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Intraspecific cone opsin expression variation in the cichlids of Lake Malawi

ADAM R. SMITH,* LINDSAY D'ANNUNZIO,*† ABBI E. SMITH,*‡ ANIT SHARMA,* CHRISTOPHER M. HOFMANN,* N. J. MARSHALL§ and KAREN L. CARLETON*

Department of Biology, University of Maryland College Park, 1210 Biology-Psychology Building, College Park, MD 20742, USA, †Joint Graduate Program in Toxicology, Environmental and Occupational Health Sciences Institute, University of Medicine and Dentistry of New Jersey, 170 Frelinghuysen Road, Piscataway, NJ 08854, USA, ‡Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892, USA, §Sensory Neurobiology Group, Queensland Brain Institute, University of Queensland, St. Lucia, Queensland, Australia

Abstract

The expression of cone opsin genes is a primary determinant of the characteristics of colour vision. Interspecific variation in opsin expression is common in African cichlids. It is correlated with foraging among cichlids from Lake Malawi, and with ambient light environment among cichlids from Lake Victoria. In this study, we tested whether gene expression varied within species such that it might be important in contributing to divergence. We hypothesized that light attenuation with depth would be correlated with predictable changes in gene expression in Lake Malawi, and that this variation would tune visual sensitivities to match the ambient light environment. We observed significant differences in cone opsin expression in three different comparisons among populations of the same species. Higher LWS expression was found in shallow versus deep *Copadichromis eucinostomus*. In *Metriaclima zebra*, individuals from Zimbawe Rock expressed significantly more SWS2B than those from Thumbi West Island, although these locales have similar ambient light environments. Finally, *Tropheops gracilior* from deeper water had significantly more variation in expression than their shallow counterparts. These results support that gene expression varies significantly between populations of the same species. Surprisingly, these results could not be explained by predicted visual performance as models predicted that differential expression patterns did not confer sensitivity advantages at different depths. This suggested that expression variation did not confer a local sensitivity advantage. Therefore, our findings were contrary to a primary requirement of the sensory bias hypothesis. As such, other explanations for intraspecific gene expression variation need to be tested.

Keywords: colour vision, cone opsin, expression, light environment, sympatry

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Introduction

Several evolutionary models have been proposed to explain speciation in sympatry. One of these is Fisher's runaway model, in which random changes in female preferences lead to rapid divergence in male traits (Fisher 1930; Lande 1981). Another is sensory drive, in which the local environment sets female sensitivities that then select for particular male traits (Endler 1992; Boughman 2002; Dangles *et al.* 2009). Both of these models predict that there should be variation in sensory systems among sister taxa and perhaps even among populations. However, these models differ in whether female sensitivities will be correlated with the local environment. The Fisherian model suggests that

Correspondence: Adam R. Smith, Fax: (301) 314 6262;
E-mail: adasmi@umd.edu

changes can occur neutrally, perhaps through drift, such that no obvious association with local environment will be observed. In contrast, the sensory drive model supposes that the local environment shapes a key sensory system and through habitat variation leads to female sensory variation. Alternatively, models of pure genetic drift require no selective processes to drive or reinforce divergence, and may occur in small, isolated populations.

African cichlid fishes are a model for rapid divergence and speciation. Replicate species flocks have arisen in each of the Great Lakes: Malawi, Tanganyika and Victoria (Kocher 2004). Lake Malawi is the most species rich of the East African rift lakes, and is estimated to have well over 500 endemic species (Kornfield & Smith 2000). Sexual selection on male nuptial colouration has been posited as one of the major forces contributing to the development of new species in this lake (Danley & Kocher 2001). Female choice for male nuptial colouration is tightly linked to the ability to perceive and distinguish different colour patterns, and therefore we expect variation in colour vision to be an important facet of speciation. More specifically, if nuptial colouration is the substrate for selection and speciation, we should observe differences in sensory traits that influence colour vision in diverging species or populations.

Work on Lake Victoria cichlids has demonstrated that sequence changes in the LWS opsin gene shift visual sensitivities (Terai *et al.* 2002, 2006; Carleton *et al.* 2005). Variation in the LWS opsin sequences is correlated with depth. Longer wavelength-sensitive alleles are found deeper where the downwelling light spectrum shifts to longer wavelengths. LWS sequences also show signs of selection at multiple locales where differing levels of turbidity influence the quality of the ambient light environment. Male colours are also correlated with depth and visual sensitivity. These results suggest that speciation has been driven by the ecological light gradient selecting for alternate visual sensitivities, which in turn select for particular male colour patterns (Seehausen *et al.* 2008). The evolution of LWS genes in Lake Victoria cichlids is an example of the sensory drive model. Similar changes in opsin sequence have been observed in closely related fishes from Malawi (Spady *et al.* 2006; Hofmann *et al.* 2009; Smith & Carleton 2010), but correlations with male colour have not been examined.

In addition to sequence diversity, changes in opsin gene expression act as a second genetic mechanism for visual tuning in African cichlids (Carleton 2009). Changes in opsin expression patterns are known to cause visual shifts in certain teleost fish species that are tied to variation in the surrounding light environment (Fuller *et al.* 2004, 2005; Shand *et al.* 2008). East African cichlids possess seven distinct opsin genes that are differentially expressed to tune the visual system to different environments. For example, the relative expression rates of SWS and LWS opsin genes of Lake Victoria cichlids are tightly linked to the ambient light environment at different geographical locations (Hofmann *et al.* 2009).

Although Malawi cichlids have seven different opsin genes, previous research suggests that individual fish usually express only three opsin genes: one for the short-wavelength sensitive single cones [ultraviolet (SWS1—λ_{max} 368 nm), violet (SWS2B—λ_{max} 415 nm), or blue (SWS2A—λ_{max} 455 nm)] and two for the medium- and long-wavelength sensitive double cones [blue-green (RH2B—λ_{max} 488 nm), two true greens (RH2Aα and β—λ_{max} 518 nm), and red (LWS—λ_{max} 560 nm); Parry *et al.* 2005; Spady *et al.* 2006]. These are typically expressed in one of a few combinations or visual palettes. The three most common visual palettes are the short-wavelength (SWS1, RH2B, RH2A), medium-wavelength (SWS2B, RH2B, RH2A) and long-wavelength (SWS2A, RH2A, LWS) sets. As the total number of expressed pigments is limited, visual systems should be tuned such that the genes expressed most closely match the local light environment.

