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Colour vision and speciation in Lake Victoria cichlids of the genus *Pundamilia*

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Abstract

Lake Victoria cichlids are one of the most speciose groups of vertebrates. Selection on coloration is likely playing an important role in their rapid speciation. To test the hypothesis that sensory biases could explain species differences in mating preferences and nuptial coloration, we studied seven populations of four closely related species of the genus *Pundamilia* that differ in visual environment and male nuptial colour. Microspectrophotometry determined that the wavelength of maximum absorption (λ_{max}) of the rod pigment and three cone pigments were similar in all four species. Only the long wavelength sensitive (LWS) pigment varied among species, with 3–4 nm shifts in λ_{max} that correlated with differences in the LWS opsin sequence. These subtle shifts in λ_{max} coincided with large shifts in male body colour, with red species having longer LWS pigments than blue species. Furthermore, we observed within and between species a correlation between water transparency and the proportion of red/red vs. red/green double cones. Individuals from turbid water had more red/red double cones than individuals from clear water. The variation in LWS λ_{max} and in the proportion of red/red double cones could lead to differences in perceived brightness that may explain the evolution of variation in male coloration. However, other factors, such as chromophore shifts and higher order neural processing, should also be investigated to fully understand the physiological basis of differential responses to male mating hues in cichlid fish.

Keywords: cichlid fish, microspectrophotometry, opsin gene, speciation, visual sensitivity

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Introduction

Cichlid fishes of the East African lakes are well known for their rapid rates of speciation (Fryer & Iles 1972; Owen *et al*. 1990; Johnson *et al*. 1996). Each of the African Great Lakes, including Malawi, Tanganyika and Victoria, harbours hundreds of endemic species (Genner *et al*. 2004). The most rapid rates of speciation have occurred in Lake Victoria, where 500 species of haplochromines have radiated in the last 15 000–250 000 years (Johnson *et al*. 1996; Nagl *et al*. 2000; Seehausen 2002; Seehausen *et al*. 2003; Verheyen *et al*. 2003). The most closely related species usually differ strikingly in male nuptial colour pattern, suggesting an important role for sexual selection in population divergence and speciation (Seehausen 1997, 2000; Seehausen & Schluter 2004).

In the haplochromine cichlids, vision can be key to females choosing conspecific mates among closely related species (Seehausen & van Alphen 1998; Jordan *et al*. 2003). Nuptial coloration has also been demonstrated to be important in intraspecific female (Seehausen *et al*. 1999; Maan *et al*. 2004) and male (Knight & Turner 1999; Seehausen *et al*. 1999) mate choice. Variation in nuptial coloration affects aggressive dominance among male cichlids (Evans & Norris 1996; Dijkstra *et al*. 2005), and is an important determinant of species coexistence (Seehausen & Schluter 2004). Vision is therefore a key sense in cichlid inter- and intraspecific communication.

Several theories have been developed to explore how the evolution of sensory systems affects the evolution and diversification of intraspecific communication. Sensory

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biases in mate choice (Endler 1992; Ryan & Keddy-Hector 1992; Ryan 1998) may arise as a result of adaptation of the sensory system to some mating-independent function such as foraging (Rodd *et al*. 2002; Smith *et al*. 2004). Visual biases in aquatic systems emerge from adaptation of the visual system to water spectral transmission due to variation in water colour, turbidity, or depth. This may drive the evolution of male mating signals, since the latter will be selected to optimally stimulate the sensory system. This implies a potential association between the photic environment, the visual system and male nuptial coloration, as has indeed been observed in fishes (Boughman 2001; Fuller 2002; Fuller *et al*. 2003; Fuller & Travis 2004).

The primary determiner of visual sensitivity is the absorption of light by the visual pigments in retinal photoreceptors. Visual pigments are comprised of a chromophore, bound to and surrounded by an opsin protein. In fishes, the chromophore can be derived from either vitamin A_1 (retinal) or vitamin A_2 (3,4 didehydroretinal) (Bowmaker 1995). There are five major classes of opsin genes in vertebrates, the rod opsin gene (RH1) and four classes of cone opsins: a medium- to long-wavelength (M/LWS) class maximally sensitive at 500–570 nm, a rhodopsin-like (RH2) class sensitive at 470–540 nm, a short-wavelength (SWS2) class sensitive at 400–470 nm and an SWS1 class sensitive at 360–450 nm (Yokoyama 2000; Ebrey & Koutalos 2001). Tuning of the visual system typically involves one of several mechanisms. (i) Opsin genes can be turned on or off to change the opsin protein present in a particular photoreceptor cell (for a review see Bowmaker 1995). (ii) The chromophore used can be shifted between A_1 and A_2. For a given opsin protein, A_2 visual pigments (porphyropsins) absorb at longer wavelengths than A_1 pigments (rhodopsins) (Bridges 1972; Harosi 1994; Parry & Bowmaker 2000). (iii) The amino acid sequence of a given opsin gene can change. Changes in the polarity of key amino acids directed into the retinal binding pocket cause shifts of a few nm up to tens of nm (Merbs & Nathans 1992; Asenjo *et al*. 1994; Yokoyama 2000; Takahashi & Ebrey 2003; Hunt *et al*. 2004).

The haplochromine cichlids of Lake Malawi as well as the related riverine cichlid, *Oreochromis niloticus*, have five classes of cone opsin genes (SWS1, SWS2a, SWS2b, RH2 and LWS) and a rod opsin gene (RH1). Typically, only three of the cone opsin genes are expressed at one time. Changes in opsin gene expression can cause large shifts in visual pigment sensitivities (30–80 nm) among species (Carleton *et al*. 2000, 2005; Carleton & Kocher 2001).

In the haplochromine cichlids of Lake Victoria, sequences of the rod opsin show evidence for positive selection (Sugawara *et al*. 2002, 2005). There is also evidence for positive selection of the LWS cone opsin gene, but little variation in the SWS2b gene (Terai *et al*. 2002). Interestingly, the LWS opsin variation is the largest of any gene studied to date in the Victorian species flock, and larger than in the genetically more diverse Lake Malawi species flock. A number of the variable sites are directed into the retinal binding pocket and involve changes in amino acid polarity. These differences may cause shifts in LWS absorption. If rapid evolution of LWS sensitivities is contributing to the rapid emergence of reproductive isolation through its effects on perception of male nuptial colours in these cichlids, we would expect to find that closely related species have fixed alternative LWS alleles, and that differences in their sensitivities correlate with differences in nuptial coloration.

