The Journal of Experimental Biology 214, 2248-2257

doi:10.1242/jeb.055566

RESEARCH ARTICLE

In ovo thyroxine exposure alters later UVS cone loss in juvenile rainbow trout

Jason C. Raine[1,*], Allison B. Coffin[1,†] and Craig W. Hawryshyn[1,2,‡]

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

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

Accepted 17 March 2011

SUMMARY

Thyroid hormones (THs) play a vital role in vertebrate neural development, and, together with the beta isoform of the thyroid hormone receptor (TRβ), the development and differentiation of cone photoreceptors in the vertebrate retina. Rainbow trout undergo a natural process of cone cell degeneration during development and this change in photoreceptor distribution is regulated by thyroxine (T4; a thyroid hormone). In an effort to further understand the role of T4 in photoreceptor ontogeny and later developmental changes in photoreceptor subtype distribution, the influence of enhanced *in ovo* T4 content on the onset of opsin expression and cone development was examined. Juvenile trout reared from the initial *in ovo*-treated embryos were challenged with exogenous T4 at the parr stage of development to determine if altered embryonic exposure to yolk THs would affect later T4-induced short-wavelength-sensitive (*SWS1*) opsin transcript downregulation and ultraviolet-sensitive (UVS) cone loss. *In ovo* TH manipulation led to upregulation of both *SWS1* and long-wavelength-sensitive (*LWS*) opsin transcripts in the pre-hatch rainbow trout retina and to changes in the relative expression of *TR*β. After 7 days of exposure to T4, juveniles that were also treated with T4 *in ovo* had greatly reduced *SWS1* expression levels and premature loss of UVS cones relative to T4-treated juveniles raised from untreated eggs. These results suggest that changes in egg TH levels can have significant consequences much later in development, particularly in the retina.

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

INTRODUCTION

Thyroid hormones (THs) are considered crucial factors for neural development in vertebrates. In humans, TH deficiency during fetal development can result in mental retardation, deafness and motor disorders (Hulbert, 2000). Evidence in mammals suggests that THs are required for neuronal proliferation, migration, synaptogenesis and myelination during brain development (Howdeshell, 2002; Horn and Heuer, 2010; Portella et al., 2010). In fish, THs also play a role in neurological development (Essner et al., 1999; Yonkers and Ribera, 2008; Yonkers and Ribera, 2009). For instance, overexpression of the thyroid hormone receptor (TR) isoform TRα1 impairs hindbrain formation in zebrafish (*Danio rerio*) embryos (Essner et al., 1999).

Thyroid hormone signaling is particularly important for early retinal development. THs are involved in differentiation and patterning of cone cells in the neural retina during early development of vertebrates, including fish (e.g. Ng et al., 2001; Roberts et al., 2006; Nunez et al., 2008). In mice, a TH gradient appears to pattern opsin photopigment expression, and hypothyroid animals have disrupted opsin expression patterns, showing an increase in short-wavelength-sensitive opsin (S-opsin) and a decrease in middle-wavelength-sensitive opsin (M-opsin) (Roberts et al., 2006; Applebury et al., 2007; Pessôa et al., 2008; Glaschke et al., 2010). Knockout studies demonstrate that TH binding to TRβ2 is necessary for proper cone subtype specification, and a recent study suggests a role for type 3 deiodinase (D3), an enzyme that inactivates TH, in cone survival and maintenance (Ng et al., 2001; Roberts et al., 2006; Pessôa et al., 2008; Ng et al., 2010).

TH signaling also regulates retinal changes during later development, including changes in opsin expression and cone distribution associated with metamorphosis in diverse fish species (Browman and Hawryshyn, 1992; Browman and Hawryshyn, 1994; Allison et al., 2003; Allison et al., 2006; Mader and Cameron, 2006; Cheng et al., 2009). Natural loss of ultraviolet-sensitive (UVS) cones from the retina in the salmon and trout (salmonid) family of fishes occurs during a developmental transition phase called smoltification (Browman and Hawryshyn, 1992; Browman and Hawryshyn, 1994; Kunz et al., 1994; Deutschlander et al., 2001; Allison et al., 2003; Allison et al., 2006). Smoltification is characterized by behavioral, physiological and morphological changes that transform juvenile fish (parr) living in freshwater streams into seaward-migrating smolts that spend much of their life in the ocean (Hoar, 1988). This process also occurs in land-locked salmonid species such as the popular fish model, rainbow trout (*Oncorhynchus mykiss*), which spend their entire lifespan in freshwater systems. In rainbow trout, loss of UVS cones is preceded by the downregulation of the *SWS1* opsin gene (Allison et al., 2003; Veldhoen et al., 2006; Raine and Hawryshyn, 2009; Raine et al., 2010). Exogenous thyroxine (T4; a thyroid hormone) induces precocious *SWS1* downregulation and UVS cone degeneration, whereas TH attenuation *via* propylthiouracil (PTU) delays *SWS1* downregulation and UVS cone loss, suggesting that endogenous T4 initiates these processes naturally during development (Browman and Hawryshyn, 1992; Browman and Hawryshyn, 1994; Kunz et al., 1994; Deutschlander et al., 2001; Allison et al., 2003; Allison et al., 2006; Kunz, 2006; Veldhoen et

al., 2006; Raine and Hawryshyn, 2009; Raine et al., 2010). Additionally, consistent upregulation of TRβ gene expression prior to T4-induced down-regulation of *SWS1* suggests that this receptor isoform plays a key role in this developmental process, similar to that found in mice (Raine and Hawryhshyn, 2009; Ng et al., 2010; Raine et al., 2010).

Because THs appear to play a significant role in early retinal development and cone differentiation in many vertebrate species, and because T4 is specifically involved in the process of UVS cone loss in salmonid fishes, we hypothesized that maternal thyroid hormones play a key role during UVS cone development, and thus altered TH content in rainbow trout oocytes would modify both UVS cone ontogeny during early retinal development and UVS cone degeneration during the parr stage of development. Manipulation of maternal TH levels in rodents has been shown previously to affect auditory function, learning and memory in adult offspring, suggesting that moderate and transient changes in TH exposure during development can have lasting physiological consequences (Sui et al., 2005; Axelstad et al., 2008). THs, both T4 and T3 (triiodothyronine, considered the most active form of thyroid hormone), are available to the developing embryo and/or fetus in vertebrates. In mammals, THs from the maternal circulation are transferred *via* the placenta, whereas in egg-laying vertebrates, such as birds and teleost fishes, THs of maternal origin are present in the egg yolk (Leatherland, 1994; Power et al., 2001; McNabb, 2006). Teleost egg TH levels appear to reflect maternal plasma TH levels at ovulation, and may be transferred to the egg through the ceolomic fluid (Leatherland, 1994; Raine and Leatherland, 2003). In teleost fishes in particular, it is not clear if these yolk THs are necessary for the development of the embryo prior to the ontogeny of endogenous TH production by the embryonic thyroid tissue (Leatherland, 1994; Raine and Leatherhead, 1999; Raine and Leatherland, 2000; Power et al., 2001; Raine et al., 2004). However, TRs are present in teleost fish embryos and it has been demonstrated that elevated rainbow trout oocyte TH content can affect later embryo TR gene expression, which suggests that both maternal THs and *in ovo* TH manipulation can affect subsequent fish development (Power et al., 2001; Raine et al., 2004). Furthermore, deiodinase activity and gene expression have been detected in fish embryos prior to development of the thyroid tissue, suggesting that regulation of TH availability occurs during early embryonic development (Raine et al., 2004; Walpita et al., 2010). Thus in the present study, it was anticipated that elevated levels of yolk T4 would be deiodinated to T3 by embryonic deiodinases, which would in turn upregulate retinal *TR*β and induce both premature UVS cone development and subsequent UVS cone loss.

