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Visual pigment composition in zebrafish: Evidence for a rhodopsin–porphyropsin interchange system*

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Abstract

Numerous reports have concluded that zebrafish (*Danio rerio*) possesses A_1-based visual pigments in their rod and cone photoreceptors. In the present study, we investigated the possibility that zebrafish have a paired visual pigment system. We measured the spectral absorption characteristics of photoreceptors from zebrafish maintained in different temperature regimes and those treated with exogenous thyroid hormone using CCD-based microspectrophotometry. Rods from fish housed at 15°C and 28°C were not significantly different, having λ_{max} values of 503 ± 5 nm (n = 106) and 504 ± 6 nm (n = 88), respectively. Thyroid hormone treatment (held at 28°C), however, significantly shifted the λ_{max} of rods from 503 ± 5 nm (n = 194) to 527 ± 8 nm (n = 212). Cone photoreceptors in fish housed at 28°C (without thyroid hormone treatment) had λ_{max} values of 361 ± 3 nm (n = 2) for ultraviolet-, 411 ± 5 nm (n = 18) for short-, 482 ± 6 nm (n = 9) for medium-, and 565 ± 10 nm (n = 14) for long-wavelength sensitive cones. Thyroid hormone treatment of fish held at 28°C significantly shifted the λ_{max} of long-wavelength sensitive cones to 613 ± 11 nm (n = 20), substantially beyond that of the λ_{max} of the longest possible A_1-based visual pigment (~580 nm). Thyroid hormone treatment produced smaller shifts of λ_{max} in other cone types and increased the half-band width. All shifts in photoreceptor λ_{max} values resulting from thyroid hormone treatment matched predictions for an A_1- to A_2-based visual pigment system. We therefore conclude that zebrafish possess a rhodopsin–porphyropsin interchange system that functions to spectrally tune rod and cone photoreceptors. We believe that these observations should be carefully considered during analysis of zebrafish spectral sensitivity.

Keywords: Retina, Teleost fish, Giant danio, Chromophore, Thyroxine

Introduction

Zebrafish (*Danio rerio*) have become an important model for visual neuroscience (reviewed in Taylor et al., 2000; Bilotta & Saszik, 2001; Li, 2001; Goldsmith & Harris, 2003). Knowledge of zebrafish photoreceptor spectral absorbance properties have important implications for methodologies that employ spectral sensitivity measures or spectral stimuli to elicit innate behaviors for the purposes of screening and evaluating the visual system mutant fish (Neuhauss et al., 1999; Bilotta et al., 2001; Van Epps et al., 2001; Krauss & Neumeyer, 2003; Neuhauss, 2003). A primary determinant of visual sensitivity is variation in the opsin amino acid sequence but in some vertebrates an additional determinant is the vitamin A_1–A_2 visual pigment interchange system.

Most marine and terrestrial vertebrates possess a vitamin A_1-based visual pigment composition, whereas many freshwater and euryhaline fishes, amphibians, and some reptiles possess an A_1–A_2 visual pigment pair system (e.g. Loew, 1995). In paired pigment fishes and amphibians, the retinal and pineal photoreceptors generally contain mixtures of the A_1–A_2 based visual pigments, and the dynamic ratio of the two can change based on environmental variables such as light regime, temperature, or during hormone manipulations (reviewed in Bridges, 1972; Levine & MacNichol, 1979; Beatty, 1984; Loew, 1995). Changes in the A_1–A_2 visual pigment composition affect the spectral properties of photoreceptors: A_2-based visual pigments have wavelength of maximum absorbance (λ_{max}) values at longer wavelengths than A_1-based visual pigments, for a given opsin protein (Loew & Dartnall, 1976; Whitmore & Bowmaker, 1989; Harosi, 1994; Koskelainen et al., 2000; Parry & Bowmaker, 2000).

Zebrafish rod and cone opsin proteins can be classified based on amino acid similarity and, like those of many other teleosts (Hisatomi et al., 1997; Carleton & Kocher, 2001; Allison et al., 2003; Dann et al., 2004), represent examples of all five vertebrate opsin classes (Raymond et al., 1993, 1996; Vihtelic et al., 1999). All copies of rod and cone opsins have been isolated from the zebrafish genome, and the λ_{max} values of each have been estimated

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by *in vitro* expression (Chinen et al., 2003). We adopt the nomenclature of Hunt et al. (2001) for cone classes, that is, ultraviolet-, short-, medium-, and long-wavelength sensitive (UVS, SWS, MWS, and LWS) cones express SWS1, SWS2, RH2, and LWS opsin genes, respectively, and rods express rod opsin RH1.