Here, we examine the visual systems of seven populations of four closely related species of the genus *Pundamilia*, which vary both in male nuptial colour and in photic environment to explore the following questions. Are there consistent differences in visual sensitivity between closely related species and/or conspecific populations? Are differences the result of opsin sequence tuning or gene expression differences? Is the LWS opsin gene unique in its diversity? How is between- and within-species variation related to photic environment, foraging behaviours and male nuptial colour? We first used microspectrophotometry (MSP) to compare visual pigment absorptions between sister taxa. Second, we sequenced the six known opsin genes to compare their variability. Third, we quantified gene expression to determine which opsin genes were expressed. Finally, we looked for evidence of covariation between visual sensitivities, gene expression, photic environment, foraging styles, and breeding colour.

Materials and methods

Cichlid species studied

Seven populations belonging to four closely related Lake Victoria cichlid species were chosen: *Pundamilia pundamilia* from shallow, clear water at Makobe Island and from shallow, turbid water at Python Island; *Pundamilia nyererei* from the same two islands but deeper water; *Pundamilia* 'luanso', a natural hybrid population between these two species from relatively shallow, highly turbid water at Luanso Island; *Pundamilia azurea* from deep, clear water at Ruti Island; and *Pundamilia* 'red head' from shallow, relatively clear waters at Zue Island. All collecting localities are along the rocky shores of the Mwanza and Speke gulfs and occur along a gradient in water turbidity (Seehausen 1997; Seehausen *et al*. 1997).

The *Pundamilia* species differ conspicuously in male mating coloration whereas geographical populations of the same species possess the same colour pattern. Males of *P. pundamilia* and *P. azurea* have primarily blue bodies in nuptial coloration, while *P. nyererei* and *P.* 'red head' have conspicuously yellow and red bodies. *P.* 'luanso' males vary from the blue coloration of *P. pundamilia* to yellowish or yellowish with small amounts of red (Seehausen 1997).

With the exception of those from Makobe Island, which were wild caught but kept in the laboratory for > 6 months, individuals used in this study were laboratory bred from initial wild-caught stocks. They were maintained at the University of Hull in 120- to 260-L tanks under 12/12 h light/dark cycles. Individuals for MSP were transported to the Institute of Ophthalmology, London, where they were kept for several weeks under an 8/16 h light/dark regime prior to sampling. Between one and four individuals were sampled for each population with two populations examined for *P. nyererei* and *P. pundamilia*, including both sexes for all species. Two to three individuals from each species examined by MSP were then used for sequencing the expressed genes from genomic DNA. In addition, one individual from each species and the hybrid population at Luanso Island were used to sequence all six opsin genes from genomic DNA. Finally, two males of *P. pundamilia*, one of *P. nyererei* from Python Island, and a pooled sample of three 14-day-old laboratory-bred F_1 hybrid juveniles were used to quantify opsin gene mRNA in the retina.

Microspectrophotometry

Fish were dark-adapted overnight, then sacrificed by cervical dislocation. All procedures were carried out under dim red light. Eyes were enucleated, hemisected and the anterior portion discarded. The retina was then separated from the pigment epithelium and either analysed immediately or lightly fixed in 2% glutaraldehyde for later analysis. Retinal samples were teased apart on a coverslip with razor blades and the dispersed tissue mounted in saline containing 5% or 10% dextran, then squashed with a second coverslip, which was sealed with wax. At least two pieces of retina were analysed from different regions of each eye, in an attempt to overcome any regional distribution of different classes of cone.

Microspectrophotometric recordings were made in the conventional manner using a Liebman dual-beam microspectrophotometer (Liebman & Entine 1964; Mollon *et al*. 1984; Bowmaker *et al*. 1991). Spectra were recorded at 2-nm intervals from 750 to 350 nm and from 351 to 749 nm on the return scan and then overlayed. This assures that baseline drifts have not occurred during the measurement. Two baseline scans were measured in an unoccupied area close to the cell, averaged, and subtracted from the intracellular recording to obtain the final spectrum. All cells were then fully bleached with white light and post bleach spectra recorded. The λ_{max} of both the absorbance spectra and difference spectra were determined by a standard computer program that best fits a visual pigment template to the right hand limb of the spectra (Mollon et al. 1984; Bowmaker *et al*. 1991). Records with low absorbance or that were clearly distorted were discarded. In almost all cases the spectra were best fitted with a pure rhodopsin, vitamin A_1–based template. However, in the case of the two *P. nyererei* from Python Island, the spectra of the two longer wavelength sensitive pigments were broader than a pure A_1 template so a mixed rhodopsin/porphyropsin template was used. Both identical and nonidentical double cones were found: identical doubles with a long-wave pigment were noted as R/R, nonidentical doubles with both a long- and middle-wave pigment as R/G.

Opsin mRNA expression

Real-time reverse transcription–polymerase chain reaction (RT–PCR) was used to determine which cone opsin genes were expressed (Carleton & Kocher 2001). Fish were sacrificed at University of Hull. Retina from both eyes were dissected, stored in RNAlater (Ambion), and sent to University of New Hampshire. For the juveniles, whole eyes from three individuals were combined. Total RNA was isolated using Trizol (Invitrogen) and quantified. One microgram of total RNA was reverse transcribed using a polyT primer and Superscript III (Invitrogen) at 42 °C to create a retinal cDNA mixture. Parallel real-time RT–PCRs were set up for each of the five cone opsin genes using the TaqMan chemistry (Applied Biosystems) with gene specific primers and probes (Carleton & Kocher 2001). Fluorescence was monitored with a GeneAmp 5700 (Applied Biosystems) over 40 cycles (95 °C, 15 s; 55 °C, 30 s; 65 °C, 1 min).

The critical threshold cycle numbers were quantified for each of the five cone opsin genes for thresholds set just above background (0.05). Relative cone opsin gene expression was determined as a fraction of the total cone opsin gene expression from:

$$\frac{T_i}{T_{\text{all}}} = \frac{(1 + E_i)^{-Ct_i}}{\sum (1 + E_i)^{-Ct_i}}$$

where T_i/T_{all} is the relative gene expression for a given gene normalized by the total measured cone opsin gene expression, E_i is the PCR efficiency for each gene, and Ct_i is the critical cycle number for each gene. PCR efficiencies were determined from a construct containing one fragment of each of the five cone opsin genes ligated together (Spady & Carleton, unpublished). Five separate real-time PCRs derived from two separate RT reactions were then averaged together for each gene expression measurement.