In previous studies, we found that light environment shaped opsin expression in cichlids from Lake Victoria (Hofmann *et al.* 2009). Fish in clear water habitats expressed more SWS2B opsin than those from murkier habitats. This increase was correlated with the amount of available light for stimulating these shorter wavelength cones. In addition, more LWS double cones were observed in fish from murky habitats (Carleton *et al.* 2005). Furthermore, hybrid-cross experiments between Malawi cichlids suggested that there is a strong genetic component to opsin gene expression with just a few genes controlling expression patterns (Carleton *et al.* 2010). However, we also found that there was some plasticity to opsin expression when fish were raised in the lab versus those caught from the wild (Hofmann *et al.* 2010). This variation was smaller, but statistically significant. Such environmental plasticity has been found in opsin expression from other species, most notably killifish (Fuller *et al.* 2004, 2005) and black bream (Shand *et al.* 2008). Clearly, both genetic and environmental mechanisms play a role in modulating expression of opsin genes in natural populations of cichlids from Lake Malawi.

In this work, we sought to determine the extent of opsin gene expression variation in natural populations and examined variation at two spatial scales. First, we profiled variation with depth at a single site in two species, *Tropheops gracilior* and *Copadichromis eucinostomus*, to determine the effects of habitat depth on local expression patterns. The differential attenuation of wavelengths through the water column should have

created an environmental gradient wherein the SWS1 and LWS opsins at the extremes of the visual spectrum should become less efficient as depth increases (Dalton *et al.* 2010; Fig. 1). We also profiled variation in *Metriaclima zebra* in two distinct geographical locations that occurred at similar depths with similar light environments. We had three primary hypotheses: (i) expression variation would be quantitatively more variable at shallow depths in *T. gracilior* and *C. eucinostomus* than in deeper waters due to the presence of a broader light spectrum allowing for a greater pigment efficiency at the ends of the spectrum; (ii) qualitative changes in these species would involve the loss of LWS in response to the narrowing light spectrum with depth; and (iii) the *M. zebra* populations would be relatively homogenous due to selection from similar light environments.

Materials and methods

Research subjects

All individuals were collected from Lake Malawi during July 2008. Fishes were sacrificed the day of capture and their eyecups were stored in RNAlater. Three species were sampled for this study: *Tropheops gracilior*, *Copadichromis eucinostomus*, and *Metriaclima zebra*. Thirty-six *T. gracilior* were collected in total at Otter Island, with 20 from 5 m and 16 from 20 m depth. Twenty-eight *C. eucinostomus* were collected at Otter Point, with 15 from 5 m and 13 from 15 m. A total of 20 *M. zebra* were collected from two geographically distinct locations: 10 from Thumbi West and 10 from Zimbawe Rock. These sampling locations are depicted in Fig. S1 (Supporting information).

Quantification of relative expression

RNA was isolated from the dissected retina using commercial Qiagen Qiashredder and RNeasy kits (Valencia, CA, USA). The isolated RNA was then reverse-transcribed to cDNA. Real-time quantitative PCR (RT-qPCR) was performed using a set of seven-cone opsin-specific primer/probe combinations. The total expression of the duplicate green genes (RH2Aα and RH2Aβ) was measured together using the aggregate RH2A primer/probe developed for prior studies (Spady *et al.* 2006). The relative expression of these two genes was then quantified using a RH2Aβ-specific primer

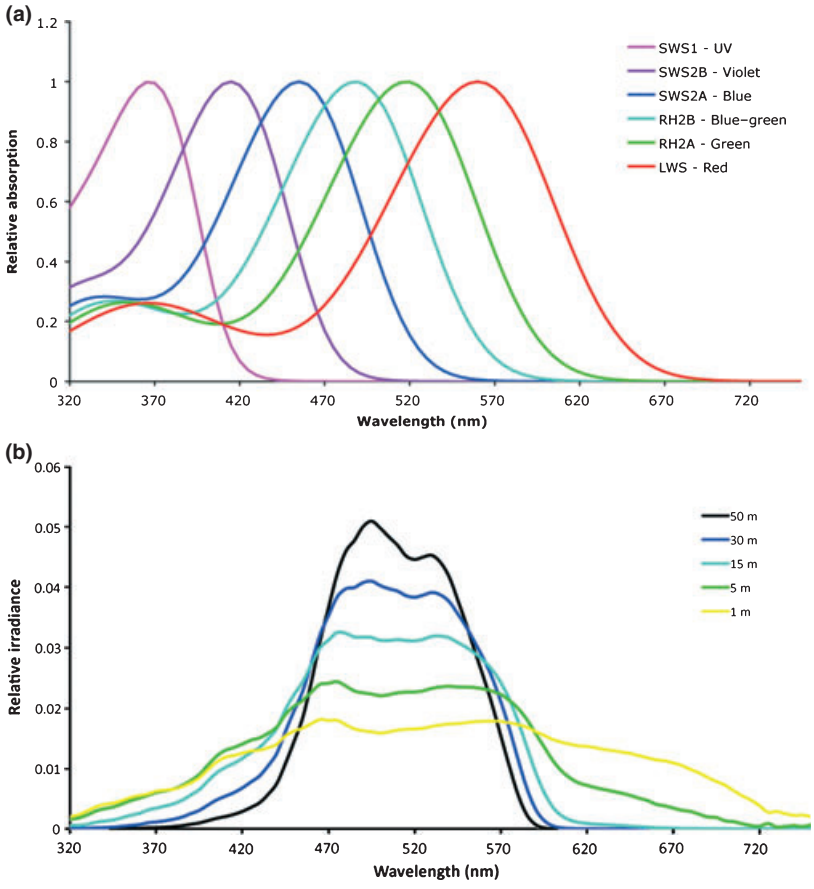

Fig. 1 Spectral absorbances of the cichlid cone opsins and the corresponding ambient light environment in Lake Malawi. (a) Idealized absorbance curves for the six-cone opsin genes investigated in this study. (b) Modelled spectral irradiance curves for 1, 5, 15, 30, and 50 m depth at Zimbawe Rock.

(F:ATTCTTGGATCCACCTTCTGTGCA, R:TACTCCAGCACCAGCATGAG) to compare RH2Aβ to aggregate RH2A expression. This seven-reaction template was run in parallel for each sample, and each sample was replicated at least twice on separate plates with separate reaction master mixes. The RT-qPCR reaction was performed using a Roche Lightcycler 480 and the critical cycle number for each gene was determined.