Over the past four decades, numerous studies have concluded that zebrafish and their congener, the giant danio (*D. aequipinnatus*), possess solely A_1-based visual pigments (Schwanzara, 1967; Levine & MacNichol, 1979; Nawrocki et al., 1985; Palacios et al., 1996; Cameron, 2002; Chinen et al., 2003). These results come from a diverse assortment of methods, including pigment extractions, partial bleaching, and high-performance liquid chromatography (HPLC). Furthermore, the λ_{max} of *in vitro* expressed opsin proteins reconstituted with A_1-based chromophore match the λ_{max} observed in suction electrode recordings and microspectrophotometry (MSP) performed on isolated photoreceptors (Levine & MacNichol, 1979; Palacios et al., 1996; Chinen et al., 2003). However, many other closely related cyprinids have been found to possess an A_1–A_2 interchange system (Schwanzara, 1967; Allen, 1971; Bridges, 1972; Tsin & Beatty, 1978; Kusmic & Gualtieri, 2000). Because there are a variety of underlying factors driving the A_1–A_2 interchange system, there could conceivably be problems related to identifying this attribute for a particular species (for examples from cyprinids see Allen, 1971; Tsin & Beatty, 1978; Tsin et al., 1981). A recent study by Saszik and Bilotta, (1999) suggested that zebrafish might possess A_2-based pigments in situations where the holding temperature is varied.

To determine if zebrafish possess an A_1–A_2 interchange system, we used thyroid hormone treatment, known to produce an A_2 visual pigment dominance in the photoreceptors of other fishes. We used MSP to measure the λ_{max} and other spectral properties of each photoreceptor class. We observed a long-wavelength shift in rod and cone photoreceptors, matching properties predicted for A_2-based visual pigments. Therefore, our data suggest zebrafish possess a paired pigment system that allows them to shift between A_1- and A_2-based visual pigments.

Materials and methods

Zebrafish (*Danio rerio*) were purchased from a local pet shop and maintained in noncirculating dechlorinated municipal water in 15-l plastic tanks. Experiments were carried out during September–October and again in November–December, 2002. A 12-h light/12-h dark (12L:12D) photoperiod was provided by standard fluorescent lights (color temperature 6500°K, for spectral irradiance measures, see Parkyn and Hawryshyn, 2000, Fig. 1). Fish were fed standard flake food and trout pellets. Ten fish were maintained in each of three different treatments: (1) cold (water temperature 15 ± 1°C); (2) warm (water temperature 28 ± 1°C, normal holding temperature for Zebrafish); and (3) warm plus thyroid hormone (TH). TH treatment was administered by adding L-thyroxine (Sigma, St. Louis, MO), dissolved in 1.5 ml of 0.1 M NaOH, to a final concentration of 300 μg l^{-1} L-thyroxine. Fish were transferred to fresh TH-treated water daily, and fish not receiving TH treatment received the vehicle only (1.5 ml of 0.1 M NaOH). Fish were maintained under these treatment conditions for 3–4 weeks prior to being sacrificed. To sample the retinae, fish were sacrificed by prolonged anesthesia with 100 mg l^{-1} Euganol (ICN Biomedicals Inc., Irvine, CA.) until euthanized (~15 min exposure). Care of fish and all procedures were in accordance with and approved by the University of Victoria Animal Care Committee under the auspices of the Canadian Council for Animal Care and thus conformed to the principles regarding the care and use of animals adopted by the American Physiological Society and the Society for Neuroscience.

Microspectrophotometry

The spectral absorbance of individual photoreceptors was measured using MSP to determine the characteristics [λ_{max}, maximum absorbance (A_{max}), half-band width (HBW), specific density, A_1/A_2 ratio] of zebrafish photoreceptors in the experimental design outlined above. All MSP procedures described below were carried out at 17°C, in a dark room and under dim red illumination. Zebrafish were dark adapted for at least 1 h, killed, and the right eyes were surgically removed and hemisected in Minimal Essential Media (Sigma). All dissection procedures were performed under infrared light. For consistency of sampling location, retinal tissue from the dorsal half of the eye was used. Pieces of retina from the dorsal retina were teased apart, placed on a 35 × 50 mm (No. 1) glass microscope cover slip and macerated. We used a charge-coupled device-based microspectrophotometer (CCD-MSP) that has been described in detail previously (Hawryshyn et al., 2001), for the measurement of spectral absorbance. In brief, this device delivered short duration flashes (500 ms) of full spectrum (300–800 nm, 150-W xenon light source-intensity regulated; Oriel, Stratford, CT), unpolarized light through the photoreceptor outer segment (beam size approximately 2 × 3 μm). The transmitted beam passed through a spectrometer (300-nm blazed grating, Acton Research Corporation, MA) onto a Peltier cooled (−45°C), back-illuminated CCD-detector (NTE / CCD-1340/400-EMB Princeton Instruments, Roper Scientific, Inc., Trenton, NJ). Photoreceptor absorbance ($\log_{10} T^{-1}$) was calculated by comparing the transmitted intensity through the photoreceptor (I_m, measurement intensity) to the transmitted intensity through an area clear of debris and in media adjacent to the photoreceptor (I_r, reference intensity) thus, $T = I_m/I_r$.

Real-time imaging of the retinal sample under infrared illumination employed a CCD camera (Canadian Photonics Laboratory, Minnedosa, MB, Canada). A Pentium computer was used as a central control unit for the CCD-MSP system, data acquisition, on-line analysis, and data storage. Photoreceptor types were identified based on their distinct morphology, and secondarily confirmed using the spectral position of the λ_{max} of the main absorbance band (α-absorption band) and the difference spectra calculated subsequent to photoreceptor bleaching. Difference spectra were used to verify that the α-absorption band was indeed a photolabile pigment and were calculated by subtracting the bleached absorbance curve (full spectrum bleach for 2–5 s) from the initial absorbance curve. We did not attempt to use partial bleaching techniques to rule out the possibility of opsin coexpression.