Opsin sequences

The sequencing strategy was similar to that used previously for other cichlid opsin studies (Carleton *et al*. 2000; Carleton & Kocher 2001). The opsin genes were amplified as either one (RH1, SWS1 and RH2a), two (SWS2a) or three (SWS2b and LWS) fragments, depending on the size of the introns. These fragments were then sequenced and used

to assemble the entire gene sequence. Previous long PCR experiments have confirmed that this procedure produces contiguous sequences in the genome (Carleton & Kocher 2001).

Following MSP analyses of each of the *Pundamilia* populations, fin clips from two individuals for each of five populations were used to extract genomic DNA. The expressed opsin genes (RH1, SWS2a, RH2, and LWS) were then sequenced to provide a direct link with MSP λ_{max} in those individuals. Because the first intron in the LWS gene is 1000 bp, only exons 2 through 6 (314 of 357 amino acids) were sequenced from the MSP individuals. The other genes were sequenced completely as described above. Finally, the LWS gene was sequenced from one individual for *P. pundamilia* and *P. nyererei* from Makobe Island.

Sequence analysis

Amino acid sequences were inferred from genomic sequences according to the known exonic structure of cichlid opsins. To identify amino acid sites of potential functional significance, the opsin sequences were mapped onto the bovine rhodopsin crystal structure (Palczewski *et al*. 2000).

Analysis of variance

To test for covariance between λ_{max} and the proportion of R/R and R/G double cones and water transparency, water depth, feeding style and nuptial coloration, analysis of variance (ANOVA) and analysis of covariance (ANCOVA) were conducted with feeding style, colour, and species as factors, and transparency and depth as covariables. These analyses examined either the combined M/LWS λ_{max} [(MWS λ_{max} + LWS λ_{max})/2], or LWS λ_{max} alone. Water transparency was measured at every island with a 30-cm Secchi disk, always in the mornings between 08:00 and 09:00 h. The long-term average was calculated from all our measurements collected over a 12-year period (1991–2003). Water depth for each population was the midpoint of the population's depth range. Feeding style was assigned categorically as planktivorous, benthivorous or omnivorous and was based on previous work (Seehausen 1996; Bouton *et al*. 1997). Nuptial coloration was assigned categorically as either red and yellow, blue, or intermediate.

Quantum catch calculations

To determine whether measured differences in λ_{max} could be visually significant, we calculated the relative quantum catch of photoreceptors with different visual pigments when viewing a red color patch. The quantum catch of a photoreceptor is given by:

$$Q = \int I(\lambda)T_w(\lambda, d)S(\lambda)T_w(\lambda, z)R(\lambda)d\lambda$$

where $I(\lambda)$ is the incident solar illumination; $T(\lambda, d)$ is the light transmission through water of depth, d; S is the spectral reflectance of the male color patch; $T(\lambda, z)$ is the light transmission to the female a distance z from the male; and R is the photoreceptor absorption properties (Vorobyev *et al.* 1998). In this model, solar illumination was described as a black body with a temperature of 5800 K (fit to the data of Neckel & Labs 1984). Water transmission was determined from *in situ* measurements in Lake Victoria from either the turbid Luanso waters or the clearer Makobe waters, using an Ocean Optics PS 1000 Microspectrometer and OCEAN OPTICS ACQUISITION software. The curve for each site was the mean from 10 spectral scans measured in 1996. Visual pigment absorbances for a pigment of a given λ_{max} and proportion of A_1/A_2 chromophore were calculated from the equations of Govardovskii *et al.* (2000). Spectral reflectance was determined from red patches of *P. nyererei* taken at Makobe Island. Fish spectral reflectance was measured with an Ocean Optics PS 1000 Microspectrometer and OCEAN OPTICS ACQUISITION software off the flanks of live fishes photographed (Kodak Elite 100 ISO transparency films) under daylight immediately after capture. This gives identical spectra to those obtained from live fish (Maan *et al.* unpublished).

Results

MSP

In all species, the majority of cone outer segments fell into three spectrally distinct classes with λ_{max} close to 455, 528, and 565 nm. Single short-wave-sensitive cones contained a pigment with λ_{max} about 455 nm. Two types of double cone were identified: nonidentical double cones (R/G) containing the long-wave and middle-wave pigments, and identical double cones (R/R) in which both members contained the 565-nm pigment. In addition, for the *Pundamilia nyererei* and *Pundamilia pundamilia* from Makobe, one cone with a 506-nm pigment was recorded in each individual. This pigment was found in nonidentical double cones along with the 565-nm pigment. Rods had a λ_{max} at 505 nm. For each class of photoreceptor, the λ_{max} of the mean absorbance spectrum, the λ_{max} of the mean difference spectrum, and the mean λ_{max} of the individual cells were determined along with the mean transverse optical density (Table 1). The table also includes the total numbers of identical (R/R) and nonidentical (R/G) double cones that were identified. Typical mean absorbance and difference spectra are shown in Fig. 1.

Three notable sources of variation were apparent from the data. First, the numbers of R/R and R/G double cones varied between species and individuals (Tables 1 and 2). In the two individual *Pundamilia* 'red head' *and* the three *Pundamilia azurea*, all 77 double cones were identified as R/

Table 1 Details of microspectrophotometric data obtained from all *Pundamilia* species