Reaction efficiencies for each primer were estimated using a plasmid construct containing a single copy of each opsin gene, although the RH2Aβ-specific primer could not anneal to the construct (Spady *et al.* 2006). Experiments using either RH2Aβ-specific or RH2A aggregate probes showed that PCR was equally efficient on a purified RH2Aβ substrate (data not shown). Therefore, we used the same efficiency for both RH2A genes. The efficiencies of all seven genes were used in conjunction with the critical cycle data from the Lightcycler to determine relative expression values for each gene. Replicate plate measures were averaged for each individual.

Statistical analyses

All analyses were performed on groups within each species (defined either by depth or by locale). Differences in gene expression were analysed using both univariate and multivariate methods. For univariate analyses, the mean relative expression of each gene was tested between groups (depth or location) using an ANOVA. Homogeneity of variances between groups for each gene was tested using the Brown–Forsythe test of unequal variances. The Brown–Forsythe test is robust to distributions that are skewed towards the tails (Brown & Forsythe 1974) and therefore was appropriate for our data. General patterns of gene expression and variance were plotted via a triplot method based on an open-source Excel spreadsheet (Tri-plot; Graham & Midgley 2000). By triangulating individual expression patterns for both short-wavelength pigments expressed in single cones (SWS1, SWS2B, SWS2A) or medium- and long-wavelength sensitive pigments expressed in double cones (RH2B, RH2A, LWS), it is possible to observe clustering patterns within the data. However, the triplot itself is simply a graphical technique and has no associated statistical *P*-value.

For multivariate comparisons, groups were compared using a discriminant function analysis (DFA). The DFA analysis tests for differences in multivariate means along principal component axes, allowing for the discrimination of groups using the multivariate characteristics that describe the largest fraction of data in the model. Our DFA model was constructed using all genes except for RH2Aα, which was excluded to alleviate linear dependence in the dataset. As RH2Aα was expressed in high levels in all individuals, it was considered as generally uninformative for the multivariate models.

To quantify the theoretical light collection of each cichlid visual pigment in the environment, quantum catches were calculated using in situ water spectral data from Zimbawe Rock (Hofmann *et al.* 2009), which is similar to that at Thumbi West or Otter Point (data not shown). Quantum catch for pigments was estimated for 1, 5, 15, 30 and 50 m using the following equation from Hofmann *et al.* (2009):

$$Q_{\mathrm{abs},i} = \int_{320}^{750} I(\lambda) T_w(\lambda, d) R(\lambda) \mathrm{d}\lambda \qquad (1a)$$

Here, $Q_{\mathrm{abs},i}$ is the absolute quantum catch for a particular photoreceptor visual pigment, $I(\lambda)$ is the incident solar irradiance at the surface, $T_w(\lambda,d)$ is the transmission of light of wavelength λ through water to a depth, d, and $R(\lambda)$ is the absorbance of the photoreceptor visual pigment based on the equations of Govardovskii *et al.* (2000). The visual pigment quantum catches were then normalized relative to each other by dividing by the sum of the total of all visual pigments to produce relative quantum catches:

$$Q_{\mathrm{rel},i} = \frac{Q_{\mathrm{abs},i}}{\sum Q_{\mathrm{absf},i}} \qquad (1b)$$

This corrected for total light intensity differences with depth so the relative efficiencies of each visual pigment could be compared.

To approximate the effects of variable gene expression on visual system sensitivity, we constructed a derived-model based on the Q_i functions. In so doing, we made two primary simplifying assumptions regarding the function of cone opsins in the retina: (i) relative gene expression is a proxy for cone cell number and therefore proportional to sensitivity; and (ii) cone mechanisms are additive and function as a luminance channel. While (i) is a relatively fixed assumption; (ii) may only hold for certain lighting conditions and visual tasks. However, this luminance-based additive model is the simplest model we can make without requiring knowledge of specific neural mechanisms. This enables us to estimate the total visual system sensitivity for a given combination of expressed opsins in a given ambient light environment. In performing this calculation, we weighted the quantum catch of a particular visual pigment by its relative gene expression (given as f_i or the fraction of the total opsin expression). Furthermore, we performed this weighting separately for the single cones (SC) and the double cones (DC) or combining all cones using the following equations:

$$\text{RT-S}_{SC} = \frac{f_{SWS1}Q_{SWS1} + f_{SWS2B}Q_{SWS2B} + f_{SWS2A}Q_{SWS2A}}{f_{SWS1} + f_{SWS2B} + f_{SWS2A}} \quad (2)$$

$$\text{RT-S}_{DC} = \frac{f_{RH2B}Q_{RH2B} + f_{RH2A}Q_{RH2A} + f_{LWS}Q_{LWS}}{f_{RH2B} + f_{RH2A} + f_{LWS}} \quad (3)$$

$$\text{RT-S}_{all} = f_{SWS1}Q_{SWS1} + f_{SWS2B}Q_{SWS2B} + f_{SWS2A}Q_{SWS2A} + f_{RH2B}Q_{RH2B} + f_{RH2A}Q_{RH2A} + f_{LWS}Q_{LWS} \quad (4)$$

Henceforth, we refer to the expression-scaled quantum catch as RT-S (Real-Time corrected Sensitivities). These calculations were repeated in *C. eucinostomus* and *T. gracilior* using ambient spectra from both 5 and 15 m to test visual sensitivities in each microhabitat, i.e. RT-S values were calculated for fish from each population (depth) for both light environments and the sensitivities were then compared within that depth. Absolute quantum catch models were analysed by ANOVA and the Brown–Forsythe test. In addition, weighted λ_{max} calculations were performed for comparison with the results from prior work (Hofmann *et al.* 2009) where the following equations were used:

$$\lambda_{max,SC} = \frac{f_{SWS1}\lambda_{SWS1} + f_{SWS2B}\lambda_{SWS2B} + f_{SWS2A}\lambda_{SWS2A}}{f_{SWS1} + f_{SWS2b} + f_{SWS2A}} \quad (5)$$

$$\lambda_{max,DC} = \frac{f_{RH2B}\lambda_{RH2B} + f_{RH2A}\lambda_{RH2A} + f_{LWS}\lambda_{LWS}}{f_{RH2B} + f_{RH2A} + f_{LWS}} \quad (6)$$

The λ_{max} calculation estimates the average visual pigment peak sensitivity weighted by gene expression, with each visual pigment represented solely by its wavelength of maximal absorption, This is simpler than the RT-S calculations, which weight quantum catch by gene expression. However, as quantum catch considers the full absorption profile of each opsin as well as the environmental light conditions, the RT-S model is more likely to be a good mathematical predictor of *in vivo* visual sensitivities.