MATERIALS AND METHODS

Experimental design

Part I: effect of altered oocyte T4 content on opsin ontogeny

Ten thousand unfertilized rainbow trout (*Oncorhynchus mykiss* Walbaum 1792) eggs and milt obtained from five 3-year-old females and five 4-year-old males, respectively, were acquired from the Alma Aquaculture Research Station (Alma, ON, Canada). The unfertilized eggs were pooled and divided equally into two treatment groups: untreated (U) and T4 treated (150 μg ml^{-1} L-thyroxine, sodium salt; Sigma-Aldrich Canada Ltd, Oakville, ON, Canada). The treatment of unfertilized eggs was performed using a previously established method that demonstrated consistent repeatable oocyte TH elevation (Raine and Leatherland, 2003; Raine et al., 2004). Initial T4 content of the oocytes treated in this manner was therefore expected to be approximately 100 ng egg^{-1} in the T4-treated group. This level of T4 was necessary to ensure that the embryo content of T4 in T4-enhanced eggs would remain at a significantly higher physiological level than that of control embryos, as TH levels have been found to decrease dramatically within the first 2 to 3 weeks of development, probably because of deiodinase activity (Raine et al., 2004). Using this established method, treatment solutions were generated by mixing T4 with an aliquot of coelomic fluid separated from the ovulated oocytes, whereas control solutions contained only coelomic fluid. Each of the two groups of oocytes were then combined with the appropriate treatment solution and incubated at 4°C for 3 h, and then fertilized.

Treated oocytes were fertilized with the pooled milt and placed in divided trays of a heath incubator supplied with constantly running water at 10°C. [Embryos ages are given as days post-fertilization (d.p.f.) and degree days (D). A degree day is defined as the average daily water temperature multiplied by the age of the embryos in d.p.f. Degree days can be used to compare the growth and development of the same species of fish reared at different water temperatures (Raine and Leatherland, 1999).] Embryos were sampled every second day from the first detection of eye pigmentation (21 d.p.f. or 210 D) to hatching (37 d.p.f. or 370 D) for histology, RT-PCR and quantitative real-time RT-PCR (qPCR) analysis. RT-PCR was used as a screening tool to identify the onset of specific opsin subtype expression in the retina such that qPCR and histological samples could target the developmental window encompassing opsin expression onset and associated cone development.

All post-hatching embryos were transferred to 80 l tanks after 54 d.p.f. (540 D) of development and feed was first offered at a full ration (4.5% g body mass day^{-1}; Silver Cup starter, Martin Mills, Elmira, ON, Canada) to all treatment groups shortly after the embryos began to actively swim and look for food (swim-up stage). Juvenile trout at the parr stage (approximately 2 g wet mass were transferred into 473 l tanks. Water temperature was slowly raised to 15°C over the course of a week. Fish were fed a full ration (Classic Sinking Feed; Martin Mills) until the commencement of the second experiment.

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.

Part II: use of a T4 implant to assess the later developmental response of UVS cones

Rainbow trout parr at an average wet mass of approximately 13 g were transferred to 80 l treatment tanks with constantly flowing and aerated water at 15°C. Forty fish from each *in ovo* treatment group (U and T4) were divided equally into two 80 l tanks to be used as a control group (C) and an exogenous T4 treatment group (Fig. 1). Fish were acclimated to the new tanks for 10 days and started on a low ration (1% g body mass day^{-1}; Classic Sinking Feed, Martin Mills) to slow growth and prevent the natural metamorphic loss of UVS cones from the retina.

After the acclimation period, a coconut oil implant was employed to expose the rainbow trout parr to exogenous T4 (Raine et al., 2010). Use of an implant allows fish to be held under optimal husbandry conditions with constantly flowing water while slow release of the exogenous hormone occurs from the implant within the coelomic cavity. A stock solution of 30 μg of T4 μl^{-1} dimethyl sulfoxide (DMSO; Sigma-Aldrich) was generated to enable homogenous mixing of T4 with the coconut oil. Parr in the T4 treatment group were injected with a 3 μg T4 μl^{-1} coconut oil solution, but control

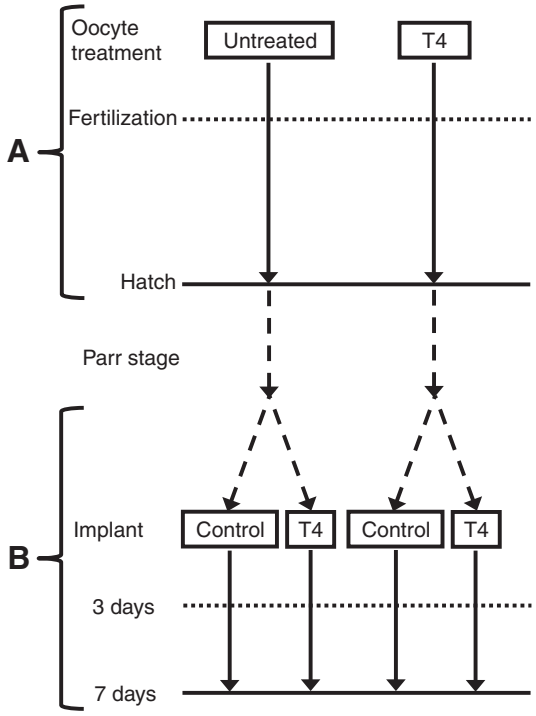

Fig. 1. A flow chart outlining the experimental design of the two-part experiment. (A) Effect of altered rainbow trout oocyte thyroxine (T4) concentration on opsin ontogeny. This first part involved rearing embryos from oocytes containing different concentrations of T4. (B) Use of a T4 implant to assess the later response of ultraviolet-sensitive (UVS) cones. The second part of the experiment investigated the effect of altered oocyte T4 content on exogenous T4 treatment-induced UVS cone degeneration during the parr stage of development. Horizontal lines in B indicate sampling time points.

fish were injected with only the DMSO vehicle. Each fish was implanted with 20 µl of coconut oil g^{-1} mean body mass. For simplicity, we have adopted a nomenclature for these treatment groups where the first designation represents the *in ovo* treatment, as mentioned previously (U or T4), and the second designation denotes the implant received by the juvenile (C or T4). For example, T4-T4 describes the treatment group that received *in ovo* T4 treatment and subsequently received a T4 implant at the parr stage.