Absorbance spectra were examined on-line and were retained for subsequent analysis based on strict compliance with the following criteria: (1) shape of the absorbance curve had the expected shape based on known templates (see below) and the λ_{max} was near the expected wavelength based on cone morphology; (2) presence of a clear baseline on the long wavelength arm; (3) absence or minimal presence of photobleaching, that is, absorption due to photoproduct; and (4) baseline noise less than 15–20 percent of A_{max}.

Determination of λ_{max} from absorbance spectra was performed off-line. Absorbance data were subjected to linear detrending and normalized relative to A_{max}. However, the main estimate of λ_{max} was determined by a minimum variance fit to the upper 20% of the

absorption spectrum based on the center of the α-peak $\pm$ 40 nm of the Govardovskii et al. (2000) template. Photoreceptor outer segment diameters were measured from several of the photoreceptors examined using Northern Eclipse 5.0 image analysis software (Empix Imaging Inc., Mississauga, ON, Canada). In the case of cones, measurements were completed on the portion of the outer segment that was immediately distal to the inner segment. We noted that there was no difference in outer segment diameter of photoreceptors from the different experimental treatments, and thus the diameter measurements were pooled.

For cone photoreceptors, we estimated λ_{max} based on calculating a mean for all photoreceptors collected. Thus, for comparisons between treatments each cone was considered to be an independent observation. In each case, the mean λ_{max} was calculated based on photoreceptors from multiple individuals. We used this approach for the comparison of cone photoreceptor λ_{max} values between treatments because the number of cones collected per fish was highly variable. For rods, where we had more samples per individual, we were also able to compare treatments using individual fish as sampling units. A comparison between treatments was made using a one-way ANOVA ($\alpha = 0.05$) followed by a Tukey's HSD *post hoc* test to determine which groups differed. To compare our results with previously reported values, we calculated mean estimates of λ_{max} for each photoreceptor class (UVS = 360 nm; SWS = 412 nm; MWS = 480 nm, LWS = 560 nm, Rod = 502.4 nm) based on the values reported in the literature (Nawrocki et al., 1985; Robinson et al., 1993; Harosi, 1994; Cameron, 2002; Chinen et al., 2003). These mean values were used as the predicted A_1-based λ_{max} values. Substituting these predicted values of A_1-based λ_{max} into Harosi's (1994) eqn. (1)

$$y = 27.914 - 2.3598x + 0.0505x^2, \quad (1)$$

where $x = A_1$-based λ_{max} in nm, and $y = A_2$-based λ_{max} in nm, we were able to predict λ_{max} values for the A_2-based visual pigments based on the same opsins for each photoreceptor class. Harosi's equation was chosen as a representative model that describes the general trend of increased shift in λ_{max} with increased wavelength, above ~400 nm (e.g. see also models proposed by Dartnall & Lythgoe, 1965; Whitmore & Bowmaker, 1989; Parry & Bowmaker, 2000). The predicted and observed values for both the A_1- and A_2-based pigments were plotted for direct comparison.

As a second line of inquiry regarding the possible presence of A_2-based visual pigments, we compared the half-band widths (HBW) of our absorbance spectra with that predicted from the A_1- (2) and A_2-based (3) HBW equations provided by Harosi (1994):

$$y = 10.17945 + 1.20985x - 0.0241x^2, \quad (2)$$

$$y = 20.74607 + 2.31334x - 0.0499x^2, \quad (3)$$

where y is the predicted HBW in 1000 cm^{-1} and x is the reciprocal λ_{max} in 1000 cm^{-1}. These equations predict a parabolic function when comparing A_1- and A_2-based pigments, with a greater difference in the width of the HBW for visual pigments with λ_{max} near 434 nm (approximately 23,000 cm^{-1}) than for visual pigments with longer and shorter λ_{max}.

Results

Microspectrophotometry was used to examine the spectral absorption properties of zebrafish photoreceptors. The rods and four cone classes have been shown to be morphologically distinct. UVS and SWS cones are single cones that differ in size and axial position, whereas the MWS and LWS cones form the accessory and principle members of double cones, respectively (Nawrocki et al., 1985; Raymond et al., 1993; Robinson et al., 1993; Vihtelic et al., 1999). Fig. 1 shows the absorbance spectra of these photoreceptors (see Table 1 for descriptive statistics), which appear to be in good agreement with the current literature (Nawrocki et al., 1985; Raymond et al., 1993; Robinson et al., 1993; Vihtelic et al., 1999; Cameron, 2002).

Visual pigment content in rod photoreceptors

To examine the visual pigment content of rod photoreceptors, we first assumed that each photoreceptor was a statistically independent entity. This is a conventional assumption for MSP analyses and is consistent with the relevant literature (Schwanzara, 1967; Levine & MacNichol, 1979; Nawrocki et al., 1985; Palacios, 1996; Cameron, 2002; Chinen et al., 2003). The mean $\lambda_{max} \pm 1$ SD values of rod photoreceptors of fish held in warm (28°C) and cold (15°C) treatments were 503 $\pm$ 5 nm ($N = 106$) and 504 $\pm$ 6 nm ($N = 88$), respectively. Zebrafish treated with TH in warm water (28°C) had a mean rod λ_{max} at 527 $\pm$ 8 nm ($N = 212$). A one-way ANOVA comparing the three treatments found a significant effect ($P < 0.001$, $df = 2$) and a multiple comparison Tukey HSD *post hoc* test showed that the two temperature treatments did not differ ($P = 0.744$) from one another but that both differed ($P < 0.001$) from the TH-treated group.

