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Variable light environments induce plastic spectral tuning by regional opsin coexpression in the African cichlid fish, *Metriaclima zebra*

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Abstract

Critical behaviors such as predation and mate choice often depend on vision. Visual systems are sensitive to the spectrum of light in their environment, which can vary extensively both within and among habitats. Evolutionary changes in spectral sensitivity contribute to divergence and speciation. Spectral sensitivity of the retina is primarily determined by visual pigments, which are opsin proteins bound to a chromophore. We recently discovered that photoreceptors in different regions of the retina, which view objects against distinct environmental backgrounds, coexpress different pairs of opsins in an African cichlid fish, *Metriaclima zebra*. This coexpression tunes the sensitivity of the retinal regions to the corresponding backgrounds and may aid detection of dark objects, such as predators. Although intraretinal regionalization of spectral sensitivity in many animals correlates with their light environments, it is unknown whether variation in the light environment induces developmentally plastic alterations of intraretinal sensitivity regions. Here, we demonstrate with fluorescent *in situ* hybridization and qPCR that the spectrum and angle of environmental light both influence the development of spectral sensitivity regions by altering the distribution and level of opsins across the retina. Normally *M. zebra* coexpresses *LWS* opsin with *RH2Aα* opsin in double cones of the ventral but not the dorsal retina. However, when illuminated from below throughout development, adult *M. zebra* coexpressed *LWS* and *RH2Aα* in double cones both dorsally and ventrally. Thus, environmental background spectra alter the spectral sensitivity pattern that develops across the retina, potentially influencing behaviors and related evolutionary processes such as courtship and speciation.

Keywords

Opsin; visual sensitivity; cichlid; spectral tuning; gene expression; plasticity

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DATA ACCESSABILITY

In situ hybridization data set, as well as sequences of primers used to determine parentage by microsatellite analysis, are made available in the supplementary material. The qPCR data, FISH probe sequences, and microsatellite genotype data are available at Dryad (doi:10.5061/dryad.kq230).

INTRODUCTION

Vision mediates numerous behaviors, such as foraging, predator evasion, and communication during species recognition and mate choice. The generation and transmission of visual signals are influenced by characteristics of the light environment (e.g., ambient light spectrum) that can vary widely both within and among habitats (Endler 1993; Levine & MacNichol 1979). Because visual systems must respond to the spectrum of light available in their environments, many species are tuned to the different ambient light spectra present in their natural habitats (Lythgoe 1979). For example, in fishes the sensitivity of double cones, pairs of partially joined cone cells in most non-placental vertebrates, is generally matched to the background light of the environment (Cummings 2004; Dalton *et al.* 2014; Loew & Lythgoe 1978; Lythgoe *et al.* 1994; McFarland & Munz 1975). Variation in visual sensitivity can in turn drive diversification of signals and preferences and ultimately lead to speciation (Boughman 2001; Briscoe *et al.* 2010; Bybee *et al.* 2012; Cummings 2007; Endler 1991; Endler & Basolo 1998; Miyagi *et al.* 2012; Seehausen *et al.* 2008).

Spectral sensitivity of the retina is chiefly determined by the visual pigments in its photoreceptors. A visual pigment is composed of an opsin protein bound to a chromophore, and altering either molecule can modify the pigment's absorbance spectrum (Harosi 1994; Sakmar *et al.* 1989). Sensitivity can also be modified by pre-receptor filtering (Bowmaker *et al.* 1997; Cronin *et al.* 2001) or by opsin coexpression in individual photoreceptors, as in the Lake Malawi cichlid fish *Metriaclima zebra* (Bowmaker *et al.* 1997; Cronin *et al.* 2001; Dalton *et al.* 2014). All African cichlids share the same seven orthologous cone opsin genes (Hofmann *et al.* 2009; O'Quin *et al.* 2010), but cone absorbance varies greatly between species due primarily to differential expression of these opsins, with opsin sequence variation playing a relatively minor role (Hofmann *et al.* 2009; O'Quin *et al.* 2010). The opsin genes include *SWS1* (pigment peak absorbance, or λ_{max}, in *M. zebra* = 368 nm, ultraviolet sensitive), *SWS2B* (423 nm, blue sensitive), *RH2B* (484 nm, blue–green sensitive), *RH2Aβ* (519 nm, green sensitive), and *RH2Aα* (528 nm, green sensitive) (Parry *et al.* 2005). Absorbance of the remaining two cone pigments, SWS2A and LWS, has not been measured in *M. zebra*. However, in *Dimidiochromis compressiceps*, a related rock-dwelling cichlid, the SWS2A λ_{max} is 447 nm (blue sensitive) and the LWS λ_{max} is 567 nm (red sensitive) (Jordan *et al.* 2006). Microspectrophotometry indicates the three *SWS* opsins are expressed in single cones, while the remaining four opsins are expressed by double cones (Carleton & Kocher 2001; Carleton *et al.* 2008; Jordan *et al.* 2006; Levine & MacNichol 1979). Using dual-labeling fluorescent *in situ* hybridization (FISH), Dalton et al. (2014) revealed that *M. zebra* expresses *RH2Aα* and *RH2B* in opposite members of nearly every double cone, and frequently coexpresses *RH2Aβ.* with *RH2B* and *LWS* with *RH2Aα.* In both cases, the coexpression of opsins produces visual pigment mixtures that shift photoreceptor sensitivity toward longer wavelengths.