Trout parr were sampled at 3 and 7 days post-implantation to determine whether *in ovo* TH manipulation altered photoreceptor distribution during T4-induced metamorphosis in juvenile fish. Following dark adaptation, fish were killed with 150 mg l^{-1} tricaine methanesulfonate (MS-222; Crescent Research Chemical, Phoenix, AZ, USA), and wet mass and fork length (tip of snout to fork in tail) measurements were recorded. Transverse severance of the tail was used to collect blood from the caudal blood capillaries using heparinized microcapillary tubes (Fisher Scientific, Ottawa, ON, Canada). Cervical transection immediately followed blood collection and the neural retina was 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 fixed in 4% paraformaldehyde for assessment of the presence of UVS cones using immunohistochemistry. The left isolated retina (N=5 fish per treatment) was preserved 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.

All fish stages were kept in a 12 h:12 h light:dark photoperiod generated using BlueMax full spectrum fluorescent lighting (Full Spectrum Solutions, Jackson, MI, USA). Fish care and treatment were performed in accordance with the Canadian Council for Animal Care regulations and the Queen's University Animal Care Committee.

RNA isolation and cDNA synthesis

To determine the onset of opsin expression in the *in ovo* treatment groups, embryos were collected and placed immediately in RNAlater (Ambion Inc.). Both eyes from each of two embryos were dissected and pooled for each total RNA sample, and four of these pooled eye samples were analyzed for each treatment. To investigate the effect of altered *in ovo* TH on subsequent exogenous T4-induced UVS cone degeneration in rainbow trout parr, the neural retina was dissected from the left eye of parr from each treatment group and preserved individually in RNAlater. Total RNA was isolated from each juvenile retina. RNA isolation and quantification procedures followed protocols developed in previous studies (Raine et al., 2004; Veldhoen et al., 2006; Raine and Hawryshyn, 2009; Raine et al., 2010). Briefly, samples were homogenized using a Retsch MM301 mixer mill (Retsch GmBH & Co. Ltd KG, Hann, Germany) and total RNA isolated with an Absolutely RNA Miniprep kit (Stratagene, LaJolla, CA, USA). Quantification of total RNA 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). Following RNA quantification, 500 ng total RNA from each RNA isolate was reverse-transcribed to cDNA using a Superscript III First Strand Synthesis SuperMix kit for quantitative real-time RT-PCR (Invitrogen, Carlsbad, CA, USA). The same pool of cDNA was used to determine the onset of opsin expression in embryo eyes, and to establish relative changes in the expression of the genes of interest with quantitative qPCR at pre-determined developmental stages.

RT-PCR screening for onset of opsin expression

The *O. mykiss* gene-specific primers used in this study, *short-wavelength-sensitive 1* and *2* (*SWS1*, *SWS2*), *long-wavelength-sensitive* (*LWS*) and *rhodopsin 1* and *2* (*RH1* and *RH2*), were developed and validated in previous studies (Table 1) (Raine et al., 2004; Veldhoen et al., 2006; Raine and Hawryshyn, 2009; Raine et al., 2010). Although the present study focuses primarily on *SWS1*, other opsins were also examined here, as previous studies showed that TH-induced downregulation of one opsin can lead to an increase in expression of other opsin(s) (e.g. Roberts et al., 2006; Pessôa et al., 2008; Cheng et al., 2009). Each 50 µl PCR reaction contained 10 µl GoTaq Flexi buffer (Promega US, Madison, WI, USA), 5 µl magnesium chloride ($MgCl_2$; 25 mmol l^{-1}), 1 µl dNTP mix, 1 µl of each gene specific primer (10 µmol l^{-1}), 2 µl of cDNA (5× dilution) and 0.25 µl GoTaq polymerase (Promega US). PCR reactions involved an initial 2 min cycle at 95°C, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. PCR products were separated on 1.5% agarose gels and visualized using GelRed (Biotium, Inc., Hayward, CA, USA) staining with an Alpha Innotech (San Leandro, CA, USA) gel imaging system.

Once the onset of opsin expression was established by RT-PCR, qPCR was used to analyze the relative expression of opsin, thyroid hormone receptor (*TR*α and *TR*β) and type 2 deiodinase (*D2*) genes during the developmental window of opsin expression onset to

Table 1. *Oncorhynchus mykiss* gene-specific primer sequences used in the present study

Gene	Forward primer	Reverse primer	Amplicon size (bp)
SWS1	GGCTTTCTACCTACAGACTGC	CCTGCTATTGAACCCATGC	258
SWS2	GGCACTGCTATCAACGTCCT	CCACTACTGAGAGAGACCATAA	244
RH1	CGTCCCTATGTCCAATGCT	AGTGGTGAAGCCTCCGATT	250
RH2	GAACGGCACAGAAGGAAGCA	AAAGAGGACCATTATCATTC	265
LWS	ATGCAGCCAGGCGACAAA	ATTCCGCAAGTGGAGACA	376
*TR*α	GCACAACATTCCCCACTTCT	AGTTCGTTGGGACACTCCAC	117
*TR*β	TCACCTGTGAAGGATGCAAG	GACAGCGATGCACTTCTTGA	152
D2	ATTTTGTATGCCGATGCACA	TACGGCGCTAACCTCTGTTT	207
L8	GGTGTGGCTATGAATCCTGT	ACGACGAGCAGCAATAAGAC	113

determine whether elevated oocyte T4 content affected opsin development and retinal TH regulation.

Quantitative real-time RT-PCR analysis

Quantitative real-time RT-PCR analysis of individual total RNA samples from each pool of embryo eyes or individual parr retina was performed using each of the *O. mykiss* gene-specific primers, including the housekeeping gene *L8* (Table 1) (Raine et al., 2004; Veldhoen et al., 2006; Raine and Hawryshyn, 2009). *L8* has been used previously as a housekeeping gene because it does not vary significantly during TH treatment or 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; Raine et al., 2010). Primer optimization reactions were run to determine the optimal concentration of primers to use in the qPCR reactions; the resulting optimal equimolar amounts of primer varied between 100 and 300 μmol l^{-1} (Raine and Hawryshyn, 2009; Raine et al., 2010). Standard curves were run in triplicate for each primer pair using serial dilutions of embryo eye or parr retinal cDNA. The $MgCl_2$ concentration varied between 3 and 5 mmol l^{-1} in the standard curve reactions, depending on the gene of interest, to generate repeatable R^2-values greater than 0.98 and reaction efficiencies between 90 and 100% (Raine and Hawryshyn, 2009; Raine et al., 2010). All experimental qPCR samples were run in duplicate.