	ROD	**SWS**	**MWS**	**LWS**	**R/G**	**R/R**
nyererei			**528.1 ± 0.6**	**564.7 ± 0.8**	**33**	**5**
Makobe			528.2 ± 0.7	565.0 ± 0.7		
n = 1			528.5 ± 2.0	564.3 ± 2.0		
			0.031/031	0.031/031		
			10	16		
nyererei	**505.2 ± 1.4**	**451.2 ± 2.9**	**535.1 ± 0.7**[a]	**568.2 ± 0.9**	**15**	**17**
Python	505.8 ± 3.4	456.8 ± 3.9	535.7 ± 0.8	567.3 ± 1.1		
n = 2	504.9 ± 2.0	450.3 ± 1.4	536.5 ± 3.9	568.1 ± 3.2		
	0.033/028	0.013/013	0.029/028	0.026/025		
	2	5	14	36		
pundamilia		**456.4 ± 3.4**	**527.2 ± 0.7**	**563.7 ± 0.5**	**33**	**0**
Makobe			527.1 ± 0.8	563.9 ± 0.8		
n = 1			526.9 ± 1.6	563.4 ± 1.8		
		0.027	0.036/036	0.028/026		
		1	10	11		
pundamilia	**505.6 ± 1.0**	**454.8 ± 3.2**	**528.6 ± 0.5**	**562.6 ± 0.7**	**10**	**7**
Python	507.7 ± 1.7	457.5 ± 2.2	528.1 ± 0.9	562.7 ± 1.1		
n = 1	504.8 ± 1.8	453.4 ± 2.8	528.2 ± 2.8	561.8 ± 2.5		
	0.025/022	0.013/013	0.029/028	0.031/030		
	3	8	7	17		
azurea	**503.3 ± 1.1**	**455.3 ± 2.0**	**526.3 ± 0.4**	**563.6 ± 1.3**	**51**	**0**
n = 3	505.4 ± 1.1	455.8 ± 1.5	526.8 ± 0.5	563.6 ± 1.2		
	503.0 ± 1.8	454.4 ± 3.5	526.4 ± 2.1	563.5 ± 1.6		
	0.022/018	0.016/015	0.038/037	0.035/034		
	17	21	48	44		
'luanso'	**504.7 ± 0.8**	**455.3 ± 1.2**	**526.5 ± 0.6**	**561.8 ± 1.2**	**42**	**36**
n = 4	507.3 ± 1.3	458.2 ± 2.1	526.6 ± 0.3	561.9 ± 1.1		
	503.6 ± 1.8	454.6 ± 1.7	526.5 ± 1.6	561.8 ± 1.8		
	0.024/020	0.018/017	0.039/039	0.038/037		
	23	18	38	110		
'red head'	**503.2 ± 1.3**	**455.7 ± 2.7**	**526.9 ± 0.3**	**566.9 ± 1.6**	**26**	**0**
n = 2	505.1 ± 1.3	455.8 ± 2.3	527.6 ± 0.4	566.9 ± 1.6		
	502.6 ± 2.2	455.1 ± 2.0	526.9 ± 1.7	567.0 ± 2.0		
	0.026/023	0.023/021	0.036/035	0.029/029		
	7	10	26	23		

Five lines of data for each species: (i) λ_{max} of mean absorbance spectrum ± standard deviation (SD); (ii) λ_{max} of mean difference spectrum ± SD; (iii) mean of the λ_{max} of the absorbance spectra of the individual cells ± SD; (iv) maximum OD from absorbance spectrum and (/) difference spectrum; (v) number of selected cells used in the analysis. [a] *nyererei* data analysed with 70% A_1. R/G and R/R are the numbers of each type of double cone identified. In both fish from Makobe, single R/G double cones were recorded with a 506-nm pigment.

G doubles, whereas in the four individual *Pundamilia* sp. from Luanso Island, both R/G and R/R double cones were found in approximately equal numbers (42:36). In the case of *P. pundamilia* and *P. nyererei*, striking differences were seen between populations from clear and turbid waters. In Python Island populations, both R/G and R/R double cones were found, again in approximately equal numbers, whereas in the individuals from Makobe Island, no R/R double cones out of 33 were identified in *P. pundamilia*, and only 5 R/R out of 38 in *P. nyererei*.

A second notable difference was between *P. nyererei* from Python and Makobe. The two fish from Python appeared to have a small percentage of porphyropsin in their cones. The absorbance spectra for the MWS cones were noticeably broader than would be expected for a pure rhodopsin and had a λ_{max} around 535 nm, about 7 nm longer than found in the Makobe fish (and all the other species). Our analysis suggests about a 30% complement of porphyropsin with a chromophore pigment pair of 528_1/556_2. The λ_{max} of the LWS cones in the Python *P. nyererei* is also longer than in the Makobe fish, again suggesting a small component of porphyropsin.

The third source of variation among the species was in the spectral location of the LWS pigment. Although the differences were on the order of only a few nm, *P. azurea*, *P.* 'luanso' and *P. pundamilia* had λ_{max} about 562–564 nm, whereas *P.* 'red head' and *P. nyererei* had λ_{max} closer to 565–571 nm (Tables 1 and 2).