Each part of an animal's visual field is viewed by a distinct region of the retina, and these regions often differ in sensitivity. Variation of spectral sensitivity within the retina has been documented in a diverse and growing number of taxa, including fish, an amphibian, a reptile, birds, mammals (reviewed by Temple 2011) as well as invertebrates such as

butterflies (Briscoe *et al.* 2003) and crustaceans (Cronin *et al.* 1996). In such species, how a visual signal appears to the viewer will depend on the spectral sensitivity of the retinal region that detects it. *M. zebra coexpresses LWS* and *RH2Aα* in the ventral retina and *RH2Aβ* with *RH2B* in the nasal retina, increasing sensitivity of these retinal regions to their corresponding viewing backgrounds (Dalton *et al.* 2014). Increasing sensitivity to the background against which an object is viewed makes that background appear brighter, thereby enhancing contrast of dark objects such as predators. The color of the viewing background typically changes with angle of view in both terrestrial and aquatic habitats (Dalton *et al.* 2014; Endler 1993; Munz & McFarland 1977). In water, broad spectrum light filters downward from above, the horizontally viewed space light approximates the color transmitted best in that water, and upwelling light may be influenced by the color of the substrate (Lythgoe 1968). Thus, the spectral sensitivity that is optimal for a given task, for example detecting a dark object, depends on the viewing angle. While different visual tasks may require different spectral sensitivities, the color of the background is consistently an important factor. In the archerfish (*Toxotes chatareus*), ventral and dorsal retinal regions are tuned to different colored terrestrial and aquatic backgrounds via mixing of multiple chromophores and probably opsins as well (Temple *et al.* 2010). Archerfish search terrestrial foliage for prey that they dislodge by shooting streams of water, and they themselves must be watchful of predatory attacks from the water or sky. In butterflies, mice, four-eyed fish, as well as cichlids and archerfish, intraretinal regionalization of spectral sensitivity correlates with spectral features in various parts of each animal's visual field (Baden *et al.* ; Briscoe *et al.* 2003; Dalton *et al.* 2014; Owens *et al.* 2012; Temple *et al.* 2010).

It is unknown whether the regional matching of sensitivity to the visual field is developmentally plastic or under strict genetic control. Plasticity could facilitate spectral tuning of retinal regions to the backgrounds present in a specific locale. If light habitat differences induce variation in development of intraretinal sensitivity patterns, this plasticity could contribute to diversification in behaviors such as mate choice and ultimately speciation (West-Eberhard 2005). The levels at which opsins are expressed in the eye are known to be developmentally plastic, but whether their intraretinal distribution changes in response to the rearing environment remains unanswered (Fuller & Claricoates 2011; Hofmann *et al.* 2010; Smith *et al.* 2012).

In this study, we asked whether the background light at different viewing angles in an animal's environment influences the pattern of spectral sensitivity that develops across its retina. We addressed this question in *M. zebra*, which has regionalized spectral tuning and inhabits a variety of different light environments (Dalton *et al.* 2014; Ribbink *et al.* 1983). Because *M. zebra* is the only Lake Malawi cichlid genome that has been sequenced (Brawand *et al.* 2014), using this species could also facilitate subsequent discovery of the genes involved in the plasticity of opsin expression patterns. To test whether the retina's spatial pattern of spectral sensitivity is developmentally plastic, we reared fish under substantially different light environments: a Full Spectrum treatment and two treatments containing red-shifted light. We have observed that expression of *LWS* is concentrated in the ventral retina of *M. zebra* (Dalton *et al.* 2014) and of two other Lake Malawi cichlids

(*Melanochromis auratus* and *Labeotropheus trewavasae*, unpublished data). The ventral expression of *LWS* may be a developmental response to increased levels of long wavelength light stimulating the ventral retina. Therefore, we used two red-shifted treatments, one lighted from above (Red) and one lighted from below (Red Inverted), to determine the effect of regional retinal stimulation on the intraretinal pattern of opsin expression. Inverting the light was intentionally an extreme treatment designed to maximize our chances of detecting whether any plasticity exists in the intraretinal distribution of opsins. We revealed regional opsin expression patterns using both FISH and quantitative PCR (qPCR).

METHODS

Fish and Light Treatments

We established breeding groups of laboratory reared F_1 derived from wild caught *M. zebra* that were collected at Mazinzi Reef in Lake Malawi. All rearing and handling of live fish was performed in accordance with approved IACUC procedures. Twenty eight broods were collected and split between at least two light treatments approximately 3 days post fertilization, which is before opsin expression begins in *O. niloticus (Carleton et al. 2008)*. Each individual grew to adulthood (6 months) in its treatment and remained there until it was euthanized for an experiment. Three distinct treatments were used to investigate how the spectrum and direction of illumination affects opsin expression in double cones. A Full Spectrum environment, illuminated from above with metal halide lights (Hamilton, 10,000 K bulbs), simulated the light spectrum present in the natural photic environment . The Full Spectrum irradiance was spectrally similar to the downwelling irradiance (Fig. 1A) measured in Lake Malawi 3 m below the surface at Thumbi West Island (Dalton *et al.* 2010). A red-shifted (Red) environment was illuminated from above with standard fluorescent lighting (Philips TL741 Plus Long Life, 32 Watt F32 T8). The Red treatment lacked ultraviolet (UV) wavelengths and had a much greater proportion of long-wavelength light compared to the Full Spectrum (Fig. 1A). The Red environment is the same environment in which we have previously observed increased *LWS* expression compared to wild-caught fish (Hofmann *et al.* 2010). Finally, a Red Inverted treatment used the same red-shifted lighting but illuminated the tanks from below. Due to safety concerns we did not invert the Full Spectrum lighting apparatus. Four tanks were used for the Full Spectrum treatment, three for the Red, and two for the Red Inverted. Treatments were either isolated in separate rooms or isolated from other light sources with black curtains. We measured downwelling irradiance just before the point where light entered the water in the Full Spectrum and Red treatments according to published methods (Dalton *et al.* 2010). Similarly, in the Red Inverted treatment we measured upwelling irradiance as well as transmittance of the aquarium glass.