Master mixes were used consistently to reduce the possibility of pipetting error. qPCR reactions used a 10 μl volume and contained Supermix-UDG (Invitrogen), ROX and 2 μl of 20-fold diluted parr retinal cDNA or 2 μl of fivefold diluted embryo eye cDNA. The thermocycling program was 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. A dissociation curve was generated at the conclusion of every thermocycling program to verify the presence of one distinct DNA peak that dissociated at a consistent temperature. An MX3000P real-time quantitative PCR thermocycler (Stratagene) was used for qPCR reactions, and data analysis was performed with the associated MXPro v. 4.0 software using the efficiency-corrected comparative quantification method (Pfaffl, 2001).

Immunohistochemistry

Both whole *in ovo* embryos and the individual right retina from parr were fixed in 4% paraformaldehyde overnight at 4°C and rinsed in phosphate buffer (PB). Embryos were incubated in 15% sucrose-PB overnight at 4°C. Finally, tissues were oriented in OCT mounting medium (VWR International, Mississauga, ON, Canada) and stored at –80°C until sectioning. Frozen blocks containing embryos were sectioned serially and transversely at 10 μm, and air dried on SuperFrost Plus (Fisher Scientific) microscope slides prior to short-term storage at –20°C. The immunostaining procedure using mouse polyclonal antibody to the N-terminal portion of the rainbow trout SWS1 opsin was similar for both tissue sections and whole retinas and followed previous protocols (Allison et al., 2006; Raine et al., 2010). Briefly, individual whole retinas or sections were incubated with PB containing 0.3% Triton X-100 (PBT) and 5% normal goat serum (Sigma-Aldrich), followed by incubation overnight at 4°C with the SWS1 opsin antibody, at 1:5000 dilution in PBT. The tissue was rinsed in fresh PB, incubated for 1 h with the fluorescent secondary antibody (Alexa Fluor 594 goat anti-mouse; Invitrogen, Carlsbad, CA, USA) diluted 1:750 in 1% goat serum and PBT, and further rinsed in PB. Coverslips were placed over the whole-mount parr retinas and sectioned embryo eyes with Prolong Gold mounting medium (Invitrogen).

Retinas were visualized with a Zeiss Axioskop 2 compound microscope equipped for epifluorescence and a Retiga EXi 12-bit CCD cooled monochromatic digital camera (QImaging, Surrey, BC, Canada). Northern Eclipse 7.0 software (Empix Imaging, Mississauga, ON, Canada) was used to capture digital images, which were cropped and adjusted for brightness and contrast using Adobe PhotoShop 7.0 (Adobe Systems, Mountain View, CA, USA).

Plasma T4 analysis

Plasma T4 levels were analyzed from both early developmental stages and T4-treated juveniles. Following blood collection, microhematocrit tubes were kept on ice, centrifuged at 1500 ***g*** at 4°C to separate blood plasma from the cellular components, and stored at –20°C. The plasma from two to three fish was pooled to generate five replicates per treatment, each containing 100 μl of plasma. T4 analysis was performed using a standard radioimmunoassay procedure (Texas Vet Med Diagnostic Laboratory, College Station, TX, USA).

Statistical analysis

One-way ANOVA was used for most data to test for significant differences among treatment means. The Holm–Sidak pairwise multiple comparison procedure was used for *post hoc* significance testing. The 3 day *TR*β qPCR data were not normally distributed and so were log transformed to allow the use of parametric statistical analysis. Kruskal–Wallis one-way ANOVA on ranks was used to establish significant differences among the means for plasma T4 levels, with Dunn's method to determine which means were significantly different from one another. For the embryo qPCR data, Student's *t*-tests were used to test for significant differences in the same gene between untreated embryos and embryos reared from T4-enhanced oocytes at each sampling time. The Mann–Whitney rank sum test was used to test for significant differences between untreated and T4-enhanced embryo *LWS* expression, as the test for equal variance of this data set failed and precluded the use of

Student's *t*-test. Statistical analysis was carried out using SigmaPlot 11 (Systat Software, Inc., Chicago, IL, USA).

RESULTS

Part I: effect of manipulated oocyte TH content on opsin expression

Opsin transcript expression was first detected by RT-PCR screening at 30 d.p.f. (300 D; Fig. 2). Both *SWS1* and *RH1* were present at 30 d.p.f. (300 D), whereas *LWS* expression was found at 31 d.p.f. (310 D) and *RH2* expression was not detected until hatching at 37 d.p.f. (370 D; Fig. 2). No *SWS2* expression was detected at any time prior to hatching. *In ovo* T4 manipulation did not appear to alter the timing of opsin expression onset. Expression of SWS1 protein was verified with immunohistochemistry at 33 d.p.f. (330 D) and SWS1 protein expression was observed in the retinas in both treatment groups, consistent with the RT-PCR results (Figs 2, 3).

Cone opsin gene expression was examined using qPCR analysis in both oocyte groups at 31 d.p.f. (310 D), at the onset of *SWS1* and *RH1* expression, as detected by RT-PCR screening. *SWS1* was significantly elevated relative to the level in untreated embryos (P=0.009; Fig. 4). The *in ovo* T4-treated group also exhibited elevated retinal *LWS* (P=0.029) transcript relative to the retinas of the untreated embryos (Fig. 4). Retinal *RH2* expression showed high variability in the T4 group and was not significantly different from *RH2* levels in untreated embryos (P=0.813; Fig. 4).

Altered oocyte TH content appeared to affect *TR* and *D2* expression in the embryonic retina during the developmental period encompassing opsin expression onset, at 25–30 d.p.f. (250–300 D). qPCR analysis of 25 d.p.f. (250 D) embryos suggested that retinal *TR*α and *TR*β may be upregulated in the *in ovo* T4-enhanced embryos relative to the untreated embryos, although no statistically significant differences were determined (Fig. 5; N=4 pools of eyes; *TR*α: P=0.090; *TR*β: P=0.237). Similarly, retinal *D2* expression also showed a slight increase in the *in ovo* T4-enhanced embryos (Fig. 5; N=4 pools of eyes; P=0.463). At 300 D, the T4-enhanced embryo retinas did not show much change in accumulation of any of the transcripts relative to the untreated embryo retinas (*TR*α: P=0.496; *TR*β: P=0.136; *D2*: P=0.814; Fig. 5).