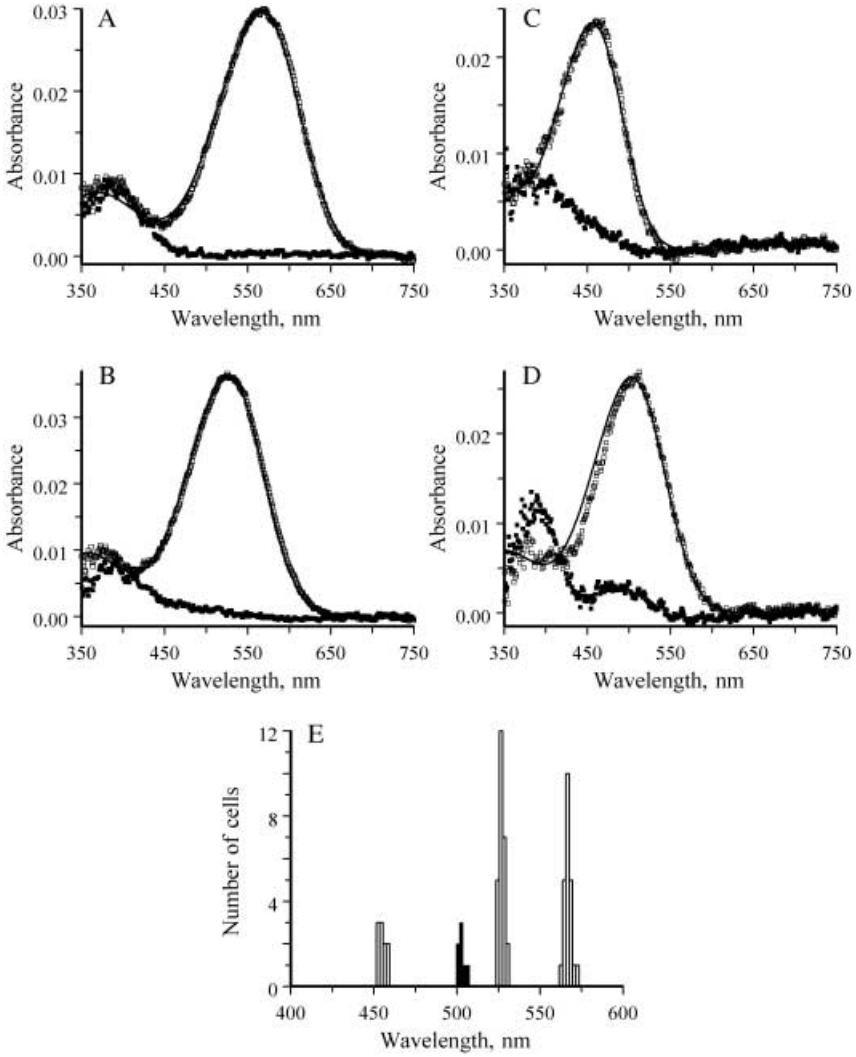

Fig. 1 Mean absorbance spectra of rods and cones from *Pundamilia* 'red head'. (A) LWS cones, (B) MWS cones, (C) SWS cones, (D) rods. Open symbols: before bleach; filled symbols: after white light bleach. Full lines are visual pigment templates (Govardovskii *et al*. 2000) with λ_{max} at 567, 527, 456 and 503 nm, respectively. (E) Distribution histogram of the λ_{max} of individual cells. Filled bars are rods. Bin size 2 nm.

Opsin mRNA expression

Pundamilia pundamilia and *P. nyererei* from Python, both have strong expression of SWS2a, RH2 and LWS cone opsin genes, presumably reflecting the 455, 535, and 565 nm visual pigments, respectively (Fig. 2). Neither of the species expressed the SWS1 gene. The expression levels for the 14-day-old juveniles from the F_1 cross have expression levels quite similar to the parental species with very little SWS1 expression. The juveniles and one *P. pundamilia* individual had a small amount (< 1%) of SWS2b expressed, although there is little evidence for a fourth visual pigment from MSP.

The expression of the LWS genes is greater than that of the RH2 genes. This agrees with MSP results, which demonstrate that *P. pundamilia* and *P. nyererei* from Python Island have both R/R and R/G cones. Assuming the two members of the double cones contain equivalent amounts of pigment and hence express equal amounts of opsin mRNA, we can calculate an RH2/LWS opsin ratio based on the MSP double cone ratios (Table 1). The RH2/LWS opsin ratios are 0.42 and 0.31 for *P. pundamilia* and *P. nyererei*, respectively, which are in reasonable agreement with the real-time PCR ratios of 0.37 ± 0.16 and 0.21 ± 0.08. Therefore, both MSP and real-time RT–PCR show that in the Python Island populations, individuals of the deeper living and red-coloured *P. nyererei* have more R/R and fewer R/G doubles cones than individuals of the shallower living and blue-coloured *P. pundamilia*.

Opsin sequences

The genomic opsin sequences and the inferred amino acid sequences obtained for the six opsin genes were quite similar amongst the seven populations (Table 3; see Table S1, Supplementary material, for accession numbers). The number of variable amino acid sites for each gene is 0 for SWS2a, 1 for SWS2b, 2 for SWS1, 3 for RH2, 4 for LWS

Table 2 Comparisons of LWS λ_{max} and double cone pigment ratios with environmental factors and male body colour

Species and locality	Sex	LWS MSP peak λ	Double cone ratio R/R	Double cone ratio R/G	Double cone ratio RR/RG cone ratio	Habitat Secchi disk (cm)	Habitat Depth (m)	Male body color		Major foraging mode
Pundamilia nyererei										
Makobe	F	565.0	5	33	0.15	225 ± 13	5.5	Red		Planktivore
Python	F	566.8	11	6	1.83	98 ± 12	3.5	Red		Omnivore
	M	571.1	6	9	0.67					
	M				1.92					
Pundamilia pundamilia										
Makobe	M	564.0	0	33	0	225 ± 13	1.5	Blue		Benthic insectivore
Python	F	562.6	7	10	0.70	98 ± 12	1	Blue		Omnivore
	M				0.71					
	M				1.03					
Pundamilia azurea										
Ruti	F	564.0	0	16	0	223 ± 31	11	Blue		Planktivore
	M	563.8	0	18	0					
	M	563.1	0	17	0					
Pundamilia 'luanso'										
Luanso	F	560.8	9	12	0.75	50 ± 7	2.5	Blue/yellow		Omnivore
	M	562.8	10	11	0.91					
	M	562.0	9	8	1.13					
	F	562.5	8	11	0.73					
Pundamilia 'red head'										
Zue	F	566.5	0	15	0	150 ± 20	1.5	Red		Benthic insectivore
	M	567.1	0	11	0					

For double cone ratios determined by MSP, R/R and R/G double cone numbers are shown along with the ratio. For double cone ratios determined by real-time PCR gene expression, the data in Fig. 2 is converted to the RR/RG ratio given here.

and 7 for RH1. In general, the variation involves conservative changes, or changes at sites that are not directed into the retinal binding pocket. The LWS gene is the one exception. It has two polymorphic sites that are directed into the retinal binding pocket and differ in amino acid polarity, F203Y and I262C. In general, most polymorphisms were between species, rather than within species even when species were sampled from different populations and very different photic environments. There was also no evidence for differences between males and females (Table 3).

The MSP data and the opsin sequences are consistent with each other. There was no sequence variation for the SWS2a gene and the MSP peak wavelengths are similar within experimental variation. There is some variation in the RH1 gene sequences, with *P.* 'red head' having six amino acid sites that differ from the other four species. Of these, only G163A is directed into the retinal binding pocket although it is a conservative change. Therefore, these sites seem unlikely to cause spectral tuning. This agrees with the rod λ_{max} that are within 2 nm of each other for all species. There is also little variation in the RH2 gene sequence. However, there is variation in the corresponding MWS visual pigment between the *P. nyererei* Python individuals and all others, which seems likely to be due to mixed chromophore usage.

The LWS gene is the only one for which there are sequence differences that are predicted to change peak sensitivities and which are consistent with between-population variation in MSP λ_{max}. Of the four variable amino acids, two (F203Y and I262C) are directed into the retinal binding pocket. Only site 262 is correlated with wavelength, with a cysteine occurring in all individuals having LWS $\lambda_{max} > 566$ nm. Therefore, this site could contribute to the small spectral shifts observed between species.

