus laevis*. *Biochim. Biophys. Acta* **1217**, 227-228.
Sjöberg, M., Vennström, B. and Forrest, D. (1992). Thyroid hormone receptors in chick retinal development: differential expression of mRNAs for α and N-terminal variant ß receptors. *Development* **114**, 39-47.
Spekreijse, H., Wietsma, J. J. and Neumeyer, C. (1991a). Ethambutol-induced color-blindness in goldfish: a behavioral, electrophysiological and morphological study. *Neurosci. Res.* **Suppl. 15**, S77-S89.
Spekreijse, H., Wietsma, J. J. and Neumeyer, C. (1991b). Induced color blindness in goldfish: a behavioral and electrophysiological study. *Vis. Res.* **31**, 551-562.
St. Germain, D. L., Hernandez, A., Schneider, M. J. and Galton, V. A. (2005). Insights into the role of deiodinases from studies of genetically modified animals. *Thyroid* **15**, 905-916.
Sullivan, C. V., Iwamoto, R. N. and Dickhoff, W. W. (1987). Thyroid hormones in blood plasma of developing salmon embryos. *Gen. Comp. Endocrinol.* **65**, 337-345.
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.
Vijayan, M. M. and Leatherland, J. F. (1989). *In vivo* effects of the steroid analogue RU486 on some aspects of intermediary and thyroid metabolism of brook charr, *Salvelinus fontinalis*. *J. Exp. Zool.* **263**, 265-271.
Wiersma, J. J. and Spekreijse, H. (1991). Biciculline produces reversible red-green color blindness in goldfish, as revealed by monocular behavioral testing. *Vis. Res.* **31**, 2101-2107.
Wikler, K. C., Szel, A. and Jacobsen, A. L. (1996). Positional information and opsin identity in retinal cones. *J. Comp. Neurol.* **374**, 96-107.
Yokoyama, S. and Yokoyama, R. (1996). Adaptive evolution of photoreceptors and visual pigments in vertebrates. *Annu. Rev. Ecol. Syst.* **27**, 543-567.