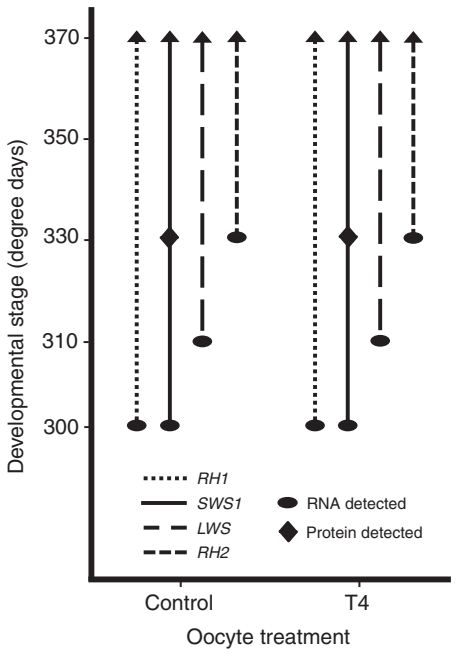

Fig. 2. Schematic diagram illustrating the ontogeny of opsin gene and protein expression in rainbow trout embryos with manipulated *in ovo* T4 content. Sampling was halted at hatching (37 d.p.f.; 370 D).

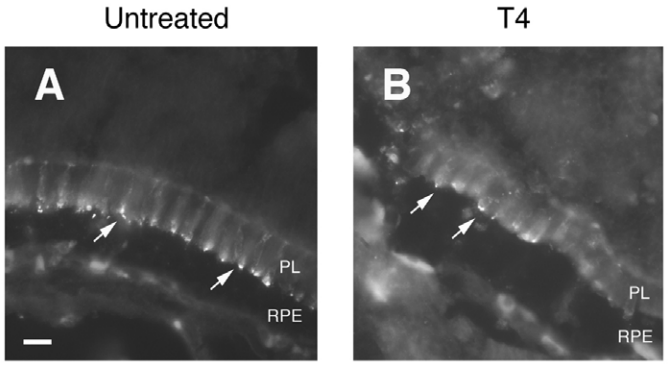

Fig. 3. Immunolabeling of UVS cones in untreated (A) and T4-treated (B) fish retina at 33 d.p.f. (330 D). Images are from radially cryosectioned retinas, and are each representative of other embryo retinas from the same treatment. PL, photoreceptor layer; RPE, retinal pigment epithelium. Arrows point to examples of UVS cone outer segments labeled with anti-SWS1. Scale bar, 10 μm (applies to both panels).

Part II: *in ovo* TH manipulation affects later opsin expression and photoreceptor distribution

Fish from treated oocytes as in part I were grown to the parr (juvenile) stage of development and treated with exogenous T4 in order to study the effect of early TH experience on later responses to T4-induced changes in opsin expression and photoreceptor distribution. Plasma T4 levels were similar in all groups at the start of this experiment and in fish that received control (DMSO) implants, regardless of previous *in ovo* TH exposure. Similarly, all fish receiving T4 implants showed elevated plasma T4 levels (P<0.05; Fig. 6).

Each gene of interest from the retinas of control- and T4-implanted juvenile fish for each *in ovo* treatment group was examined by qPCR (Fig. 7). The expression level of each gene of interest from each treatment group was calculated relative to the expression level of the same gene in the control implanted fish reared from untreated oocytes, and these resulting fold changes in gene expression analyzed statistically for each gene at each of the 3 and 7 day sampling times (Fig. 7). T4 implantation for 3 days led to decreased *SWS1* transcript levels in both *in ovo* treatment groups relative to untreated parr that had not had TH manipulation *in ovo* (U-C group), although the transcript levels in T4-implanted fish reared from untreated oocytes was not significantly lower at this time (Fig. 7A; U-T4: P=0.098). Interestingly, *SWS1* expression was reduced in fish reared from T4-elevated oocytes that only received control implants at the parr stage (Fig. 7A; T4-C; P=0.003), and exogenous T4 treatment of these parr further decreased *SWS1* levels 3 days post-implantation (Fig. 7A; T4-T4: P<0.001). After 7 days of treatment, although, *SWS1* expression levels remained significantly lower than in the untreated fish that were implanted with DMSO only (U-C reference group), these groups were no longer significantly different from each other.

SWS2 expression levels were also dependent on both *in ovo* and later TH manipulation. All treatment groups exhibited significantly higher levels of *SWS2* expression relative to control implanted parr reared from untreated oocytes at 3 days post-implantation (Fig. 7A; U-T4: P=0.003; T4-C: P<0.001; T4-T4: P=0.005). By 7 days post-

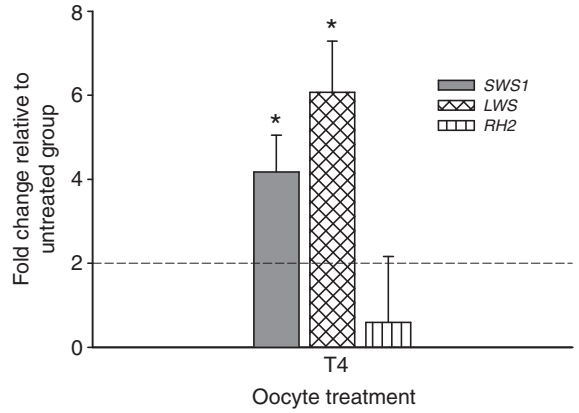

Fig. 4. Quantitative real-time RT-PCR analysis of expression of retinal opsins genes during rainbow trout development (310 D) in response to enhanced T4 content of oocytes (N=4 pools of embryo eyes; values are means ± s.e.m.). *Significantly different from the untreated embryos (P<0.05).

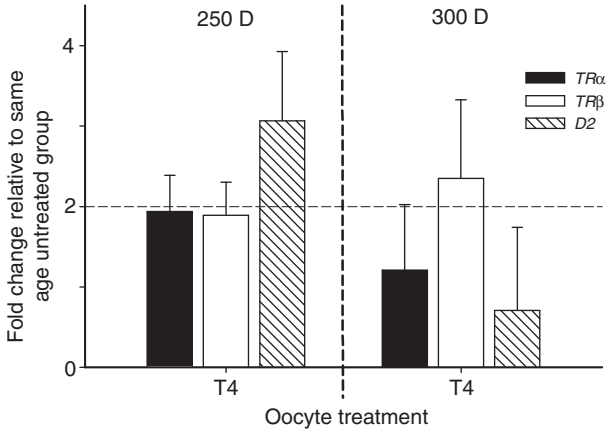

Fig. 5. Quantitative real-time RT-PCR analysis of gene expression of thyroid hormone (TH) regulatory elements during rainbow trout development (250 and 300 D) in response to enhanced T4 content of oocytes (N=4 pools of embryo eyes; values are means ± s.e.m.).

implantation, only *SWS2* transcript levels in T4-C and T4-T4 parr retina remained significantly elevated relative to U-C parr (Fig. 7B; U-T4: P=0.080; T4-C: P=0.009; T4-T4: P<0.001). Exogenous T4 treatment did not cause increased *SWS2* expression in the retinas of previously untreated parr, suggesting a synergistic effect of prior TH manipulation on the retinal response to later T4 exposure.