Although the shapes of the treatment light spectra are not smooth like that of the natural light (Fig. 1A), their effects on the visual system depend on how they stimulate the photoreceptors, which is determined by the number of photons the visual pigments absorb. Therefore, we compared treatment spectra and Lake Malawi irradiance in terms of how they stimulated the different visual pigments of the *M. zebra* visual system. To do so, we computed the relative quantum catch of visual pigments containing the *M. zebra SWS1*,

SWS2B, *RH2B*, *RH2Aβ*, *RH2Aα*, and *LWS* opsins bound to an A1 chromophore (Fig. 1B). Quantum catch calculations followed Hoffman et al. (2010), incorporating *M. zebra* lens transmittance (Dalton *et al.* 2010), and for the Red Inverted treatment, transmittance of the aquarium glass. The relative quantum catch of the Full Spectrum was similar to that of Lake Malawi irradiance, though LWS stimulation was slightly higher in the Full Spectrum treatment. In comparison, both the Red and Red Inverted spectra yielded almost no quantum catch by the SWS1 pigment, markedly less by RH2B, and substantially more by LWS. The three treatments were similar in overall intensity but substantially dimmer than the natural environment. Total quantum catch of all six pigments for the Full Spectrum, Red, and Red Inverted irradiances (from 350 to 700 nm) were 3.71×10^{15}, 1.97×10^{15}, 1.77×10^{15} photons/cm^2/s, respectively. Irradiance previously measured in Lake Malawi, 3 m below the surface at Thumbi West Island (Dalton *et al.* 2010), produced a total quantum catch of 9.28×10^{19} photons/cm^2/s.

Opsin Expression

To determine the level and the retinal distribution of cone opsins, we used both FISH and qPCR. Performing FISH on whole retinas provided gene expression data on each individual double cone cell across the entire retina. We previously used FISH to determine the intraretinal distribution and coexpression of *RH2B*, *RH2Aα*, and *LWS* (Dalton *et al.* 2014), so in this study we use FISH for these opsins in order to compare results. We have shown *LWS* is expressed regionally, and its ventral location allowed us to manipulate the light received by this region during development. Compared to FISH, qPCR on dorsal and ventral retinal hemispheres is a quicker method for obtaining gene expression data, albeit at lower spatial resolution. Therefore we used qPCR to confirm the FISH results and to examine the effects of the light treatments on the remaining cone opsin genes.

We performed dual-labeling FISH on seven individuals from the Full Spectrum treatment, six from the Red treatment, and nine from the Red Inverted according to published procedures (Allison *et al.* 2010; Barthel & Raymond 2000). The use of fluorescent dyes allows simultaneous detection of mRNA from multiple opsins without signal interference, which can occur with colorimetric precipitate reporters. Briefly, fish were euthanized, and retinas were extracted and fixed in 4% paraformaldehyde. Opsin was detected with Dig- and Fluorescein-labeled riboprobes, and signal was enzymatically augmented with sequential Tyramide Signal Amplification (Invitrogen). A coding sequence probe was used for *LWS* opsin, while a 3' UTR probe was used for *RH2Aα* and *RH2B* to differentiate them from each other and the closely related *RH2Aβ* opsin. Dot blots have confirmed that these probes are specific to their respective genes (Dalton *et al.* 2014). We used Alexa Fluor 594 and 488 dyes (Invitrogen) to detect Dig and Fluorescein probes, respectively. The excitation and emission spectra of these dyes are sufficiently separated to eliminate significant dye crosstalk. Retinas were viewed with a Leica DM 5500B epifluroescence microscope equipped with L5 and TX2 filter cubes. Because *LWS* expression was previously found to vary dorsoventrally, we sampled along the dorsoventral axis, dividing each retina into five equal sized regions along this axis. In each region 50 double cones were examined for the presence or absence of specific opsin transcripts. We probed 18 retinas for transcripts of both *LWS* and *RH2Aα* and one retina for *LWS* only. To determine whether *LWS* and *RH2B*

are expressed in opposite members of the double cone, we probed three retinas for this gene combination. The FISH data from the Red individuals are from Dalton et al. (Dalton *et al.* 2014).

We performed qPCR according to published protocols (Carleton 2011; Spady *et al.* 2006). Briefly, retinas of five fish from each treatment were isolated and divided into dorsal and ventral halves. Total RNA was extracted from each half using the Qiagen RNeasy kit and reverse-transcribed using Superscript III (Invitrogen) and a polyT primer. The cichlid cone opsins (*SWS1*, *SWS2B*, *SWS2A*, *RH2B*, *RH2A*, and *LWS*) were quantified separately with gene-specific primers and probes using the Taqman Universal PCR mix (LifeTech) on a Roche Lightcycler 480. Here the *RH2A* primers and probes matched both *RH2Aα* and *β*. A separate set of primers that matched only *RH2Aβ* was then used to estimate *RH2Aα* expression by subtracting the *RH2Aβ* level from using the *RH2A* sum. Relative PCR efficiencies for each opsin were computed using a construct containing a tandem array of segments of each opsin gene (Spady *et al.* 2006). Absolution efficiencies for *RH2A* and *RH2Aβ* were determined from a dilution series. The relative expression of each opsin gene was calculated from the PCR efficiencies (E_i) and critical cycle numbers (C_{ti}) for each gene i. Each of the cone opsins was normalized to the expression level of the alpha subunit of transducin (GNAT2), the G protein activated by opsins in cones. The normalized cone opsin expression was then calculated as:

$$\frac{T_i}{T_{GNAT2}} = \frac{(1+E_{GNAT2})^{C_{tGNAT2}}}{(1+E_i)^{C_{ti}}}$$

where i is the cone opsin genes of interest and E_{GNAT2} and C_{tGNAT2} are the PCR efficiency and critical cycle number for GNAT2. All Taqman primers and probes are listed in Supplementary table S1.