We collected a large number of rod absorbance spectra from several individual fish (>18 rods/fish), which permitted us to use each fish as the sampling unit. This eliminates the issue of pseudoreplication, that is, that multiple photoreceptors sampled from the same individual are not independent observations. Thus, in a second step of data analysis, we focused on the results of 12 fish that were examined over a four-day period (4 fish at 28°C, 5 fish at 28°C + TH-treated, and 3 fish held at 15°C). This allowed us to calculate a mean rod λ_{max} for each fish, and to use each fish as a statistically independent entity (see Fig. 2). Fish held at 28°C (not TH-treated) had a mean $\lambda_{max} \pm 1$ SD of 503.5 $\pm$ 1.14 nm ($N = 4$), those held at 15°C had a mean $\lambda_{max} \pm 1$ SD of 504.0 $\pm$ 1.18 nm ($N = 3$), while the mean λ_{max} of the TH-treated fish (at 28°C) was 527.9 $\pm$ 2.9 nm ($N = 5$, Fig. 2). A one-way ANOVA showed that there was a significant effect ($P < 0.001$, $df = 2$) of treatment on the rod λ_{max} values and the Tukey's *post hoc* test again showed that the temperature treatments did not differ ($P = 0.953$) while both warm and cold treatments differed ($P < 0.001$) from the warm TH-treated treatment. Rod λ_{max} values in TH-treated fish matched the value we had predicted for a shift from an A_1- to an A_2-based rod opsin pigment based on Harosi's (1994) formula solved using previously determined zebrafish A_1-based rod opsin λ_{max} (Fig. 3).

Zebrafish rod absorbance spectra collected from fish held at 28°C (not TH-treated) had a mean half-band width (HBW) similar to the values reported for giant danio rods, which were A_1-based (Harosi, 1994) (Table 1). The mean HBW of rods from TH-treated zebrafish was larger than untreated zebrafish and this matched the shift predicted by Harosi's (1994) eqns. (2) and (3). The mean A_{max} of rods from TH-treated zebrafish (0.015 $\pm$ 0.0007) was lower than untreated zebrafish (0.019 $\pm$ 0.0006; Table 1), consistent with the lower molar extinction coefficient of A_2-based visual pigments (Brown et al., 1963). Measurements of rod outer segment diameter for both TH-treated and control groups (Table 1) were similar to previously reported values (Connaughton & Dowling, 1998).