The expression of other members of the TH signaling pathway, *TR*α, *TR*β and *D2* did not significantly change at 3 days post-implantation in any group treated with exogenous T4 (Fig. 7A). By 7 days post-implantation, both *in ovo* treatment groups with T4 implants (U-T4, T4-T4) demonstrated equivalent and significant increases in *TR*α and *TR*β but not *D2* transcript accumulation in the retina compared with vehicle-implanted parr (Fig. 7B; *TR*α and *TR*β, U-T4 and T4-T4: P<0.001; *D2*, U-T4: P=0.047; D2, T4-T4: P=0.154). No significant changes in accumulation of *TR*α, *TR*β or *D2* transcripts were evident in the T4-C group relative to the U-C group, 7 days post-implantation (Fig. 7B).

Immunohistochemical staining of whole neural retinas using a polyclonal antibody to SWS1 was performed 7 days post-implantation to examine topographical changes in UVS cone distribution (Fig. 8). Natural UVS cone loss had begun in the ventral retinas of fish from both treatment groups (data not shown), so the analysis focused on the dorsal hemisphere. A complete UVS cone mosaic was present in the dorsal retina of all fish that received vehicle-only implants, regardless of previous TH manipulation (Fig. 8A,C). Similarly, a full complement of UVS cones was present in the retinas of U-T4 (Fig. 8B) parr. However, substantial UVS cone loss was observed in the dorsal retina of T4-T4 (Fig. 8D) fish (Fig. 7). Interestingly, the retinal mosaic in treated fish appeared to be disrupted.

DISCUSSION

The manipulation of rainbow trout oocyte T4 content resulted in a change in the pattern of retinal gene expression both during embryonic development and later in development during T4-induced changes in opsin expression in juvenile trout. Relative opsin expression levels were altered with *in ovo* T4 treatment and juveniles reared from treated eggs demonstrated an increased sensitivity to later T4-induced downregulation of *SWS1* and loss of UVS cones. These results suggest that TH affects the ontogeny of opsin expression in the embryonic retina, and that maternal TH levels *in ovo* affect embryonic development and can have lasting effects on the retina of the organism.

The first clear expression of both *SWS1* and *RH1* in the retina occurred at 300 D and was followed a short time later by *LWS* expression at 310 D. *RH2* expression occurred next, at 330 D, and *SWS2* was not detected before hatching in any rainbow trout embryos. This order of opsin expression corresponds to that previously reported for a number of salmonid species, including rainbow trout (Cheng et al., 2007). The detection of SWS1 in the central ventral retina using immunohistochemistry is supported by the appearance of *SWS1* expression in the same region of the retina using *in situ* hybridization in rainbow trout and other salmonids (Cheng et al., 2007). This developmental sequence of cone opsin expression is also found in mammals (reviewed by Lukáts et al., 2005). In mice, S-opsin (most closely related to salmonid *SWS1*) is the first to be expressed during development, followed by L-opsin (of the M/LWS class), and the three opsins in humans (SWS and two M/LWS family member) follow a similar developmental course (Wang et al., 1992; Xiao and Hendrickson, 2000; Lukáts et al., 2005). The ontogenetic pattern of rainbow trout (salmonid) opsin expression differs from that of other fish species (Raymond et al., 1995; Stenkamp et al., 1996; Bumsted et al., 1997; Raymond and Barthel, 2004). In goldfish, RH1 appears first, followed by LWS, RH2, SWS2 and SWS1 opsins (Raymond et al., 1995; Stenkamp et al., 1996). Eel and winter flounder (a flatfish), both species considered to undergo true metamorphosis, express only *RH2* during the premetamorphic stage, whereas *SWS2* expression appeared only after metamorphosis in juveniles (Mader and Cameron, 2004; Cottrill et al., 2009). In juvenile flatfish, *RH1* and *LWS* expression also appeared after metamorphosis (Mader and Cameron, 2004). Regarding the developmental sequence of opsin expression, therefore, the rainbow trout retina more closely resembles that of mammals than of other fishes. Further study with antibodies specific to each rainbow trout opsin will be necessary to confirm that the RT-PCR data reflect subsequent protein expression.

Although no difference in the timing of opsin ontogeny between *in ovo* treatment groups was observed using RT-PCR, qPCR analysis demonstrated that there were significant changes in opsin gene expression levels between treatments. *SWS1* and *LWS* were elevated in T4-treated embryos relative to the untreated controls,

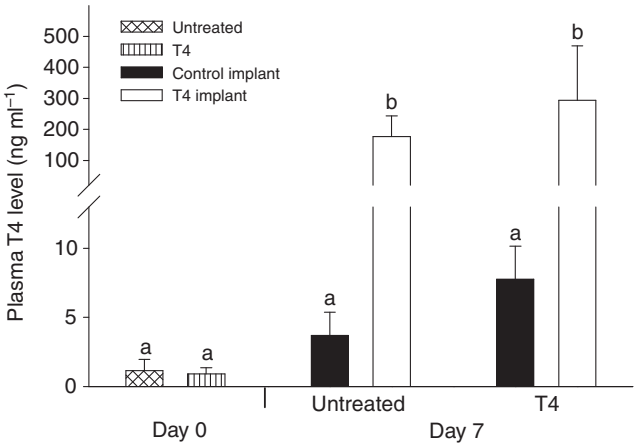

Fig. 6. Plasma T4 levels in rainbow trout parr prior to implantation (day 0, left) and 7 days after control and T4 implants (*N*=3–5 pools of blood plasma; values are means ± s.e.m.). Common letters indicate no significant difference between means; *P*<0.05 was considered significant.