Genotyping

Our FISH results revealed substantial variation among Red Inverted individuals in the plasticity of LWS expression, suggesting a strong genetic effect. Therefore, to determine the relatedness of the Red Inverted individuals examined by FISH, we genotyped them and the parents of all broods in this treatment using a set of 16 microsatellites. Microsatellites were selected to be unlinked (see Supplementary table S2 for sequences and locations). Many are located at QTL peaks near a candidate gene for opsin expression. For some loci, the forward primer was fluorescently labeled with 6-FAM or 5'Hex dyes with labeled forward and unlabeled reverse primers used for PCR amplification. For other loci, the forward primer was unlabeled but included a 5' CAG tag (GCAGTCGGGCGTCA) that matched a fluorescently labeled CAG primer. Unlabeled forward and reverse primers and a small amount of labeled CAG primer were then PCR amplified (Schable *et al.* 2002; Schuelke 2000). PCR products were sized on an ABI 3730 by comparison to a Rox labeled size standard and converted to size using Genemapper. Each specimen was genotyped by microsatellite allele size. The genotypes were then used to determine parentage through process of elimination.

Data Analysis

The purpose of the Red Inverted treatment was to see if lighting the fish from below would increase *LWS* expression in the dorsal retina and decrease *LWS* expression in the ventral retina. To test this, we computed the total percent of double cone members expressing *LWS* in the dorsal and mid-dorsal regions for each individual. We used an arcsine transformation of these percentages, and then performed an ANOVA and used Tukey's test to compare treatment effects with α=0.05. The procedure was repeated for the ventral and mid ventral regions. A separate ANOVA was performed on the FISH data from the mid region. To check for other patterns in the data, we also performed an ANOVA on the combined *LWS* FISH data using treatment and region as factors, and Tukey's test to compare means (α = 0.05). The qPCR results were analyzed in the same manner, except that we log- transformed the qPCR data to satisfy assumptions of the ANOVA. In addition, we performed two Pearson's correlation analyses. In one we compared fish standard length with *LWS* expression in dorsal regions in the Red Inverted individuals examined by FISH. In the other we compared the log-transformed *LWS* data for the ventral and dorsal retinas of the fish examined by qPCR, analyzing each treatment group separately. All statistical analyses were performed in SAS 9.2 (Cary, N.C.).

RESULTS

Fluorescent *in situ* hybridization

The rearing light environment had a significant effect on the distribution of *LWS* expression in the retina as determined by FISH (p=0.002). Only in the Red Inverted treatment did we find individuals that expressed *LWS* frequently in the dorsal retina (Figs 2J-2R, 3, Supplementary Table S3). Light treatments did not significantly affect frequency of *LWS* expression in the mid retina (p=0.21) or the ventral retina (p=0.09). When all retinal regions were combined, *LWS* expression was higher in the Red Inverted compared to the other two treatments based on a post-hoc Tukey test ($p < 0.05$). The frequency of double cone members expressing *LWS* did not differ between the Red and Full Spectrum treatments. Consistent with Dalton et al. (2014), fish from all treatments had *LWS* expression that varied between regions of the retina (post-hoc Tukey test, $p < 0.05$). Retinal regions fell into three groups: ventral and mid ventral regions had the highest *LWS* expression, followed by the mid retina, while the mid dorsal and dorsal both had the least *LWS* expression.

The morphology of the square cone mosaic did not vary between retinal regions or light treatments. Four pairs of double cones always surrounded each single cone. In the double cones two genes were expressed consistently. One member of nearly every double cone across the retina expressed *RH2Aα.* This included 99.8% +/− 1.0% of double cones (n=4,500) in 18 fish. In rare double cones, *RH2Aα* was detected in both members or was not detected in either member. Similarly, in 99.9% +/− 0.5% of double cones (n=750), one member expressed *RH2B*. These results suggest that the predominant pattern of opsin expression, in which *RH2Aα* and *RH2B* occupy opposite members of virtually every double cone, was unaffected by rearing under red-shifted light or by inverting that light. In addition, all fish frequently coexpressed *LWS* with *RH2Aα* in the ventral retina. When present, *LWS*

was coexpressed with *RH2Aα* 98.8% of the time and was never coexpressed with *RH2B*, consistent with previous observations (Dalton *et al.* 2014).

LWS was frequently expressed in the dorsal retina of some but not all Red Inverted individuals. We investigated whether variation in the plasticity of dorsal *LWS* expression was associated with parentage, sex, or body size. After genotyping the nine Red Inverted individuals examined by FISH, as well as the parents of all broods in this treatment, we detected no correlation between plasticity and parentage. There were 5-6 pairings in total (identity of one dam was incompletely resolved). Each of the four individuals with strong dorsal *LWS* had a different dam. In addition, eight of the nine individuals from the treatment had the same sire, and three of these had strong dorsal *LWS*. Furthermore, individuals from the same broods exhibited varying levels of plasticity. In two broods, one individual expressed *LWS* in the dorsal retina much more frequently than its one or two siblings did. From these results we found no observable correlation between plasticity and parentage. However, heritability of *LWS* expression plasticity might be detected with a larger sample size. Dorsal *LWS* plasticity was not associated with sex (all but two individuals were determined to be male). We also found no significant correlation between standard length of fish and plasticity of dorsal *LWS* expression ($p=0.11$).