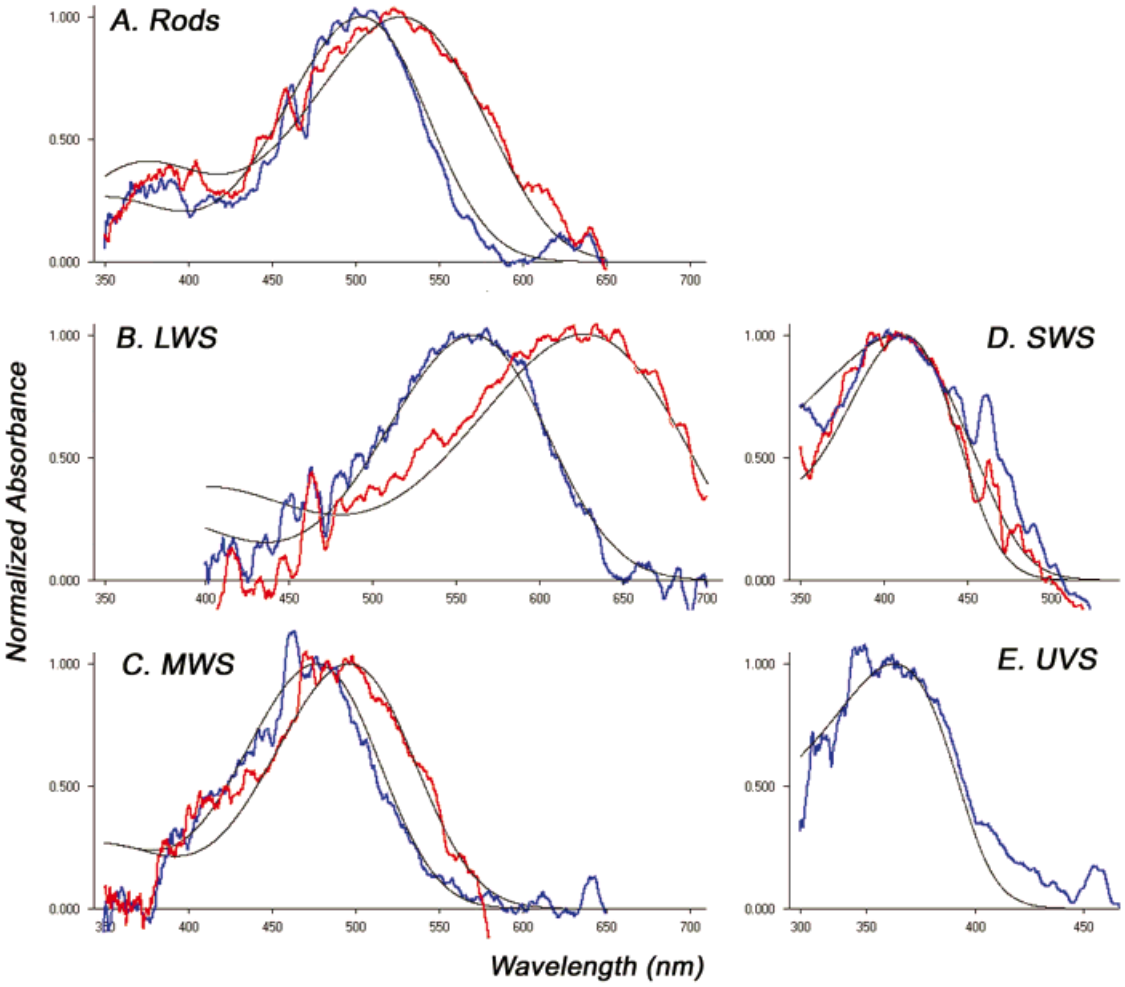

Fig. 1. Representative absorbance spectra collected using microspectrophotometry of individual photoreceptors isolated from zebrafish. Normalized data from control and thyroid hormone (TH) treated fish are represented by red and blue lines, respectively, and the templates used to fit the data are represented by the smooth black lines. The data from control fish were similar to previous findings from zebrafish, whereas TH treatment resulted in photoreceptors with A_2-based pigments. The latter was determined by the absorption maxima being shifted to longer wavelengths, and broader half–band widths and this was especially apparent in LWS cones and rods, as would be expected in chromophore shifts. A: rod photoreceptors; B–E: represent long-, medium-, short-, and ultraviolet-wavelength sensitive (LWS, MWS, SWS, & UVS) cones, respectively. In panel E only zebrafish not treated with TH are represented. Note that the scale of the abscissa changes between some panels.

Visual pigment composition in cone photoreceptors

MSP on cone photoreceptors from fish that did not receive TH revealed λ_{max} values that closely matched the average λ_{max} from previous MSP studies (Fig. 3; Nawrocki et al., 1985; Cameron, 2002). In particular, these results were comparable to those from a recent study examining zebrafish opsins expressed *in vitro* and reconstituted with A_1-based chromophore (Chinen et al., 2003). The LWS cone λ_{max} we measured (565 ± 10, $N = 14$) was similar to that reported by Cameron (2002) and longer than that reported by Nawrocki et al. (1985). The values we obtained for SWS cone λ_{max} fell between those found by these authors.

The LWS cone photoreceptors from zebrafish treated with TH had a mean λ_{max} value (613 ± 11 nm, $N = 20$) that was significantly higher ($P < 0.001$, $df = 32$, *t*-test) than observed in zebrafish not treated with TH. The λ_{max} of the MWS cones from TH-treated fish were also shifted to significantly ($P = 0.005$, $df = 10$, *t*-test) longer wavelengths from a mean of 482 ± 6 nm ($N = 9$) for the control fish to a mean of 505 ± 16 nm ($N = 3$) for the TH-treated fish (Table 1, Fig. 3). The small change in λ_{max} that one expects when comparing A_1- and A_2-based visual pigments for the SWS cone (Whitmore & Bowmaker, 1989; Harosi, 1994; Parry & Bowmaker, 2000) was confirmed in the present study with no difference evident between the control (411 ± 5 nm, $N = 18$) and the TH-treated fish (412 ± 4 nm, $N = 4$) (Table 1, Fig. 3).

Cone absorbance spectra collected from zebrafish at 28°C (not TH treated) had mean HBW values (Table 1) that were broader for cone classes with shorter λ_{max}, as expected (Harosi, 1994; Hawryshyn et al., 2001). The HBW of the cone spectra from TH-treated fish were broader than the HBW of untreated zebrafish for each cone class. This change was greater at shorter wavelengths (Table 1), as would have been expected if the TH-treated fish possessed A_2-based pigments (Harosi, 1994). Differences in HBW values were predicted by taking the difference of values based on eqns. (2)