Analysis of variance

Table 2 provides data on all individuals for which there is MSP or mRNA expression data and compares the LWS λ_{max} and either the R/R to R/G double cone ratio or mRNA

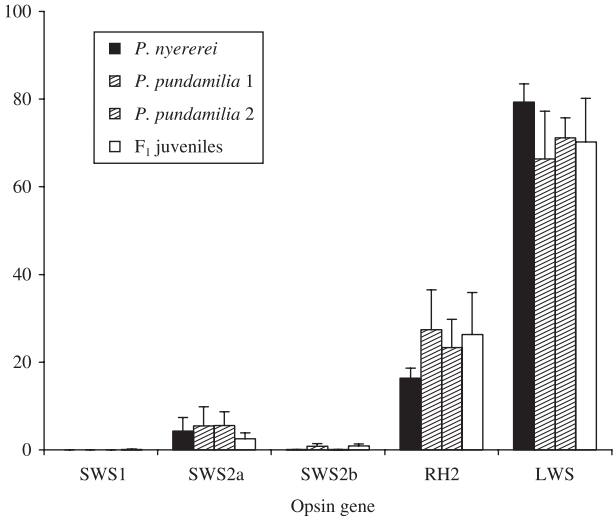

Fig. 2 Real-time opsin expression for *Pundamilia nyererei* and two *Pundamilia pundamilia* individuals from Python Island. Also shown is data from 14-day-old juveniles from an F_1 cross between *P. nyererei* and *P. pundamilia*. Opsin expression is given as percent of total opsin expression. Error bars are 1 SD.

expression double cone ratio with ecological characteristics (photic environment, foraging mode), and male nuptial colour. In single-factor ANOVAS/ANCOVAS, portions of the variance in double cone ratios were explained by water transparency ($n = 17$, $F = 14.5$, $P = 0.002$, $r^2 = 0.46$) and species ($n = 17$, $F = 3.3$, $P = 0.047$, $r^2 = 0.37$), but not by male colour, feeding style, or depth ($P > 0.1$). Higher R/R to R/G double cone ratios (i.e. more red cones) were observed for individuals from more turbid sites. When water transparency was nested in species, transparency remained a significant predictor of the cone ratio, besides species (transparency $F = 5.3$, $P = 0.040$; species $F = 10.2$, $P = 0.015$). Using transparency, depth, colour, and feeding in one ANCOVA, only transparency significantly explained a portion of the variance ($P = 0.039$, all others $P > 0.3$). When transparency was nested in species, depth explained a portion of the R/R to R/G double cone variance besides transparency (depth $F = 10.2$, $P = 0.015$; transparency F8.9, $P = 0.006$). In addition, when transparency was treated as a factor (4 levels), and nuptial colour was nested in it, nuptial colour explained a portion of the double cone ratio variance with individuals from red species having higher R/R to R/G ratios than individuals of blue species ($F = 9.7$, $P = 0.001$).

Portions of M/LWS λ_{max} variance were explained significantly by nuptial colour ($n = 14$, $F = 6.2$, $P = 0.016$, $r^2 = 0.45$) and species ($F = 4.5$, $P = 0.029$, $r^2 = 0.52$), but not by feeding style, transparency, or depth ($P > 0.6$) in one-factor ANOVAS/ANCOVAS. Individuals of species with red nuptial colour had long wave shifted M/LWS λ_{max} compared to species with blue nuptial colour. When species was nested

Table 3 Comparisons of opsin gene sequences for each individual sequenced

Species and locality	Sex	LWS MSP peak λ	13	203	217	262	RH2 MSP peak λ	56	255	263	S2b 282	S2a MSP peak λ	S1 166	RH1 MSP peak λ	42	104	158	159	162	163	173
Consensus			V	Y	T	I		S	I	I	P		G –		C	V	G	V	I	G	L
P. nyererei	M		A	.	A	C		.	.	.	.		. –		.	I	.	.	.	.	.
Makobe	F	565		F	.	.	528														
P. nyererei	F	567		.	A	C	532	.	.	.		451		505	.	.	.	.	.	.	.
Python	M	571		.	A	C	538	.	.	.					.	.	.	.	.	.	.
P. pundamilia	M		.	F	.	.		SG	.	.	.		. –		.	I	.	.	.	.	.
Makobe	M	564		F	.	.	527					456									
P. pundamilia	F	563		F	.	.	529	SG	IV	.		455		506	.	IV	.	.	.	.	.
Python	M	563		F	.	.		.	IV	.					.	IV	.	.	.	.	.
P. azurea	M		.	.	.	.		.	V	V	.		S –		.	IV	.	.	.	.	.
Ruti	M	564		.	.	.	527	.	V	V		456		503	.	I	.	.	.	.	.
	M	564		.	.	.	527	.	V	V		456		503	.	I	.	.	.	.	.
P. 'luanso'	M		.	F	.	.		.	.	.	.		S –		.	.	.	.	.	.	.
Luanso	F	562		F	.	.	527	.	.	.		455		505	.	.	.	.	.	.	.
	M	562		F	.	.	527	.	.	.		455		505	.	.	.	.	.	.	.
P. 'red head'	M		A	.	A	C		.	.	.	A		. I		A	.	A	F	V	A	V
Zue	F	567		.	A	C	527	.	.	.		456		503	A	.	A	F	V	A	V
	M	567		.	A	C	527	.	.	.		456		503	A	.	A	F	V	A	V

Individuals are grouped by species and locality. Sequences are compared to the consensus sequence with identity indicated by a dot. The sequences for gene SWS2a (S2a) are identical for all the individuals studied and so no sites are listed. Sites are numbered according to bovine rhodopsin. Sites directed into the retinal binding pocket are shaded. MSP peak wavelengths (in nm) are listed for those genes that are expressed in the adult fish. SWS2b (S2b) and SWS1 (S1) are not expressed as visual pigments and so were not quantified by MSP nor sequenced in the MSP individuals.

in colour, species no longer significantly explained any amount of variance ($P = 0.22$). With transparency, depth and colour in a single ANCOVA, only colour explained a portion of the variance ($P = 0.005$), but there was a trend for transparency to explain some variance as well ($P = 0.061$), with individuals from turbid water having M/LWS λ_{max} at longer wavelengths. When transparency and depth were nested as covariables in colour, transparency and colour both explained portions of the M/LWS λ_{max} variance (transparency $F = 8.5$, $P = 0.013$; colour $F = 7.1$, $P = 0.031$) whereas depth did not ($F = 1.4$, $P = 0.19$). When LWS λ_{max} alone was used instead of M/LWS λ_{max}, very similar results were obtained with regard to effects of nuptial colour, but effects of transparency were weaker.