Quantitative PCR

We performed qPCR on the dorsal and ventral retina to confirm our FISH results for *LWS* and to examine whether the light environment affects the expression level of the six other cone opsin genes. The qPCR results were in agreement with the FISH results for *LWS* and revealed plasticity in *RH2Aβ*, *RH2B*, and *SWS1* (Fig. 4, Table 1).

LWS transcript levels were higher in the Red Inverted treatment than in the Full Spectrum treatment ($p=0.02$). In addition, *LWS* expression was stronger in the ventral than in the dorsal retina when all treatments were combined ($p<0.0001$). Two of the five Red Inverted individuals had high levels of *LWS* in the dorsal retina, while dorsal *LWS* was low in all other fish regardless of treatment. Although dorsal *LWS* transcript levels were higher in the two Red Inverted fish, dorsal *LWS* was not significantly higher for the Red Inverted fish as a group ($p=0.13$). To examine whether the stronger *LWS* expression in the dorsal retina might be the result of certain individuals expressing more *LWS* across the entire retina, we performed a correlation analysis of the qPCR data (Fig. 5). Dorsal and ventral *LWS* expression was positively correlated in both the Red ($r = 0.93$, $p = 0.022$) and Red Inverted ($r = 0.99$, $p = 0.002$) but not in the Full Spectrum light treatment ($r = 0.81$, $p = 0.097$). However, as ventral *LWS* expression increased, dorsal *LWS* expression increased only slightly in the Red while it increased greatly in the Red Inverted (Fig. 5). Thus, dorsal LWS expression appears to be induced by disproportionate stimulation of the dorsal retina by light that is rich in long-wavelengths and lacking in UV.

The Red treatment induced an increase of *SWS1* in the ventral relative to the dorsal retina ($p=0.045$) as well as higher expression of *SWS1* across the whole retina (dorsal and ventral combined, $p=0.01$). In addition, *RH2B* was highest in the Red treatment ($p=0.01$), while *RH2Aβ* expression increased in the Red Inverted environment ($p<0.001$). *SWS2B* expression also appeared higher in the Red Inverted, but this result was not quite significant ($p=0.057$).

SWS2A was not expressed by any of the individuals, and expression of *RH2B, RH2Aβ,* and *RH2Aα* did not differ between ventral and dorsal hemispheres.

DISCUSSION

Plasticity of Intraretinal Patterns of Spectral Sensitivity

Spectral sensitivity can vary regionally within the retina and potentially influence behaviors mediated by these different retinal regions (Endler & Basolo 1998; Lythgoe 1979). Previous work has shown that the average level of opsin in a whole retina is plastic and can respond to the light environment during development (Fuller & Claricoates 2011; Hofmann *et al.* 2010; Smith *et al.* 2012). In this work, we have demonstrated that the retina's pattern of spectral sensitivity is developmentally plastic with respect to the light environment. Expression of opsins can be altered regionally by changing the orientation and spectrum of illumination. In *M. zebra* we previously observed *LWS* almost exclusively in the ventral retina, but here we show that fish illuminated by a red-shifted light from below frequently express *LWS* in the dorsal retina (Figs 2-5, Table 1). Substantial dorsal *LWS* expression has not been observed in any other *M. zebra* individuals, whether laboratory-reared or wild-caught (Dalton *et al.* 2014). In addition, illumination from above with a red-shifted light induced upregulation of *SWS1* in the ventral retina relative to the dorsal retina (Fig.4, Table 1). To our knowledge this is the first report of distinct retinal regions exhibiting independent developmental plasticity of opsin expression by their photoreceptors.

The rearing light environment affected the levels at which four of the seven opsins were expressed by the whole eye (dorsal and ventral regions combined). If genes are paired according to the cell types that express them, the shorter-wavelength sensitive gene of the pairs was upregulated in the Red environment, while longer-wavelength sensitive genes were upregulated in the Red Inverted treatment. That is, *SWS1* and *SWS2B* are expressed in single cones, *RH2B* and *RH2Aβ* are expressed in one member of a double cone, and *RH2Aα* and *LWS* in the opposite member. Both *SWS1* and *RH2B* were most highly expressed in the Red environment, while the levels of *RH2Aβ* and *LWS* were highest in the Red Inverted (Fig.4, Table 1). There was also a tendency for *SWS2B* expression to be highest in the Red Inverted treatment, though this trend did not quite reach significance. For each opsin that was plastic, its response was sensitive to the angle of lighting. It should be noted, however, that we were unable to examine the effect of inverting the Full Spectrum light because safety constraints prevented us from inverting the Full Spectrum lighting apparatus. All increases in expression were induced by either the Red treatment, which was lighted from above, or the Red Inverted, but never by both red-shifted light treatments. Thus, differences in the spectrum and angle of illumination both affected opsin expression levels.

Regulation of Opsin Expression and Ecology

Taken together, our FISH and qPCR results suggest that both the spectrum and the retinal location of incoming environmental light influence the retinal pattern of spectral sensitivity by altering the distributions and levels of opsins. Given that spectral sensitivity is known to influence visual behaviors, it is expected that variation in spectral sensitivity between regions of the retina will affect behaviors mediated by these different retinal regions (Levine

& MacNichol 1982; Levine *et al.* 1979; McFarland & Munz 1975). *M. zebra* females are approached from below by courting males and therefore initially view these prospective mates with the dorsal retina (personal obs.). The egg spots (yellow anal fin patches thought to mimic eggs) of male cichlids have been reported to influence several behaviors, including male-male aggression, mate choice, and fertilization (Egger *et al.* 2011; Hert 1991; Theis *et al.* 2012; Wickler 1962). Modeling indicates that coexpression of *LWS* and *RH2Aα* in the ventral retina could enhance discrimination of *M. zebra* colors, including egg spots (Dalton *et al.* 2014). In addition, ventral *LWS* increases sensitivity to the background viewed by this retinal region, potentially aiding the detection of distant dark objects such as predators. Numerous other examples of intraretinal variation in opsin expression have been documented among vertebrates and invertebrates, and in several of these the opsin distributions correlate with the environment (Baden *et al.* ; Briscoe *et al.* 2003; Cronin *et al.* 1996; Owens *et al.* 2012; Temple 2011). However, few studies have directly examined the relationship between behavior and intraretinal differences in spectral tuning (Temple 2011), so this remains an opportunity for investigation in cichlids and many other animals.