In addition to calculating relative quantum catch and RT-S using downwelling irradiance in Lake Malawi, we calculated these same quantities using downwelling irradiance in Lake Victoria (Hofmann *et al.* 2009). The latter used spectra taken near Makobe Island, a relatively clear water site in Lake Victoria to calculate quantum catch and compare it with that at Zimbawe Rock, Malawi. In both cases:

$$I(\lambda)T_w(\lambda, d) = I_{surface}(\lambda)\exp(-\alpha_\lambda d) \quad (7)$$

where $I_{surface}(\lambda)$ was irradiance measured at the water's surface, which was taken at Zimbawe Rock, α_λ is the attenuation coefficient measured previously for these locations in each lake (Hofmann *et al.* 2009), and d is the depth. This enabled a comparison of the depth effects in Lake Malawi with our previous results, which showed that gene expression differences change significantly with depth in Lake Victoria.

Results

Variation in gene expression

This is the first study where we have attempted to distinguish RH2Aα and β in qPCR studies from wild caught samples. The Rh2Aβ gene was either absent or weakly expressed (<3% of total opsin expression) in all samples. Therefore, the measure obtained from the aggregate RH2A primer/probe pair was considered indicative of RH2Aα expression. This makes sense in comparison with previous protein expression studies where the RH2Aα gene was shown to have a longer wavelength sensitivity (528 nm) compared with that of RH2Aβ (518 nm) (Parry *et al.* 2005; Spady *et al.* 2006). RH2Aα gene expression is in agreement with MSP studies for Lake Malawi species where green cone peak absorbances were 525–535 nm (Jordan *et al.* 2006).

We found clear evidence that populations of the same species can differ in gene expression. Significant differences in gene expression were observed among populations in both *M. zebra* and *C. eucinostomus* (Fig. 2a,b), but not in *T gracilior* (Fig. 2c). Significant differences in the variability of gene expression within a population were found in both *M. zebra* and *T. gracilior*, but not in *C. eucinostomus* (Fig. 3). More specifically, *M. zebra* displayed differential levels of variation in short-wavelength sensitive pigments (Fig. 3a), whereas *T. gracilior* had significant variation differences for both single-cone (Fig. 3e) and double-cone pigments (Fig. 3f).

Significant mean differences were observed between the Thumbi West and Zimbawe Rock populations of *M. zebra*. Expression differences were found for three genes (Fig. 2a). Both SWS1 ($F = 5.629$, $P = 0.011$) and RH2Aα ($F = 7.575$, $P = 0.013$) were expressed in greater quantities in the Thumbi West population, although there was no difference in the degree of variation between populations ($P > 0.05$). The fish from Zimbawe Rock expressed more of the violet-sensitive SWS2B gene than the fish from Thumbi West (Welch's ANOVA, $F = 13.474$, $P = 0.001$), and there was a larger variance of expression in the Zimbawe Rock population ($F = 11.500$, $P < 0.001$). Multivariate models could discriminate the fish from Zimbawe and Thumbi West based on relative opsin expression (Wilks' $\lambda = 0.286$, $P = 0.001$).

For *C. eucinostomus*, relative gene expression was qualitatively different among the populations. The fish

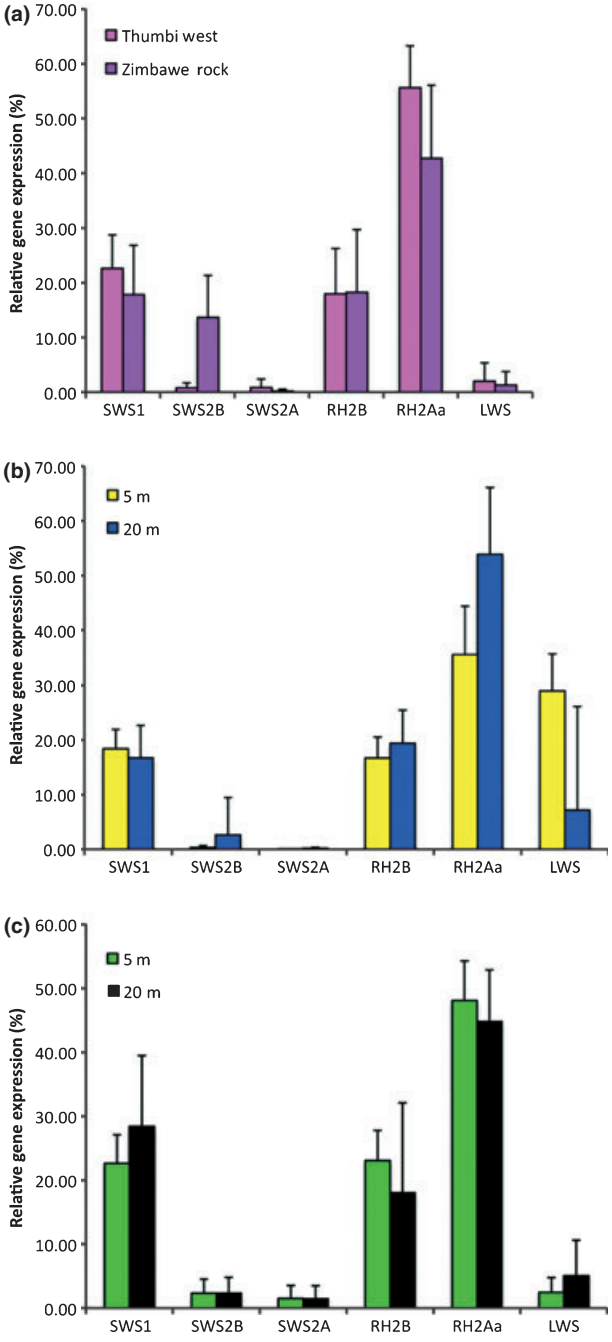

Fig. 2 Mean relative opsin expression patterns for populations of (a) *Metriaclima zebra*, (b) *Copadichromis eucinostomus*, and (c) *Tropheops gracilior*.

from 5 m depth expressed LWS, whereas fish from 15 m did not (Fig. 2b). Statistical differences were observed for both the green-sensitive RH2Aα ($F = 21.2$, $P < 0.001$; Fig. 2b) and the red-sensitive LWS ($F = 17.5$, $P < 0.001$; Fig. 2b) genes. Fish collected at 15 m expressed more RH2Aα, whereas the fish from 5 m expressed more LWS. The higher level of RH2Aα in fishes from 15 m was equal to the sum of RH2Aα and LWS in the shallow fish. For this reason, a multivariate model based on the expression of this pair of double-cone genes easily differentiates fish from the two depths (Wilks' $\lambda = 0.098$, $P < 0.001$). The qualitative LWS shift in *C. eucinostomus* was similar to the SWS2B effect in *M. zebra* as the *C. eucinostumus* at depth did not express LWS, whereas those at the surface did (Fig. 3c,d).