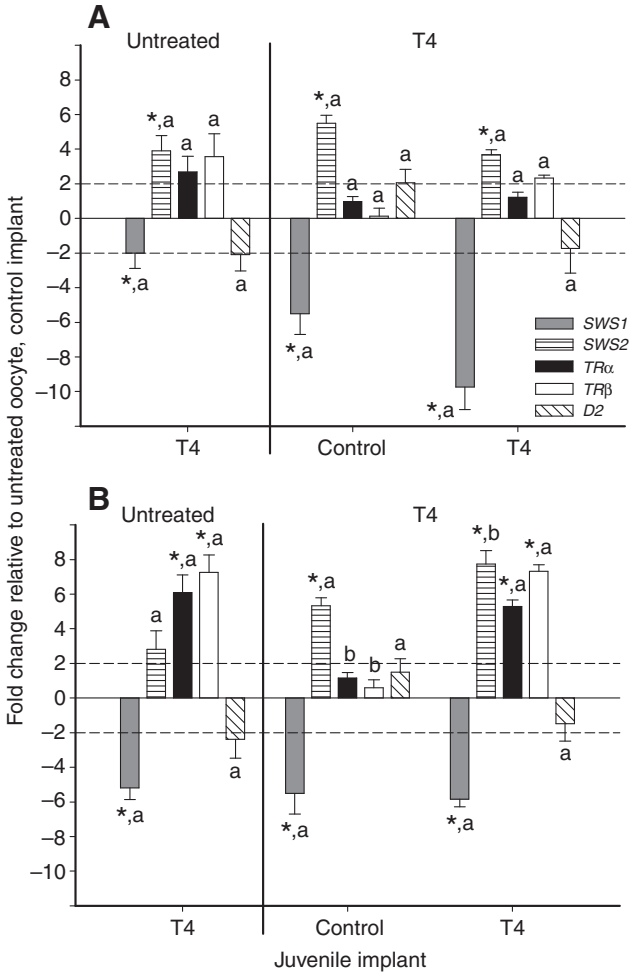

Fig. 7. Expression of retinal thyroid hormone receptors (*TR*α, *TR*β), deiodinase 2 (*D2*) and cone opsins (short-wavelength-sensitive 1 and 2; *SWS1* and *2*) genes in response to exogenous T4 treatment of rainbow trout parr reared from oocytes with altered T4 content. (A) At 3 days post-implantation. (B) At 7 days post-implantation (*N*=5 retinas; values are means ± s.e.m.). *Significantly different (*P*<0.05) for the gene of interest in a treatment from that of the control parr reared from untreated embryos (U-C group). Common letters indicate no significant difference between treatment means for the same gene of interest. Dashed lines represent 2-fold changes in gene expression relative to the untreated oocyte, control group.

which might suggest that these genes had been 'turned on' before those same genes in the untreated embryos. Subtle differences in the timing of the onset of opsin expression may not have been detected in the present study if genes were turned on at different times between sampling points. Rearing these trout under the same treatment conditions but using a cooler water temperature would provide enhanced spatial separation in the ontogeny of opsin expression. Rainbow trout possess a broad range of thermal tolerance, which can be used to alter the metabolic rate and thus growth and developmental rate of these cold-blooded fish. Manipulation of water temperature to alter developmental rate has been successfully utilized previously (Raine and Leatherland, 1999; Li et al., 2006; Li et al., 2007; Raine et al., 2004; Raine et al., 2007). Alternately, these results could suggest that in the treated fish there were increased numbers of *SWS1* or *LWS* transcripts per cell, or that there were greater numbers of photoreceptors expressing these transcripts relative to the untreated embryos.

Changes in *TR* and *D2* expression corresponded to differences in *in ovo* T4 exposure during embryonic development. Analysis of retinal *TR*α, *TR*β and *D2* expression prior to opsin ontogeny at 250 D demonstrated increased expression of all three of these genes in embryos exposed to elevated T4 *in ovo* compared with the untreated embryos. Increased *TR*β expression persisted until at least 300 D, just prior to the onset of *SWS1* expression. Upregulation of *TR*β in response to T4 treatment has been demonstrated previously in the retina of trout parr (Raine and Hawryshyn, 2009; Raine et al., 2010). However, this phenomenon was usually correlated with downregulation of *D2*, presumably to regulate the high T4 levels through a decrease in T4 to T3 conversion. It is possible that high T3 levels are required at this developmental stage and that the upregulation of *D2* found in these groups provides a necessary increase in T4 conversion to T3.

The relative upregulation of *SWS1* and *LWS*, combined with the upregulation of *TR*β expression prior to the onset of opsin expression, suggests that *TR*β may be involved in regulation of these two opsin genes. In TRβ2 knockout mice, M-opsin (RH2 homolog) and S-opsin (SWS1 homolog) patterning across the retina is disrupted; S-opsin is overexpressed and M-opsin is not expressed (Ng et al., 2001; Forrest et al., 2002; Roberts et al., 2006; Applebury et al., 2007). Pharmacologically or genetically induced hypothyroidism in prenatal mice disrupts cone differentiation in the same manner as TRβ2 knockout and demonstrates that both the ligand (T3) and receptor (TRβ2) are required for normal cone differentiation and spatial patterning (Roberts et al., 2006; Pessôa et al., 2008; Lu et al., 2009; Glaschke et al., 2010). These results suggest that in mice, TRβ2 suppresses S-opsin expression. The results of the current study suggests an alternative TH-mediated regulatory mechanism in rainbow trout, as upregulation of *TR*β was correlated with increased expression of *SWS1*, implying an instructive or permissive role for *TR*β in driving *SWS1* expression rather than the suppressive role seen in mice. However, similar to these other species, the parr stage trout reared from T4-enhanced oocytes appeared to exhibit a disruption of the retinal mosaic when whole SWS1-immunostained retinas were examined. Although the current study was not designed to thoroughly examine the spatial patterning of UVS cones, the current results, combined with our previous studies showing that TH regulation, and specifically *TR*β,

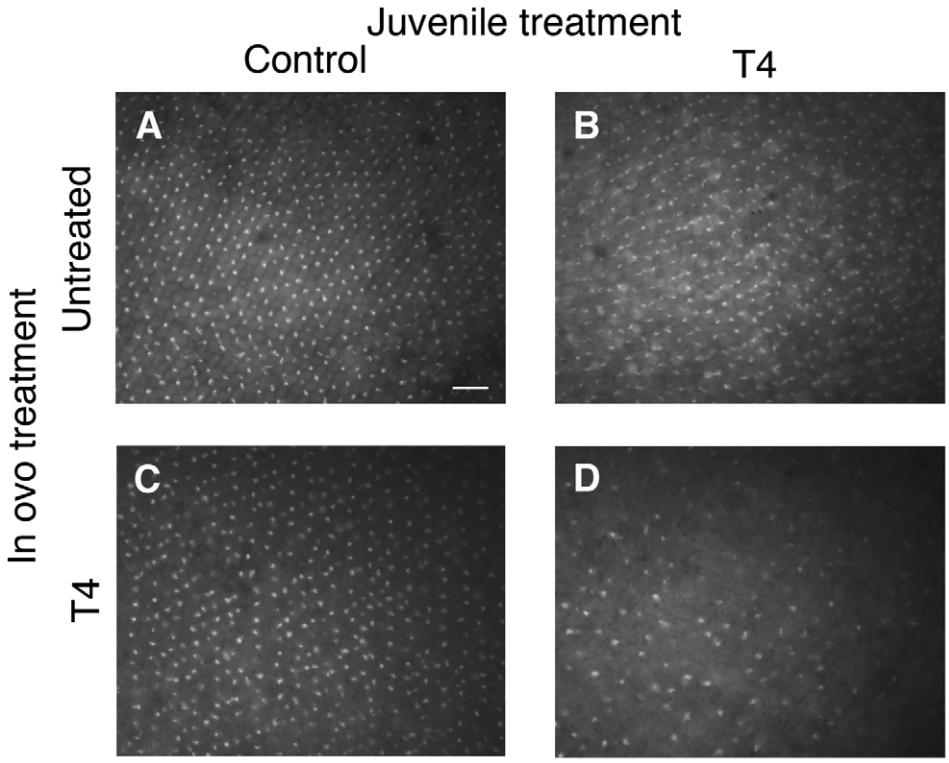

Fig. 8. SWS1-immunostained retinas in fish that were either untreated *in ovo* (A,B) or treated *in ovo* with T4 (C,D). These fish were then implanted as juveniles with either vehicle only (control; A,C) or T4 (B,D). Images are from the dorsal temporal quadrant of whole-mount retinas 7 days after implantation and are representative of other retinas in the same treatment. Scale bar, 30 μm (applies to all panels).

is involved in the spatial patterning of UVS cones across the retina during rainbow trout smoltification suggest that TH-mediated patterning of cone photoreceptors is a conserved vertebrate trait (Raine and Hawryshyn, 2009; Raine et al., 2010).