Phenotypic plasticity allows species to adapt to a variety of environmental changes that occur over broad spatial and temporal scales, contributing to diversification and speciation (West-Eberhard 1989; West-Eberhard 2005). Several species, including black bream (Shand *et al.* 2002), rainbow trout (Allison *et al.* 2006), and European eels (Cottrill *et al.* 2009) are known to change cone opsin expression as they progress through their life cycle and move to different photic habitats. For the black bream there is evidence that the opsin expression changes are a plastic response to changes in light environment (Shand *et al.* 2008). Studies have found that several species alter cone opsin expression during development in response to manipulation of the rearing light spectrum. When reared in environments containing more long wavelength light, expression shifts toward opsins sensitive to longer wavelengths in black bream (Shand *et al.* 2008), killifish (Fuller & Claricoates 2011), barramundi (Ullmann *et al.* 2011), and to some degree in African cichlids (Hofmann *et al.* 2010; Hornsby *et al.* 2013; Smith *et al.* 2012). However, these studies looked at average responses across the retina. To our knowledge only *M. zebra* and the threespine stickleback have been examined for plasticity of intraretinal opsin distributions. Sticklebacks exhibited little plasticity, but they were subjected to light environment changes only after reaching adulthood (Flamarique *et al.* 2013). We have not tested cichlids to determine whether they remain plastic as adults or are only plastic through some critical period of development.

We modified the rearing light environment substantially with our treatments in order to demonstrate that intraretinal variation in spectral sensitivity is developmentally plastic (Fig. 1). To determine the influence of this plasticity on *M. zebra* visual ecology, it will be necessary to characterize spatial and temporal variation in Lake Malawi light environments and examine its effects on the development of intraretinal opsin distributions. Our preliminary observations suggested stimulation of the ventral retina by a long-wavelength enriched spectrum might induce *LWS* expression. In addition, other cichlid species increased LWS expression in habitats that were enriched with long-wavelength light due to their location near the lake surface or under fluorescent lights (Hofmann *et al.* 2010; Smith *et al.* 2011; Smith *et al.* 2012). In the current study, the frequency of ventral *LWS* expression in *M.*

zebra as determined by FISH was unaffected by the spectrum or angle of illumination. Although qPCR results suggested that ventral *LWS* was upregulated in both red-shifted treatments (Fig. 4), there was substantial variation between individual responses, and this result was not statistically significant. In addition, the upregulation of *SWS1* we observed in the Red environment differs from the responses of other cichlid species in our earlier studies (Hofmann *et al.* 2010). The differences between previous and current results may be due to genetic differentiation between species, as most of the plasticity in opsin expression that we have observed to date has differed among the species examined.

As research has progressed on cichlid visual systems it has become apparent they are quite variable on multiple scales. In *Oreochromis niloticus*, expression levels of all seven opsins, lens transmission, and spectral sensitivity change during the first six months of development in the laboratory (Carleton *et al.* 2008; Sabbah *et al.* 2012). *O. niloticus* is a riverine species and a representative of the ancestral outgroup to the diversity of cichlids that have evolved in Africa's great lakes. Adults of the different species in these lakes differ greatly in spectral sensitivity as they express different combinations of the opsins (Parry *et al.* 2005), which has been attributed to heterochronic shifts in development (Carleton *et al.* 2008; O'Quin *et al.* 2010; O'Quin *et al.* 2011; Spady *et al.* 2006). Differences in opsin expression also occur between and within cichlid populations (Dalton *et al.* 2014; Hofmann *et al.* 2009; Smith *et al.* 2011). In this study, developmental plasticity in the distributions and levels of opsins varied among siblings. Although we did not find plasticity to be associated with dam or sire, genetic effects on plasticity of opsin expression in *M. zebra* cannot be ruled out from this relatively small sample. Parents of the experimental fish could have been heterozygous for one or more contributing genetic loci, and undetected epigenetic factors may be acting as well.

Conclusion

In summary, we have shown that the location as well as the relative level of opsins in the *M. zebra* retina can change in response to the environmental light environment. During development, the light illuminating retinal regions affects their opsin expression and therefore their spectral sensitivity. Because backgrounds in nature differ with angle of view, their differences may contribute to the development of variation in spectral sensitivity between retinal regions. In addition, light environments can vary temporally and spatially, between and within populations. The variation in *M. zebra* light environments throughout the year and between locations requires further documentation. Such environmental differences may influence the development of variation in spectral sensitivity at the population and species levels in cichlids and other animals, potentially affecting ecological and evolutionary processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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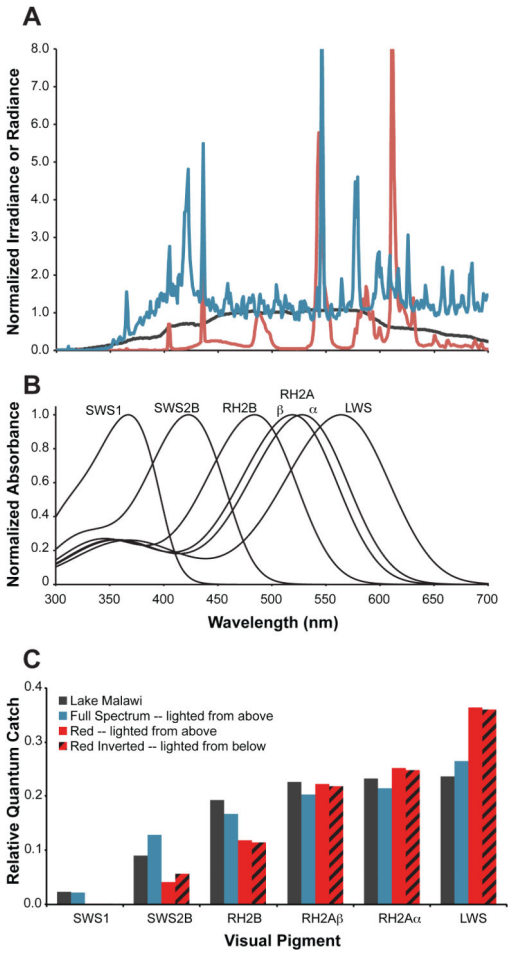