No depth-specific differences were found for cone opsin gene expression in *T. gracilior* ($P > 0.05$; Fig. 2c), and the multivariate model could not discriminate the two depth classes based on gene expression (Wilks' $\lambda = 0.705$, $P = 0.0517$). However, differences in the variance of gene expression were found for the UV-sensitive SWS1 gene ($F = 10.45$, $P = 0.003$), the blue-green-sensitive RH2B gene ($F = 23.47$, $P < 0.001$) and the LWS gene ($F = 6.285$, $P = 0.017$; Fig. 3e,f). Expression of all three genes had higher variance for fish collected at 20 m than for the fish collected at 5 m.

Variation in quantum catch

Using light transmission spectra collected from downwelling light transmitted through the water column to calculate relative quantum catch for the different visual pigments (eqn 1b), we found three distinct trends for changes in pigment quantum catch over the 1–15 m range in which fish were collected for this study: (i) the SWS1 and SWS2B pigments became less efficient with depth; (ii) the RH2B and RH2Aα became more efficient; and (iii) the SWS2a and LWS pigments quantum catch had no change (Fig. 4). However, these changes in quantum catch were much smaller than what we would have predicted. We extended our depth series to 50 m to estimate changes in efficiency at the suspected extremes of the distributions in *M. zebra* and *T. gracilior* (Ribbink *et al.* 1983; data not available for *C. eucinostomus*). Even over a depth range from 1 to 50 m, the change in the relative efficiency of different pigments for four of the six cone opsins was proportionately small with the exception of SWS1 and SWS2B (Fig. 4). These results are further supported by calculations of the absolute quantum catch for each pigment (eqn 1a), as the attenuation rate of cone catches with depth was similar for all pigments with the exceptions of SWS1 and SWS2b (Fig. S2, Supporting information). This result is particularly surprising for the LWS pigment, as its absorbance curve overlaps a primary region of spectral attenuation with depth (Fig. 1).

Significant differences were found for gene expression weighted quantum catch, RT-S_{SC} in *Metriaclima zebra* from Thumbi West and Zimbawe Rock (Fig. 5a). The additional SWS2B opsin expression increased both the RT-S and the variance of expression in the short

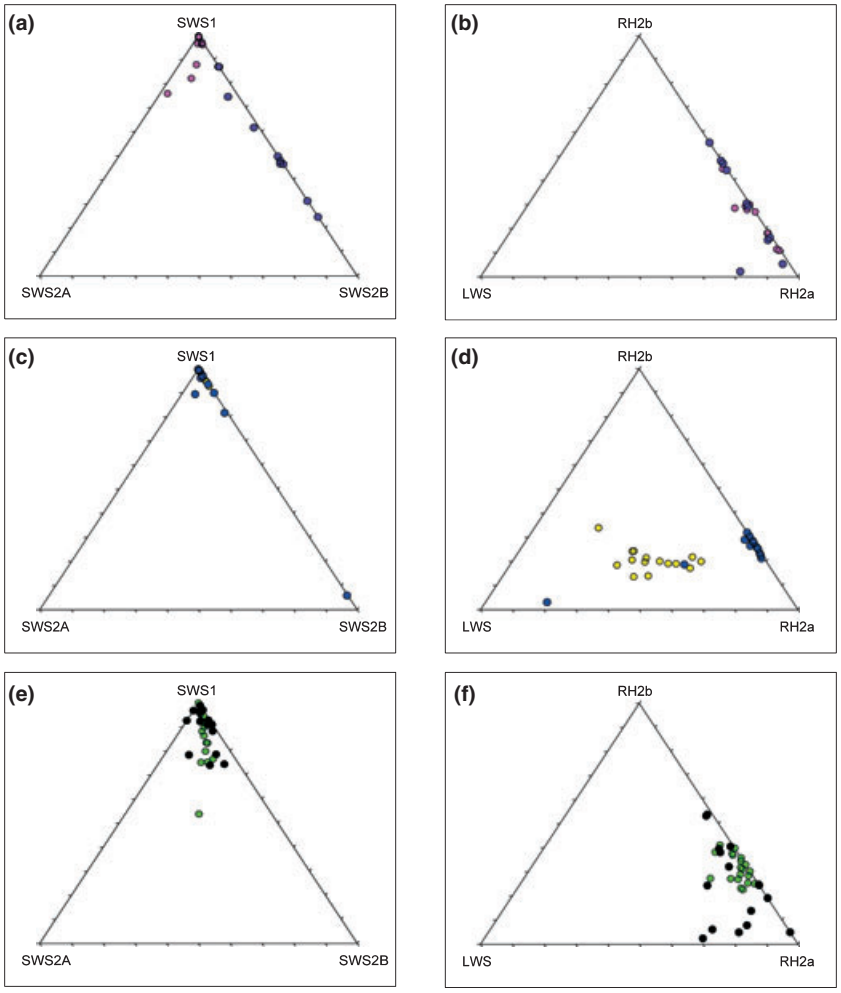

Fig. 3 Gene expression triplots for all individuals included in this study. (a) Single-cone and (b) double-cone pigments for *Metriaclima zebra* (pink = Thumbi West, violet = Zimbawe Rock). (c) Single-cone and (d) double-cone pigments for *Copadichromis eucinostomus* (yellow = 5 m, blue = 15 m). (e) Single-cone and (f) double-cone pigments for *Tropheops gracilior* (green = 5 m, black = 20 m).