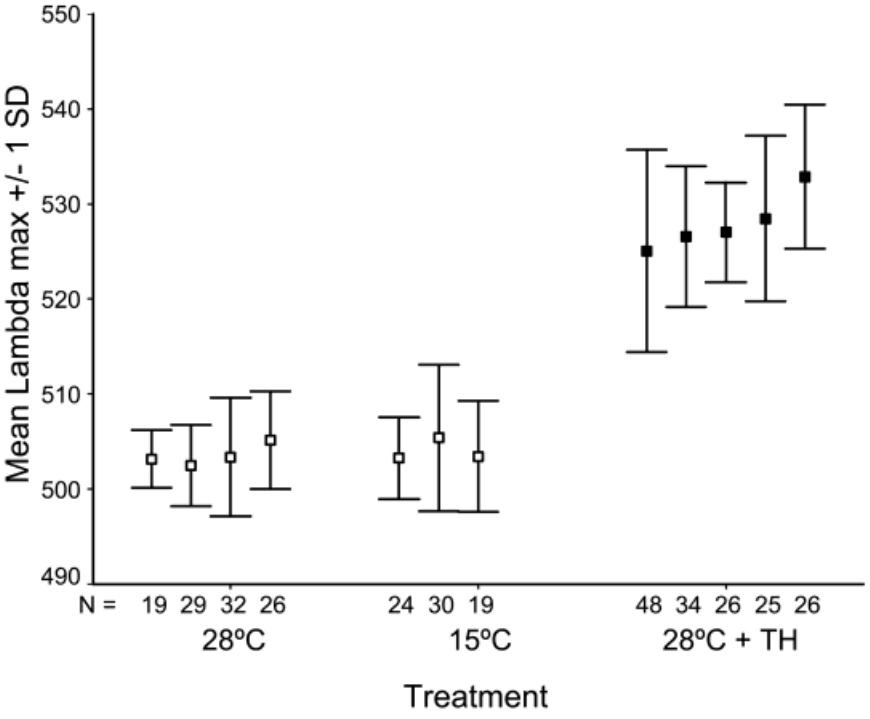

Fig. 2. Mean wavelength of maximum absorbance values (λ_{max}) of the rod photoreceptors from 12 individual zebrafish as determined by microspectrophotometry. Zebrafish from the same cohort were maintained in one of three conditions: at 28°C, 15°C, or at 28°C and receiving thyroid hormone (TH) treatment. Temperature did not significantly affect mean rod λ_{max} values, whereas TH treatment shifted the means to significantly longer wavelengths compared to untreated fish at 28°C ($P < 0.001$, $df = 2$). Means from untreated fish are represented by open symbols, and fish receiving TH treatment are represented by filled symbols. The number of rods measured per individual fish appears below the abscissa.

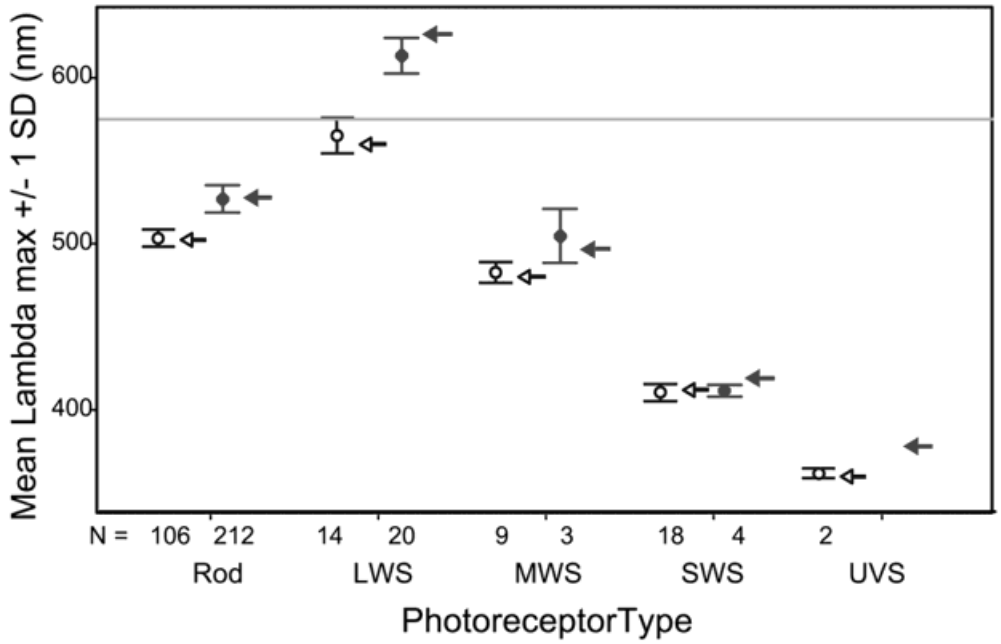

Fig. 3. Mean wavelength of maximum absorbance values (λ_{max}) of photoreceptors measured from control and thyroid hormone treated (TH) zebrafish maintained at 28°C. Data was grouped into five photoreceptor classes: rods and long-, medium-, short-, and ultraviolet-wavelength sensitive (LWS, MWS, SWS, & UVS) cones. The λ_{max} values determined for photoreceptors from untreated zebrafish (empty circles) closely matched the mean of results from previous studies (indicated by empty arrows). Photoreceptors from TH-treated zebrafish had mean λ_{max} (filled circles) that were shifted to longer wavelengths. These closely matched values one predicts when photoreceptors switch from an A_1- to A_2-based chromophore (filled arrows). The difference observed in this switch was greater at longer wavelengths, as expected. The difference was found to be significant for rods, LWS, and MWS cones. The shifts could not be explained by changes in opsin expression as all rod and cone opsins and their associated λ_{max} have been identified from zebrafish genome (Chinen et al., 2003). Furthermore, the λ_{max} determined for LWS cones from TH-treated fish exceeded the largest possible value for an A_1-based pigment (thin horizontal line at 575 nm, see text), and thus it represents an A_2-based pigment. The data for UVS cones from TH-treated fish did not meet our criteria for acceptance and thus are not presented. The sample size per treatment for each photoreceptor class appears below the abscissa.