In ovo-treated embryos allowed to develop to the parr stage and subsequently treated with exogenous T4 demonstrated that *in ovo* changes in TH content affected later opsin expression and photoreceptor distribution. Exogenous T4 treatment of the *in ovo*-untreated group showed the expected pattern of gene expression after 3 and 7 days, found in previous studies: downregulation of *SWS1*, upregulation of *TR*β and downregulation of *D2* (Veldhoen et al., 2006; Raine and Hawryshyn, 2009; Raine et al., 2010). *In ovo* T4-treated parr showed a similar pattern of gene expression following juvenile T4 treatment, but interestingly, *SWS1* was downregulated fourfold to fivefold more than in the U-T4 fish, suggesting that *in ovo* TH manipulation sensitized the animals to later T4 challenge. This suggests that although there are no obvious effects of altered yolk TH content on rainbow trout retinal and general somatic development, there may be long-term consequences that can reduce the survival and overall fitness of affected individuals. Thus, although maternal hyper- and hypothyroidism in mammals has clear and immediate effects on progeny and their health, it may be that comparable maternal thyroidal status in fish could result in unforeseen problems later in life.

Surprisingly, T4-C parr displayed considerable downregulation of *SWS1* expression relative to U-C parr even though plasma T4 levels were similar in fish from both vehicle-injected treatments. Furthermore, the decreased *SWS1* levels in the T4-C parr did not correspond to increased *TR*α and *TR*β expression, unlike the negative correlation between *SWS1* and *TR*s seen in T4-implanted fish. These results suggest a potential decoupling of *SWS1* and *TR* expression in fish that experienced early TH manipulation but not later T4 exposure.

The most striking manifestation of early TH manipulation was the precocious loss of UVS cones from the dorsal retina of T4-T4 parr. All fish, including U-C animals, had lost ventral UVS cones but only the T4-T4 animals were missing dorsal UVS cones 7 days after implantation. Thus, *in ovo* exposure of rainbow trout embryos to elevated T4 content in the yolk appears to have increased the responsiveness of the retina to exogenous T4 treatment and influenced subsequent UVS cone loss. TH-induced UVS cone loss has been shown to occur *via* programmed cell death, suggesting that early TH exposure may sensitize photoreceptors to later TH-related cell death signaling events (Kunz et al., 1994; Allison et al., 2006).

As in the present study, *SWS1* downregulation in the retina of T4-treated parr raised from untreated oocytes occurs within 1 week and it generally takes at least an additional 3 weeks for UVS cone loss to be evident across the central retina, when detected using immunostaining with anti-SWS1 and measurement of UV photosensitivity (Browman and Hawryshyn, 1994; Raine et al., 2010). The considerably greater downregulation of *SWS1* gene expression in T4-treated parr reared from T4-enhanced oocytes relative to the *SWS1* downregulation seen with T4 exposure of previously untreated parr, was coupled with the unprecedented premature loss of UVS cones from the central retina within one week in the T4-T4 fish. This suggests that the degree of *SWS1* downregulation may be correlated with the rapidity and/or degree of UVS cone loss in the retina and requires further investigation.

There was an upregulation of *SWS2* in the implanted fish of all treatment groups when compared with the control fish reared from untreated oocytes. This upregulation of *SWS2* corresponded with the downregulation of *SWS1* as seen before, and with increased levels of *TR*s. It has been postulated that SWS2 opsin replaces SWS1 opsin in corner cones in the rainbow trout retina and that the increase in *SWS2* expression that often accompanies exogenous T4 treatment of salmonid parr is indication of this opsin switch (Veldhoen et al., 2006; Cheng and Flamarique, 2007; Raine and Hawryshyn, 2009;

Raine et al., 2010). Although the present study did not examine opsin co-expression or switching, the upregulation of TR gene expression accompanying upregulation of *SWS2* expression in this and other studies suggests that T4 may be involved in *SWS2* expression in this system and requires further investigation (Raine and Hawryshyn, 2009; Raine et al., 2010).

There were no gross morphological differences in retinas from fish treated with TH either *in ovo* or as juveniles. However, we did not examine each retinal cell type in detail, so it is possible that TH manipulation resulted in changes to many cell types within the retina. Studies in mice with altered thyroid hormone signaling demonstrate that effects in the retina are specific to cone photoreceptors, suggesting that TH treatment in rainbow trout may similarly influence opsin expression and photoreceptor subtype distribution without additional retinal effects (Glaschke et al., 2010; Ng et al., 2010).

How THs might affect photoreceptor ontogeny or UVS cone loss in later parr-stage fish is not clear. Several studies provide evidence that TH acts on the retina to initiate *SWS1* downregulation and subsequent UVS cone degeneration (e.g. Allison et al., 2006; Raine and Hawryshyn, 2009; Raine et al., 2010). Consistent patterns of *TR*α, *TR*β and *D2* expression correspond to changes in *SWS1* expression and UVS cone loss in isolated whole and quartered neural retina (Raine and Hawryshyn, 2009; Raine et al., 2010). However, further investigation is necessary to determine whether TH acts directly on photoreceptors or other retinal cells, rather than through secondary TH-mediated changes in other tissues, to alter opsin gene expression.

THs have been demonstrated to be necessary for both induced and natural UVS cones loss in rainbow trout parr, and the results of this study suggest that THs also influence opsin expression during early retinal development. Furthermore, these findings demonstrate that any factors affecting the maternal contribution of THs to the developing embryo may have long-term consequences on the organism.

LIST OF ABBREVIATIONS

D	degree days
D2	deiodinase 2
LWS	long wavelength sensitive
PL	photoreceptor layer
qPCR	quantitative real-time RT-PCR
RH	rhodopsin
RPE	retinal pigment epithelium
SWS	short wavelength sensitive
T3	triiodothyronine
T4	thyroxine
TH	thyroid hormone
TR	thyroid hormone receptor
U	control
UVS	ultraviolet sensitive

ACKNOWLEDGEMENTS

This study was funded by the Canada Research Chair program, Canada Foundation for Innovation, Ontario Innovation Trust, and NSERC Discovery grant (C.W.H.). The authors would like to thank Michael Burke and the Alma Aquaculture Research Station for supplying the eggs and milt used in the current study, and Helen Kerr for technical support.

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