Figure 1.

Light treatments. A) Overhead irradiance spectra of Lake Malawi (gray), Full Spectrum (blue), and Red (red) environments. To facilitate comparison, spectra were normalized to their values at 487 nm and y-axis was truncated. The Full Spectrum peaked at 9.6 at 546 nm and the Red peak was 8.8 at 611 nm. B) Absorbance spectra of *M. zebra* visual pigments. C) Computed quantum catch by visual pigments of Lake Malawi, Full Spectrum, Red, and Red Inverted spectra, taking *M. zebra* lens transmittance into account. Overhead irradiance was measured 3 m below the surface at Thumbi West Island, Lake Malawi.

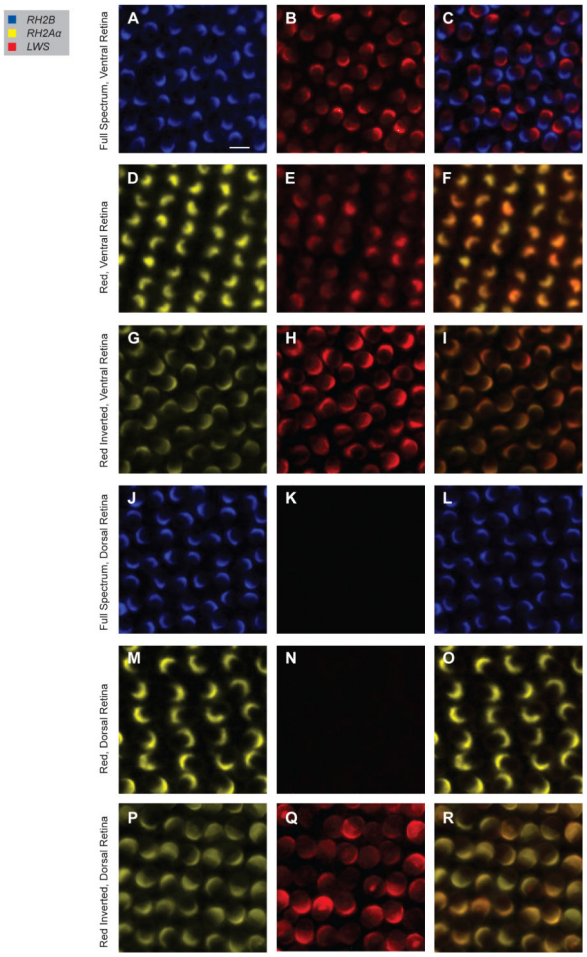

Figure 2.

Opsin expression revealed by dual-labeling fluorescent *in situ* hybridization in ventral (A-I) and dorsal (J-R) regions of the retina. A-C) Individual reared in Full Spectrum light expressed *RH2B* (A, blue) and *LWS* (B, red) in opposite members of double cones (C, merged image). D-F) Individual reared in Red treatment coexpressed *RH2Aα* (D, yellow) and LWS (E, red) in the same members of double cones (F, merged image). G-I) Individual reared in Red Inverted light also coexpressed *RH2Aα* (D, yellow) and *LWS* (E, red) in the same double cone members. J-L) The same individual shown in A-C expresses *RH2B* but not *LWS* in dorsal retina. M-O) The same individual shown in D-F expresses *RH2Aα* but not *LWS* in the dorsal retina. P-R) The same individual shown in G-I coexpressed *LWS* with *RH2Aα* in the dorsal (R) as well as ventral retina. Scale bar, 10 μm.

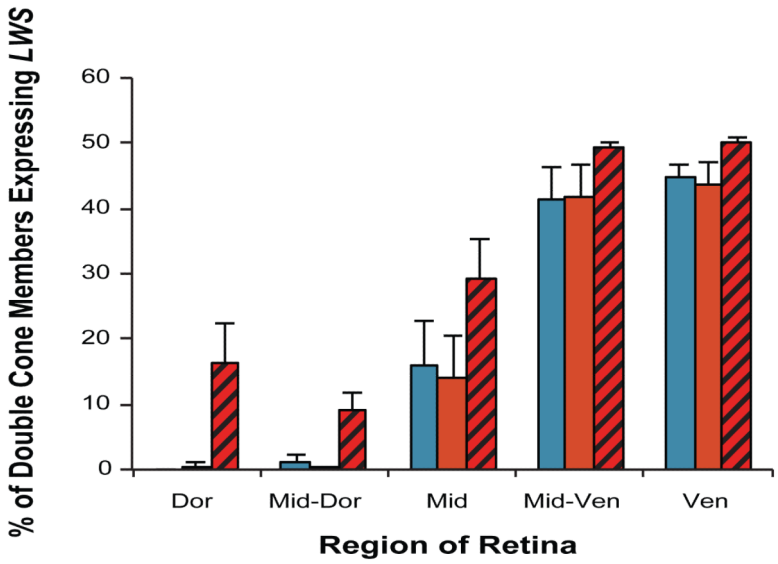

Figure 3.