Correspondingly, the only variable that significantly explained between-species variance in colour in univariate analyses of variance was M/LWS λ_{max} ($n = 14$, MWS $F = 5.7$, $P = 0.034$; all others: R/R to R/G ratio, transparency, depth, feeding, $P > 0.2$). M/LWS λ_{max} remained a significant predictor of colour when nested in transparency ($F = 4.9$, $P = 0.023$). R/R to R/G ratio was not a significant predictor of colour even when it was nested in transparency ($P = 0.3$). The same results were obtained when LWS λ_{max} was used instead of M/LWS λ_{max}.

In summary, variation in double cone ratios was primarily explained by water transparency, whereas the effects of water transparency on variation in λ_{max} were more subtle. On the other hand, variation in male nuptial colour was best explained by variation in λ_{max}.

Quantum catch calculations

The quantum catch of a photoreceptor viewing the red colour of *P. nyererei* is indeed sensitive to the observed variation in visual pigment λ_{max}. For an A_1 pigment, the relative quantum catch will increase 10% for a 4 nm shift in λ_{max} from 563 to 567 nm. This increase is independent of whether transmission spectra from clearer Makobe Island or murkier Luanso are used. Such a change could cause a significant difference in neural response.

The effects of chromophore shifts are even greater. If a 563-nm pigment partially shifts in combination with mixed chromophores to peak at 580 nm, the photoreceptor quantum catch will increase by a factor of 1.44. If the chromophore shift is nearly complete and absorption peaks at 600 nm, these factors increase to 1.89. Chromophore-induced quantum catch increases occur in both clearer and murky waters. Therefore, chromophore shifts provide a far greater increase in photoreceptor output than the changes in opsin gene sequence.

Finally, a change in which opsin gene is expressed can also cause a significant difference in photoreceptor quantum catch. For cones that switch from G (530 nm) to R (563 nm) pigments, their quantum catch will increase by 2.6 times (Makobe Island water transmission) or 3.4 times (Luanso Island water transmission) when viewing a red patch. This would make obvious improvements in brightness detection of red patches.

Discussion

Visual system comparisons between species

This is the first comparison of visual system physiology and opsin gene sequences between closely related species of African cichlid fish and between populations of the same species from different photic environments. On the whole, visual pigment absorptions appear similar between species and populations. All utilize a rod pigment and three cone pigments. The similarity between populations in λ_{max} of the RH1, SWS2a and RH2 pigments is in agreement with the lack of functionally significant opsin sequence variation in the corresponding genes. The only sizable λ_{max} difference in RH2 pigments is likely a chromophore effect and not the result of sequence variation. In contrast to these three pigments, the LWS pigment showed small but consistent differences between species in λ_{max} that coincide with LWS opsin gene sequence differences. Two of the four variable sites in this gene are directed towards the retinal binding pocket and differ in amino acid polarity, F203Y and I262C. Comparisons between the MSP absorptions and the sequences suggest that I262C contributes the majority of the spectral shift. The effect of this site has not previously been studied and will require testing with site directed mutagenesis.

The variability of LWS but not the SWS2a or SWS2b genes is curious considering that the SWS2a–SWS2b–LWS opsin genes are in a tandem array in which the genes are separated by 5–6 kb (Carleton & Kocher 2001). Comparisons of the nearly invariant SWS2 sequences and the variable LWS sequences suggest that selection must be acting on a very narrow chromosomal scale and supports the idea of Terai *et al*. (2002) that variation in the LWS opsin gene is unique.

Chromophore effects

The majority of individuals in this study had MSP spectra that could be fit using A_1 templates. While this is not a quantitative means of determining chromophore usage, it suggests that these laboratory-reared and/or wild-caught but laboratory-adapted individuals were primarily using A_1 chromophores. There is some variation in λ_{max} between individuals of *Pundamilia nyererei* from Python Island (all laboratory reared), which can be ascribed to different admixtures of A_1/A_2 chromophores. Although the opsin genes were identical, there were 3–6 nm shifts in both the RH2 and LWS pigments. This is consistent with previous

MSP studies of Lake Victoria cichlids, which found that certain species incorporated A_2 chromophores in their visual pigments (van der Meer & Bowmaker 1995). In the present study, it is notable that porphyropsins were found only in *P. nyererei* derived from a relatively deep and turbid water population, whereas neither the deep but clear water Makobe population of *P. nyererei*, nor the shallow but turbid water populations of *Pundamilia pundamilia* and *Pundamilia* 'luanso' showed any evidence of porphyropsins.

The A_1 LWS λ_{max} in our laboratory-maintained fish varied between 562 and 567 nm. Based on the established relationships between rhodopsins and porphyropsins, the corresponding A_2 pigments would be 605 and 613 nm (Parry & Bowmaker 2000) or 626 and 633 nm (Whitmore & Bowmaker 1989; Harosi 1994). Therefore, A_2 chromophore shifts could magnify species differences in LWS λ_{max}. Such chromophore shifts could be interpreted as a means of increasing long-wave sensitivity in deep and/or turbid water. Fishes are known to switch phenotypically between A_1 and A_2 usage, depending on ambient light levels (Munz & McFarland 1977; Beatty 1984). Ambient light in our laboratory was bright compared to that in the natural environment of the deeper living of our populations. Thus examination of wild-caught specimens may show that heritable variation in the propensity to shift to A_2 chromophore usage is important in adaptation to local environments and may differ between species.

MSP comparisons with opsin mRNA expression

The MSP data support the idea that there are three primary cone pigments in these fishes. This is similar to previous results on Lake Victoria cichlids (van der Meer & Bowmaker 1995) as well as *Astatotilapia burtoni* from Tanganyika (Fernald & Liebman 1980) and numerous species from Malawi (Carleton *et al*. 2000; Parry *et al*. 2004; Jordan & Loew, personal communication). A complement of three cone visual pigments is further supported by the cone opsin gene expression results, which show that just three cone opsin genes are expressed. The known spectral ranges of these cone opsin gene classes suggests the following correspondence: 455 nm pigment from SWS2a, 528 nm pigment from RH2, and 565 nm pigment from LWS. These spectral correspondences have recently been confirmed for cichlid opsin genes (Parry *et al*. 2005; Spady *et al*., unpublished). There is no evidence for differences in gene expression between these sibling species of Lake Victoria cichlids as observed between less closely related Lake Malawi species (Carleton & Kocher 2001).