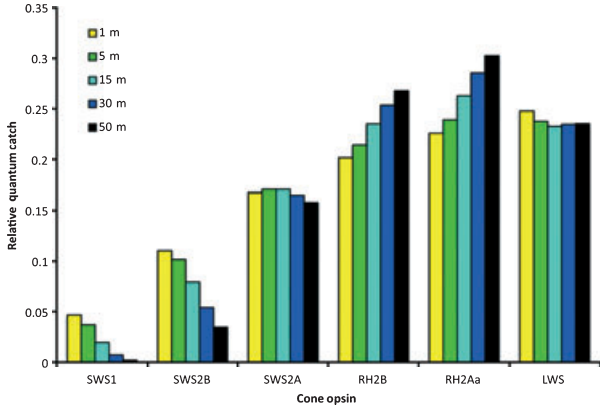

Fig. 4 Relative quantum catch of the cichlid visual pigments at a series of five depths at Zimbawe Rock. The sum of gene expression for single-cone genes (SWS1, SWS2B and SWS2A) and for double-cone genes (RH2B, RH2A and LWS) are normalized to one.

wavelength sensitive single cones in the individuals from Zimbawe Rock in comparison with those at Thumbi West (Welch's ANOVA: $F = 19.566$, $P = 0.001$). This increase in short-wavelength sensitivity is accompanied by a decrease in both double cone RT-S_{DC} ($F = 19.768$, $P < 0.001$) and total RT-S_{all} ($F = 14.088$, $P = 0.001$) when compared with *M. zebra* from Thumbi West.

We calculated RT-S for *C. eucinostomus* and *T. gracilior* using light spectra measured for water depths of 5 and 15 m, corresponding to the depths where the populations were sampled. Statistical tests found no difference between depths for single cones (RT-S_{SC}), double cones (RT-S_{DC}), or the sum of those mechanisms (RT-S_{all}) for either species ($P > 0.05$; Fig. 5b,c). While there was no variation between depths for the mean, there was a significant difference in the spread of the data between depths for *T. gracilior*, as individuals from 20 m had significantly more variation in their double cone (5 m: $F = 5.817$, $P = 0.024$; 15 m: $F = 6.470$ $P = 0.016$) and total (5 m: $F = 8.175$, $P = 0.007$; 20 m: $F = 7.709$, $P = 0.001$) sensitivity. This is in accordance with the increased variance in the expression of RH2B and LWS in these fish.

We calculated relative quantum catch (eq 1b) for cichlid opsins in both Lake Malawi and Lake Victoria. The

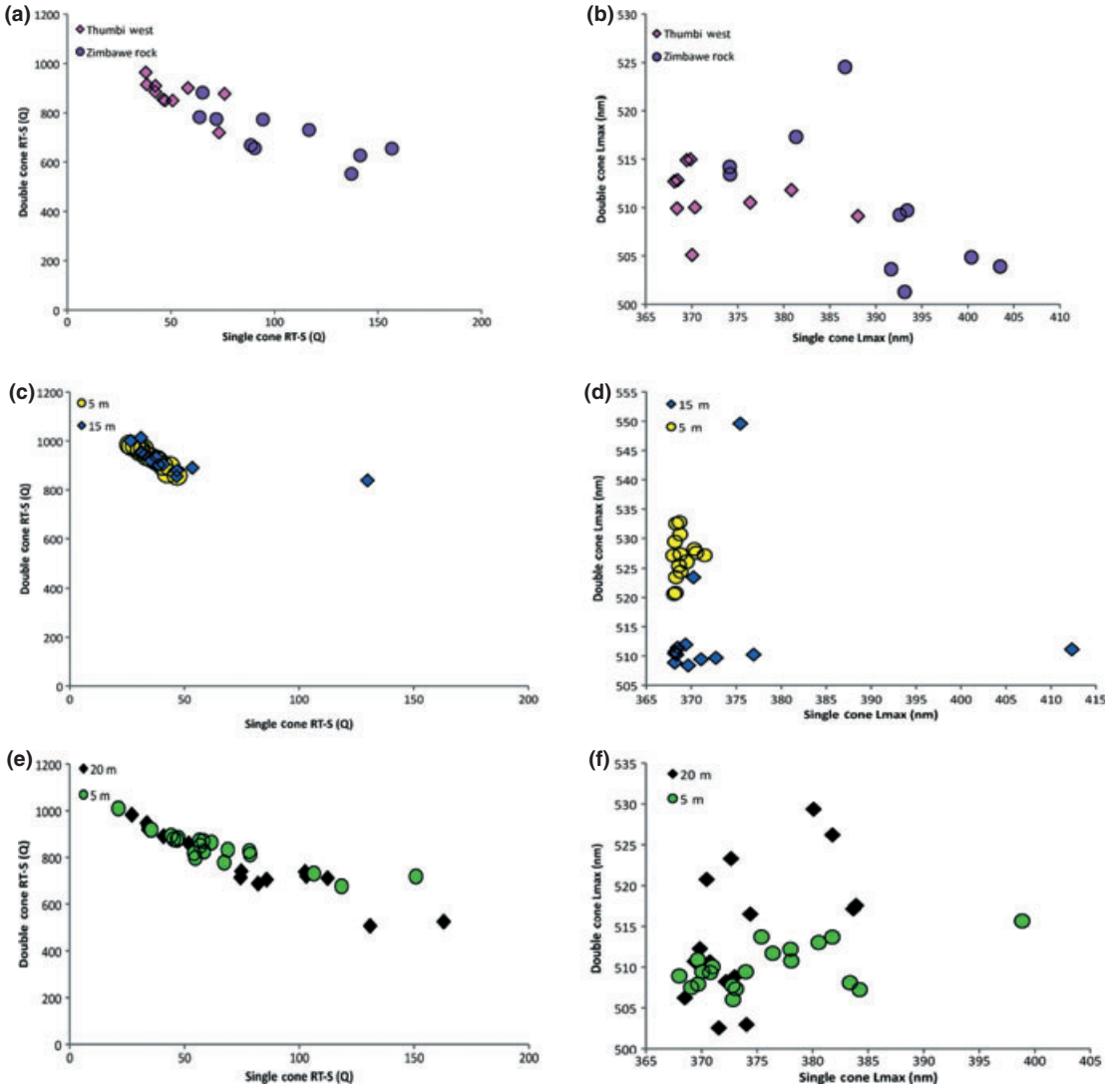

Fig. 5 Comparison of the expression-weighted RT-S models at 5 m depth applied in this study to the predicted single- or double-cone maximal absorbances calculated in previous studies (Hofmann *et al*. 2009). (a) RT-S and (b) predicted λ_{max} calculations for *Metriaclima zebra*. (c) RT-S and (d) predicted λ_{max} calculations for *Copadichromis eucinostomus*. (e) RT-S and (f) predicted λ_{max} calculation for *Tropheops gracilior*.

variation in relative quantum catch as a function of depth is shown in Fig. 6a for several of the opsins. In Lake Victoria, the relative quantum catch of the LWS pigment increased rapidly with depth, whereas the SWS1 and SWS2B pigment quantum catch neared zero at depths as shallow as 5 m. In contrast, the clear waters of Lake Malawi allowed for relatively invariant pigment quantum catches to a depth of 10 m and beyond (Figs 4 and 6a). Therefore, there were no real changes in quantum catch as cichlids moved to deeper depths in Malawi.