Frequency of *LWS* expression in double cone members, determined by *in situ* hybridization within five retinal regions. Fish were reared in the Full Spectrum (blue, n=7), Red (red, n=6), or Red Inverted (hatched red, n=9) light environment. Data from the Red individuals are from Dalton et al. (Dalton *et al.* 2014). Bars indicate standard error.

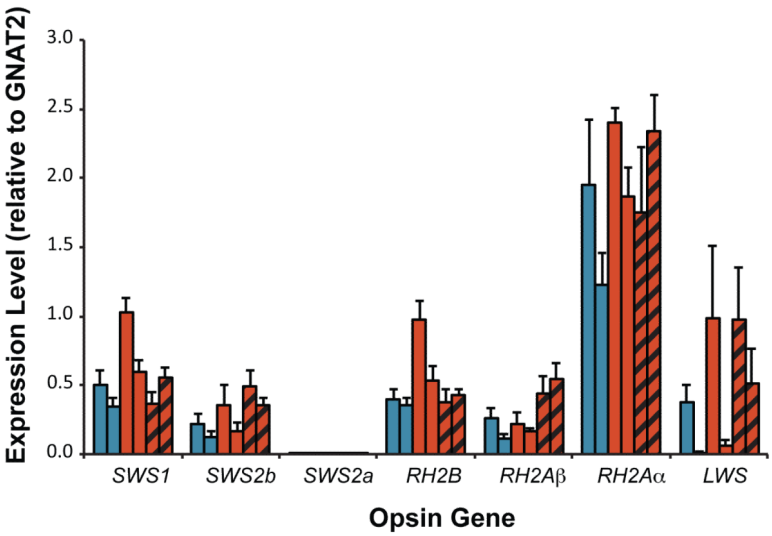

Figure 4.

Proportional cone opsin expression in the dorsal and ventral retina determined by qPCR. Fish were reared in the Full Spectrum (blue, n=5), Red (red, n=5), or Red Inverted (hatched red, n=5) light environment. Pairs of bars are shown for each environment; the left bar indicates ventral opsin expression and the right indicates dorsal. Error bars indicate standard error.

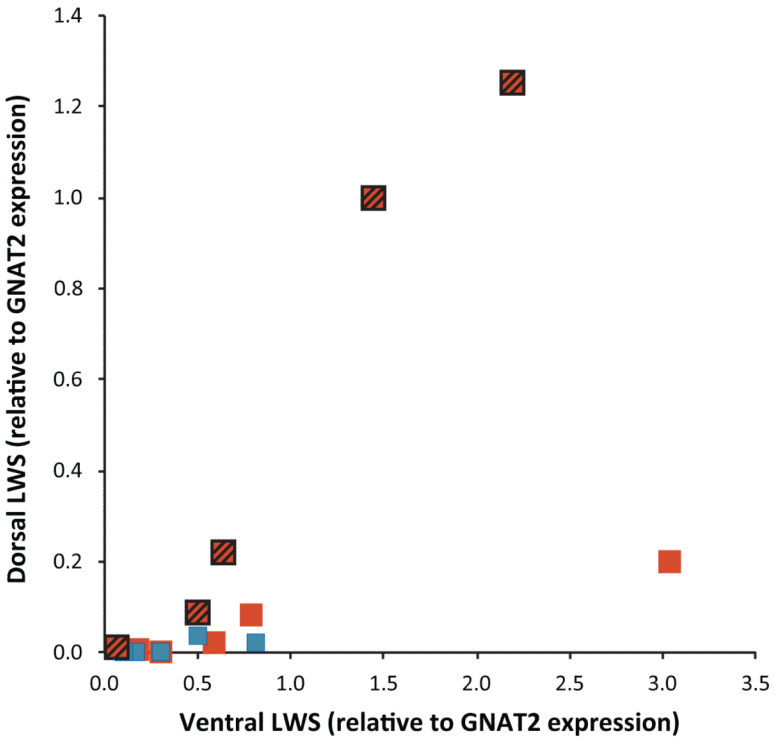

Figure 5.

Relationship between dorsal and ventral *LWS* expression determined by qPCR. Pearson's correlation was significant for the Red (red, n=5, r = 0.93, p = 0.022), and Red Inverted (hatched red, n=5, r = 0.99, p = 0.002) but not the Full Spectrum light treatment (blue, n=5, r = 0.81, p = 0.097).

Table 1

Comparison of opsin expression in Full Spectrum (FS, n=7), Red (R, n=6), and Red Inverted (RI, n=9) light environments and retinal hemispheres. Expression was determined by qPCR and analyzed by ANOVA with a post-hoc Tukey test ($p < 0.05$); NS = non-significant. Horizontal lines group genes that are expressed in the same cell type.

Opsin	Response	Dorsal vs Ventral	Interaction
SWS1	Up in R (0.0101)	NS (0.5811)	Dor < Ven in R (0.0454)
SWS2B	NS (0.0570)	NS (0.3289)	NS (0.8687)
RH2B	Up in R (0.0115)	NS (0.4795)	NS (0.1615)
RH2Aβ	Up in RI (0.0007)	NS (0.3550)	NS (0.1186)
RH2Aα	NS (0.2436)	NS (0.7890)	NS (0.1263)
LWS	Up in RI relative to FS only (0.0195)	Dor < Ven (<0.0001)	NS (0.1275)