Table 1. *Spectral data for zebrafish (Danio rerio) photoreceptors examined in this study*

Photoreceptor type	Mean λ_{max} in nm ± SD[a] (N)	Mean HBW[b] in cm^{-1} ± SD (N)	Difference in HBW[c] (cm^{-1})	Mean A_{max} ± SD (N)	Mean OSD in μm ± SD (N)	Specific absorbance[d] (μm^{-1})
Rods						
Control (A_1-based)	503 ± 5 (194)	4007 ± 743 (106)	378	0.019 ± 0.0006 (186)	2.4 ± 0.3 (53)	0.0079
TH-treated (A_2-based)	527 ± 8 (212)	4385 ± 680 (212)		0.015 ± 0.0007 (160)		0.0063
Cones						
LWS-double cones						
Control (A_1-based)	565 ± 10 (14)	4109 ± 258 (14)	55	0.019 ± 0.0004 (14)	2.6 ± 0.3 (16)	0.0073
TH-treated (A_2-based)	613 ± 11 (20)	4164 ± 492 (20)		0.014 ± 0.0006 (15)		0.0054
MWS-double cones						
Control (A_1-based)	482 ± 6 (9)	4291 ± 128 (8)	261	0.015 ± 0.0005 (9)	2.4 ± 0.2 (12)	0.0063
TH-treated (A_2-based)	504 ± 19 (3)	4552 ± 198 (2)		0.014 ± 0.0006 (3)		0.0058
SWS-single cones						
Control (A_1-based)	411 ± 5 (31)	4478 ± 665 (29)	967	0.019 ± 0.0006 (31)	2.8 ± 0.8 (8)	0.0070
TH-treated (A_2-based)	412 ± 4 (4)	5445 ± 616 (4)		0.014 ± 0.0006 (4)		0.0052
UVS-single cones						
Control (A_1-based)	361 ± 3 (6)	n/a[e]		0.021 ± 0.0005 (6)	3.9 ± 0.3 (6)	0.0054

HBW, Half-band widths; A_{max}, maximum absorbance; OSD, Outer segment diameter; TH, thyroid hormone; LWS, MWS, SWS, & UVS are long-, medium-, short-, & ultraviolet-wavelength-sensitive cones, respectively.
[a]Mean wavelength of maximum absorbance values (λ_{max}). N is sample size.
[b]Half-band widths.
[c]Calculated by subtracting the A_1-based HBW from the A_2-based HBW (in cm^{-1}).
[d]Calculated by taking ratio of mean A_{max} over mean outer segment diameter.
[e]UVS cone HBW were not calculated due to the unreliability of the short wavelength arm of the absorbance curve, which goes below 330 nm. In this portion of the spectrum the quantal flux of the xenon light source is limited.

and (3). The difference in HBW we observed at the short- and long-wavelength spectral extremes examined in this study (55 and 967 cm^{-1} for LWS and SWS cones, respectively) closely matched the values predicted by Harosi's equations (25 and 1012 cm^{-1}). A_{max} was lower in cones from TH-treated zebrafish (Table 1), as would have been predicted for A_2-based pigments (Hawryshyn et al., 2001) which have a molar extinction three-quarters that of A_1-based pigments (Brown et al., 1963). The measurements of cone outer segment diameter (Table 1) were similar to previously reported values for cones (Connaughton & Dowling, 1998); however, our measurements extend the previous data set to differentiate between UVS and SWS cones.

Discussion

Zebrafish have become an important model organism for many aspects of visual neuroscience, and knowledge of the potential variation in photoreceptor spectral sensitivity is required to permit effective experimental design and appropriate interpretation of results. In the present study, we provide data that convincingly demonstrate zebrafish have a paired pigment system and therefore can have a substantially broader range of spectral sensitivity than was previously believed. Our MSP results on photoreceptors from fish that did not receive TH treatment showed λ_{max} values that closely match the average λ_{max} from previous MSP experiments, that is, a vitamin A_1-dominated visual pigment composition (Fig. 3). However, even small differences (statistically insignificant) in λ_{max} seen in this study are of note because this data may serve some utility in modelling spectral sensitivity (Cameron, 2002). The criteria for accepting spectra in our analysis were relatively strict and our sample sizes, in most cases, were larger than previous reports. The reported differences between all studies, including the present one, could be biologically relevant particularly in the case of MWS and LWS cones where the expression of different opsin variants (Chinen et al., 2003) could account for the observed differences.

Previous results for zebrafish photoreceptor λ_{max} are known to represent A_1-based visual pigments determined using partial bleaching on visual pigment extracts (Schwanzara, 1967; Nawrocki et al., 1985), half-bandwidth comparisons (Nawrocki et al., 1985; Harosi, 1994; Cameron, 2002), and HPLC analysis (Palacios et al., 1996; Taylor et al., 2000). Our results from fish not receiving thyroid hormone are consistent with these findings and recent experiments where zebrafish opsins were expressed *in vitro* and reconstituted with A_1-based chromophore (Chinen et al., 2003).