The changes in gene expression in *C. eucinostomus* caused very little difference in RT-S for shallow versus deep locations in Lake Malawi (Fig. 5c). However, there were significant differences in depth-dependent relative quantum catch between the two lakes. In light of these large differences, we wanted to see if this variation would cause significant differences in RT-S at different depths in Lake Victoria. These calculations did indeed find large differences in the double cone RT-S_{DC} and total RT-S_{all} for *C. eucinostomus* based on light environment from shallow (5 m) vs deep (15 m) locations (Fig. 6b).

Discussion

If visual sensitivities are important in mate choice and ultimately speciation, then we should be able to detect

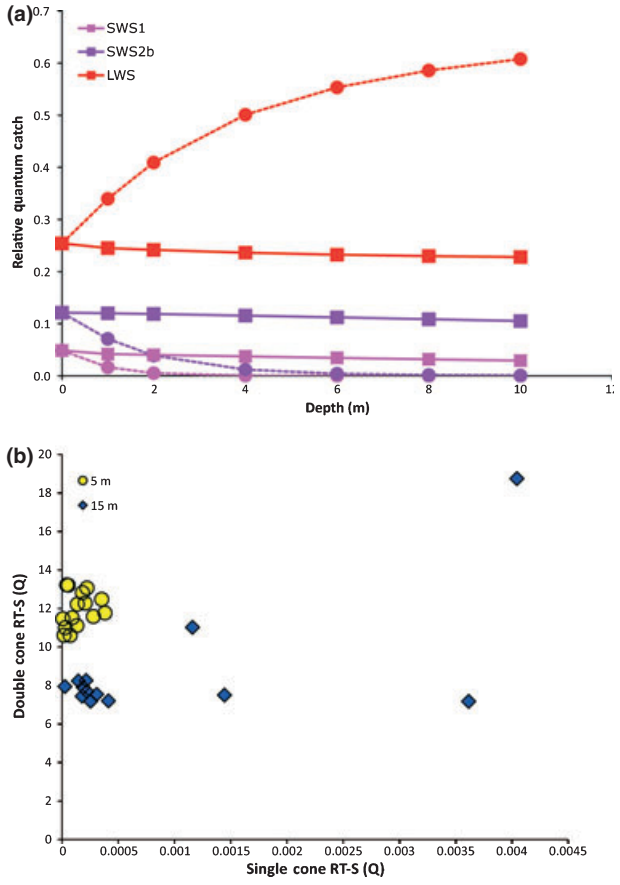

Fig. 6 Comparison of the biological effects of light gradients in Lake Malawi and Lake Victoria. (a) Variation in relative quantum catch for the SWS1, SWS2B, and LWS pigments from 1 to 10 m depth in Lake Malawi (squares with solid lines) and Lake Victoria (circles with dashed lines). Quantum catches are normalized to the full six-cone template as in Fig. 2, but SWS2A, RH2B, and RH2Aα have been excluded for clarity. (b) Comparison of RT-S models for *Copadichromis eucinostomus* from 5 m (yellow) and 15 m (blue) under the ambient light environment observed at 10 m depth in Lake Victoria. This should be compared to panel 4C for the equivalent comparisons in the light environment of Lake Malawi.

differences among populations and species. It has recently been shown that differences do occur among species (Hofmann *et al.* 2009). However, this work is the first demonstration that variation in opsin gene expression can occur within species on small spatial scales. We found pronounced qualitative gene expression differences in both *Copadichromis eucinostomus* and *Metriaclima zebra*, whereas quantitative variation was present in *Tropheops gracilior*. The *T. gracilior* and *C. eucinostomus* depth samples are in such close geographical proximity (~100 m distance along the lake bottom) that they would typically be considered fully sympatric. As such, the degree of gene expression variation in Malawian cichlids may be of a much greater magnitude than we once thought.

In previous work, variation was examined in Lake Victoria and a link was found between gene expression and light environments (Hofmann *et al.* 2009). Fishes from locations with red-shifted ambient light environments expressed higher amounts of the medium- and long-wavelength sensitive RH2A and LWS genes. Large variation in SWS2 gene expression also occurred with changes in the SWS2B/SWS2A ratio that correlated with quantum catch of the single cones as it varied over four orders of magnitude. Short-wavelength attenuation is so great in some locales that it renders single-cone photoreceptors essentially inactive, and this is reflected in the overall gene expression patterns (Hofmann *et al.* 2009).

On the basis of these previous results in Lake Victoria, we expected the differences we observed at local spatial scales in Lake Malawi to be linked to differences in local light environments. We began with three hypotheses that follow logically from the hypothesis that visual systems should be tuned to the local environment. We expected (i) greater variance in gene expression in fishes from shallow water, where the light spectrum is broader; (ii) differences in opsin gene expression with depth as a result of differences in light stimulation of cone pigments; and (iii) no differences in gene expression among the two *M. zebra* populations as the populations occur at similar depths in locales with similar light environments. However, the gene expression patterns observed in this study are inconsistent with each of the three hypotheses we put forth.

Instead of observing increased variation in shallow water fishes, where the light spectrum is broader, we found evidence that gene expression is more variable in deep-dwelling individuals of *Tropheops gracilior*. Our initial hypothesis regarding the variance of gene expression was predicated on the idea that selection on visual systems at depth would be stronger due to spectral attenuation. More specifically, we expected that genes outside the primary three-cone colour palette would not be observed due to a penalty incurred by a decrease in quantum catch. Calculations of visual efficiency between the shallow *T. gracilior* and their relatively variable deep counterparts indicate that variable gene expression does not cause a decrease in sensitivity due to the high transmission of light in Lake Malawi (Fig. 5e).