Correlations between vision and the environment

Although the differences in visual systems between species are subtle, there are some interesting correlations among vision, colour, and ecology. The most striking is between the R/R to R/G double cone ratio and water transparency, where the proportion of R/R double cones increases from clearer to more turbid water. Strikingly, this is observed not just between species, but also between populations of the same species. Increased turbidity causes a reduction in light levels, but also disproportionately reduces transmission of shorter wavelengths, as a result of wavelength-selective light scattering. It has long been suggested that in teleosts, the combined sensitivity of double cones is tuned to the maximum transmission of the water (Munz & McFarland 1977; Lythgoe 1979; Bowmaker *et al*. 1994; Lythgoe *et al*. 1994). Measurements of water spectral properties in Lake Victoria suggest that peak transmissions at sites with turbid water are at longer wavelengths than at sites with clearer water (Seehausen *et al*. 1997). The increased number of LWS-expressing cones with sensitivities at 560–570 nm will cause the overall sensitivity of the double cones to be shifted towards longer wavelengths, which should increase the distance at which objects can be detected in more turbid water. Since the fish used in the present study were laboratory bred under identical conditions, the apparent retention of differences in the double cone ratio strongly suggests that the distribution of double cones has a strong genetically determined component.

The relationship between λ_{max} of the double cone pigments and photic environment was more subtle, but pointed towards shifts to maximally transmitted wavelengths too. Individuals from populations with more turbid water tended to have slightly more red-shifted M/LWS λ_{max}. However, because of a strong relationship between λ_{max} and male nuptial coloration, the relationship with water transparency became apparent in an ANCOVA only when transparency was nested in male colour. We did not discover any relationship between visual pigment sensitivity and foraging mode. Taken together, these results suggest that variation in the visual system, and in the pigments of the double cones in particular, has evolved in response to local variation in the ambient environment. Changes in the proportion of R/R double cones permit a bolder response to environmental selection than changing opsin sequences. The same may be said of chromophore shifts, but more work is required to test the hypothesis that populations differ in their heritable propensities to incorporate different chromophores.

Both A_1/A_2 chromophore shifts and increases in the number of R/R double cones could also have environmental contributions in addition to genetic control. For a given visual pigment, it is possible that shifting the chromophore is more facile than changing the opsin sequence in response to varying environmental conditions. If chromophore shifts do provide the environmental response, this might explain why λ_{max} has only a weak correlation

with water transparency. Future sampling is needed to examine wild-caught fish in their natural environment and to compare them with laboratory-reared specimens to determine the extent of environmental effects.

Implications for speciation in cichlid fish

The association between M/LWS λ_{max} and male colour that we observed in this study is remarkable. Three populations (two species, *P. nyererei* and *Pundamilia* 'red head') that use red male nuptial colour, had a longer wavelength LWS pigment ($\lambda_{max} > 565$ nm) than four populations (two species and a hybrid population) that use blue male nuptial colour ($\lambda_{max} < 565$ nm). A significant relationship was observed regardless of how the additional variables were partitioned. Although the differences in LWS λ_{max} observed are small, and barely significant given the limits of accuracy of MSP, they are consistent with the LWS opsin sequences. This supports the interpretation that observed individual λ_{max} variation, and species differences, are real and genetically determined.

In addition, calculations suggest that λ_{max} shifts of 4 nm do increase photoreceptor quantum catch by 10% when viewing red patches. Therefore, small shifts in λ_{max} can cause significant differences in photoreceptor response. This might affect brightness or contrast between patches within a fish, or between the fish and its background. Significant differences in behavioural responses to light sources of different colour have indeed been found between *P. pundamilia* and *P. nyererei*, which are consistent with our data (Maan *et al*. submitted). Chromophore shifts cause even greater increases in quantum catch, up to a factor of 2. Such shifts could play an important role in visual perception and cichlid mate choice and need further study in field-caught individuals.

Changes in opsin gene expression, and a resulting increase in R/R double cones, also lead to increased photoreceptor quantum catch by factors of 2.6–3.4. This could cause improvements in brightness detection. However, three lines of evidence make it appear unlikely that variation in the proportion of R/R cones provide an explanation for species differences in mating preferences. First, turbid water populations of blue species have much higher proportions of R/R cones than clear-water populations of red species. Yet, in laboratory mate choice experiments, females of blue species prefer blue males and females of red species prefer red males (Seehausen 1997). Second, the other red species that we studied, *P.* 'red head' shows no increase in the proportion of R/R cones over blue species at all. Third, differences in cone number will probably have no effect on colour vision. In humans, studies have shown that even though L/M cone ratios may vary by factors of 3, colour sense (as tested by the wavelength of unique yellow) remains essentially constant (Brainard *et al*. 2000). If the opponency in fish visual processing is similar to humans, then changes in cone numbers should not impact colour sense and therefore colour preference. This may be one reason why there is no correlation between double cone ratio and male coloration.

In conclusion, we have identified a number of heritable differences between populations and species including striking differences in R/R double cone numbers between populations within and between species, and subtle spectral shifts in LWS pigment λ_{max}, between species. These differences are likely due to local adaptation to spatially variable photic conditions. They could cause differences in visual perception of different hues, particularly in their brightness, and could at least partially determine cichlid mating preference. These factors need to be considered along with others such as chromophore shifts and higher order neural processing in exploring the proximate mechanisms that cause differences in cichlid mate choice and speciation.

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Supplementary material

The supplementary material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC2735/MEC2735sm.htm

Table S1 GenBank Accession nos for opsin genes sequenced in this work. The accession number for the genes which were sequenced are noted by their last three digits (with the first five digits being AY673xxx) except for two LWS sequences where the last six digits are given (being AYxxxxxx). If sequences were identical for more than one individual, then they were included in the same GenBank submission.

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This work grows out of our interests in evolution and speciation. Karen Carleton is interested in the molecular basis of cichlid fish visual sensitivities and the role of visual diversity in driving speciation. David Hunt, Jim Bowmaker and Juliet Parry are currently involved in a study of the visual systems in a diverse array of vertebrates with a focus on the molecular mechanisms of spectral tuning and the evolution of photopic vision. Ole Seehausen is interested in adaptive radiation, speciation, and the evolution of mate choice.