Temperature

Temperature did not significantly affect the λ_{max} of rods in our study: fish maintained at low temperatures appeared to possess primarily A_1-based visual pigments (Fig. 2). An earlier experiment comparing fish maintained at 28°C and 19°C also showed that temperature did not significantly effect the proportion of A_1- and A_2-based visual pigments (i.e. the fish had predominantly A_1-based visual pigments) in rods ($P = 0.175$, $df = 284$). On the other hand, the same cohort of fish (receiving identical light regime, water, treatment vehicle, and diet) showed evidence of A_2-based visual pigments when treated with TH. Previous studies have shown that giant danio raised at low temperatures also possess A_1-based visual pigments (Levine & MacNichol, 1979; Palacios et al., 1996).

Our study shows no effect of temperature on visual pigment composition. The temperature treatments effectively enveloped the temperatures experienced by zebrafish in their natural environ-

ment, although other factors such as day length could have additive effects. It is noteworthy that the previous reports regarding temperature effects have focused on temperate species (see Beatty, 1984). The tropical, thermally stable environment of zebrafish may have obviated temperature as an important selection factor with respect to the evolution of chromophore interchange systems.

Thyroid hormone

TH treatment resulted in significantly longer λ_{max} values in the rods, MWS, and LWS cones (Figs. 2 & 3), consistent with zebrafish possessing a paired pigment system. The shifts seen in this study were consistent with those seen during TH treatment of another cyprinid, the goldfish (Tsin & Beatty, 1979). The degree of λ_{max} shift is described by well-defined functions (Whitmore & Bowmaker, 1989; Harosi, 1994; Parry & Bowmaker, 2000); visual pigments with larger λ_{max} produce a greater shift towards longer wavelengths. As expected from these functions, there was little change in the λ_{max} of SWS cones, an increase of 20–25 nm in the λ_{max} of rods and MWS cones, and an increase of about 60 nm in the LWS cone. Further, results derived from rod recordings matched the classic 503_1-527_2 pigment pair of many vertebrates with labile visual pigments (Schwanzara, 1967; Levine & MacNichol, 1979; Beatty, 1984; Harosi, 1994). Thus, the difference in λ_{max} between control and TH-treated fish matched that expected for a shift from A_1- to A_2-based chromophores seen in many paired pigment fishes.

The shifts in λ_{max} we observed cannot be explained solely by changes in opsin expression because all copies of the rod and cone opsins, along with their A_1-based λ_{max}, have been identified from zebrafish genome (Chinen et al., 2003). This is true for at least the rods and LWS cones, where the mean λ_{max} values observed were 527 and 613 nm, respectively. These values were substantially longer than the longest λ_{max} reported for these visual pigments reconstituted with A_1-based chromophore (501 and 558 nm, respectively) (Chinen et al., 2003).

Other aspects of our results add strong support to the conclusion that our TH-treated zebrafish had A_2-based pigments in their rods and cones. Templates required to fit the absorbance spectra of TH-treated fish were broader, that is, they had larger HBW as measured in wave-numbers (see Table 1), than those for untreated fish. The effect was most apparent for the SWS cone, where we observed little change in $\lambda_{max,}$ but a substantial broadening of the HBW. The differences in HBW between treatments were smaller in photoreceptor classes with longer λ_{max}; this was also consistent with the established relationship of HBW for A_1- and A_2-based pigments (see Table 1, Harosi, 1994). Furthermore, each photoreceptor class from TH-treated fish had lower A_{max} values, consistent with lower molar extinction coefficient of A_2-based pigments (Brown et al., 1963). Finally, we note that biophysical considerations indicate that the longest possible λ_{max} for an A_1-based pigment, regardless of opsin sequence, is near 580 nm (Blatz & Liebman, 1973). Indeed, no A_1-based pigment has been observed with a λ_{max} greater than approximately 570 nm. Reconstitution of *in vitro* expressed opsins and their mutated variants from a variety of vertebrates with A_1-based chromophore do not achieve λ_{max} greater than approximately 565 nm (Yokoyama & Radlwimmer, 2001). Therefore, even if one speculates that every copy of the LWS opsin has not been found in the zebrafish genome, which is unlikely (see Chinen et al., 2003), our observation that the λ_{max} of LWS cones was 613 ± 11 nm ($n = 20$) allows us to confirm that our TH-treated fish possessed A_2-based pigments.

Conclusion

Despite their importance to visual ecology, the genes underlying the chromophore interchange system remain unknown. The shift from A_1- to A_2-based pigments, which entails the addition of a double bond to the terminal ring of the chromophore, is presumed to require an unidentified 3,4-dehydrogenase. Considering the expanding genetic tools available for zebrafish, mutational screens or analyses of differential gene expression could provide promising methodologies to discover this pathway.

Our results demonstrate that A_2-based visual pigments are present in zebrafish. This should be carefully considered when assessing their visual sensitivity. The presence of A_2-based pigments would be expected to have substantial effects on both scotopic and photopic functional measurements, broadening the absorption band, lowering the absolute sensitivity, and shifting the spectral sensitivity to longer wavelengths (Kennedy, 1957; Allen et al., 1973; Allen & Munz, 1983; Whitmore & Bowmaker, 1989). Thus, our results could be relevant to mutational screens, toxicology screens, hormone treatments, and functional assessments of zebrafish retinal development and regeneration.

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