Perhaps more surprisingly than the nature of expression variance with depth is the observation that the presence of LWS in shallow *C. eucinostomus* would not cause a decrease in the predicted visual sensitivity of these fish if they were transplanted in deeper waters (Fig. 5c). The light attenuation with depth in Malawi is such that <10% of the total LWS absorbance spectrum

falls outside the ambient irradiance at 15 m. When combined with the 15–30% expression of LWS in the retina, this leads to a rough sensitivity decrease of <3% at depth. In the context of the natural range of RT-S variation between individuals, this decrease is statistically negligible. In other words, these data indicate that there is very little difference in the sensitivity of photoreceptors expressing LWS and RH2 opsins in Lake Malawi associated with depth (Fig. 4) and therefore the presence or absence of LWS in *Copadichromis eucinostomus* does not confer a sensitivity advantage to either group. Conversely, if these same *C. eucinostomus* were introduced into Lake Victoria where a pronounced sensitivity advantage is associated with the LWS pigment, we would expect the shallow individuals to display higher visual sensitivities. Our RT-S model supports this hypothesis, as the addition of the LWS pigment in the shallow fishes increases double-cone sensitivity relative to their deeper counterparts (Fig. 6b). In other words, while gene expression variation associated with total spectral sensitivity is apparent due to the steep light gradients in Lake Victoria, the relatively invariant light environment in Lake Malawi makes our initial sensitivity hypotheses less plausible (Fig. 5c).

We hypothesized that there would be no expression difference between the *Metriaclima zebra* populations at Thumbi West Island and Zimbawe Rock given the similar ambient light environments in their respective habitats. On the contrary, the Thumbi West population expressed a typical short-wavelength sensitive palette (SWS1, RH2B, RH2A), whereas the Zimbawe Rock population expressed a modified tetrachromatic palette (SWS1, SWS2B, RH2B, RH2A). Perhaps most surprisingly, the *M. zebra* populations represented the only system we observed in which our RT-S models were able to predict a specific difference in chromatic sensitivities between the two groups. The addition of the SWS2B pigment in the Zimbawe Rock population augments the total single-cone catch in these fish. However, given the similar ecology of these two populations, we are currently unable to tie this sensitivity difference to any known environmental or behavioural correlate.

Comparisons of gene expression in both *C. eucinostomus* and *M. zebra* from two locales showed a change from expression of three visual pigments to four. This could have profound implications for cichlid visual perception. Both represent modifications of the classic short trichromatic UV, blue-green, and green template observed in many of the mbuna (Hofmann *et al.* 2009). In *C. eucinostomus*, LWS is added to the double-cone complement, creating a three pigment double-cone system. This extends the theoretical sensitivity of the *C. eucinostomus* visual system further into the red. Conversely, the addition of SWS2B to create a single-cone complement with two distinct pigments in *M. zebra* would not broaden the theoretical total spectral range of visual sensitivity. Rather, it broadens single-cone sensitivities in the direction of the double cones, effectively augmenting sensitivity in intermediate spectral ranges. However, more important than the broadening of spectral sensitivities is the potential for contrast (opponent) mechanisms that arise from the addition of these pigments. If these pigments are wired opponently, they may create a tetrachromatic visual system from the original trichromatic one. Tetrachromatic opponency was recently demonstrated by Lisney *et al.* (2010) through electrophysiological recordings in the tilapia *Oreochromis niloticus*. They found three potential opponent mechanisms based on four-cone pigments present in this cichlid retina, including an opponent interaction between the SWS2B and SWS2A pigments.

Two primary facets of the expression data described here suggest that ongoing selection for divergent visual systems within species is plausible in Malawian cichlids: (i) the highly variable opsin expression in the deep *T. gracilior* indicates that there is sufficient sensory variation available for selection to act upon; and (ii) the localized tetrachromatic visual systems in the other two species demonstrate that there is profound sensory diversification within the lake. These results are consistent with both sensory drive and Fisherian selection and drift. However, we are not currently able to tie these data to any known environmental or behavioural variables that would exert definitive selection. A previous work has demonstrated a correlation between feeding mode and SWS1 expression in Lake Malawi cichlids (Hofmann *et al.* 2009). The observation of SWS2B expression in *M. zebra* from Zimbawe Rock is actually contrary to this idea. Strong currents at Zimbawe Rock prevent food items from settling out of the water column as they do at Thumbi West (Kocher, personal communication). Therefore, *M. zebra* from Zimbawe should be more planktivorous than their counterparts at Thumbi, and we would expect selection to drive a SWS1-dominated visual system at this site (Hofmann *et al.* 2009). However, *M. zebra* at this site have more SWS2B, and hence less SWS1 expression than their counterparts at Thumbi. As such, evidence for the sensory drive hypothesis remains weak in the Malawi cichlids. Therefore, Fisherian mechanisms or neutral drift may be the more plausible explanations for phenotypic divergence in Lake Malawi.

The tetrachromatic visual systems in particular support the Fisherian runaway or neutral drift hypotheses. The addition of a fourth major pigment to the retinal mosaic is a considerable change to the visual system as a whole. Such a dramatic shift in expression patterns suggests that stabilizing selection on the trichromatic

short palette is fairly relaxed, and relaxed selection may result in diversification through random drift. The derivation of divergent phenotypes that result from drift without any explicit environmental consequences is amenable to the development of subsequent Fisherian selection. Therefore, we posit that in the clear waters of Lake Malawi, intraspecific visual diversification largely functions outside sensory drive and results from less rigid selective processes. To test this hypothesis, we hope to identify the loci controlling opsin gene expression (Carleton *et al*. 2010) so that we can then test these loci for evidence of selection or drift.

This study demonstrates that cone opsin gene expression in the cichlids of Lake Malawi is much more complex than previously thought. The magnitude of expression variation differs in fishes occupying different local habitats. Also, distinct qualitative differences in gene expression are present in some species. Taken together, these observations offer the possibility that Fisherian sexual selection may be linked to colour vision in Malawian cichlids. Furthermore, the observation of two distinct tetrachromatic visual systems in different species presents a novel view of how visual systems adapt. Rather than switching from one trichromatic visual palette to another, visual tuning may occur by adding expressed genes to the current visual palette. This system has profound implications for both ecology and neuroscience, as the logistics of collecting and processing tetrachromatic information can be quite different from those for a trichromatic system (Zana *et al*. 2001). As such, visual divergence in Malawian cichlids may function through genetic and neural mechanisms that remain to be found.

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ARS is primarily interested in the evolution of sensory systems and the interplay of genetic, neural, and behavioural mechanisms. LD'A and AS are both pursuing professions in the medical field. AES is interested in reproductive biology and molecular mechanisms of fertility. CMH performs research on the evolution of communication traits and the reconstruction of ancestral states. NJM studies the visual ecology of various organisms and the interaction between environment and visual sensitivities. KLC investigates the molecular mechanisms that control colour vision in the cichlids of Lake Malawi and the selective forces that act on these traits.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Raw opsin gene expression measures for all individuals used in this study

Table S2 Population means and variances for the fishes used in this study

Fig. S1 Map of sampling sites

Fig. S2 Absolute quantum catch of cone opsin pigments with depth in Lake Malawi

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