The Journal of Experimental Biology 213, 1453-1463

doi:10.1242/jeb.036897

Electrophysiological assessment of spectral sensitivity in adult Nile tilapia *Oreochromis niloticus*: evidence for violet sensitivity

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Accepted 4 January 2010

SUMMARY

The cichlid fish radiations of the African Great Lakes are an important model for evolutionary biology. Cichlids have diverse colour vision systems and predominately express three cone visual pigments. However, rare populations of spectrally distinct cones have been found in a number of species, but it is not known whether they contribute to spectral sensitivity. Adult Nile tilapia, *Oreochromis niloticus*, an ancestral outgroup to the cichlid radiations in the Great Lakes, have three cone types: short-wavelength sensitive (SWS), medium-wavelength sensitive (MWS) and long-wavelength sensitive (LWS) cones, but evidence from microspectrophotometry and cone opsin gene expression suggests they may also have violet-sensitive (VS) cones. We used electrophysiology to assess spectral sensitivity in this species and found evidence of four sensitivity peaks in the ranges 380–420, 440–480, 500–600 and 600–680 nm, with maximal sensitivity at longer wavelengths. The continued presence of a 380–420 nm peak under long-wavelength chromatic adapting backgrounds indicates that this is due to a VS cone mechanism not the β-band of the LWS cone mechanism. Differences in spectral sensitivity curves recorded at different times of year revealed evidence of A1/A2 shifts. The presence of notches in the sensitivity curves and a multiple-mechanisms model used to assess cone contributions indicated that the curves are the result of four cone mechanisms (VS, SWS, MWS and LWS cones) and that chromatically opponent processes occur between mechanisms. The spectral transmittance of the lens steeply declines between 410–380 nm, limiting the short-wavelength limb of the VS cone. As adults, Nile tilapia appear to possess the necessary retinal mechanisms for colour vision. While maximal sensitivity to longer wavelengths is an adaptation to the wavelengths of light predominantly available in their natural habitats, their broad sensitivity range suggests that Nile tilapia possess a flexible, generalised visual system able to adapt to changes in visual environment in their highly variable natural habitat.

Key words: chromatic opponency, cichlid, compound action potential, cone mechanism, electroretinogram, fish, lens transmission, visual ecology, visual pigment.

INTRODUCTION

The cichlid fishes of the East African Great Lakes are the largest and most recent vertebrate radiation on the planet (Fryer and Iles, 1972) and are an important model for evolutionary biology (Kornfield and Smith, 2000; Kocher, 2004). Each lake contains hundreds of separately evolved, endemic species that exhibit enormous diversity with regard to many aspects of their biology, including body size and form, feeding behaviour and associated ecomorphological trophic specializations, breeding behaviour and colour patterns (Fryer and Iles, 1972; Keenleyside, 1991; Barlow, 2000; Kocher, 2004). More recently, it has become apparent that the visual systems, especially the visual pigments, of these fishes are also highly diverse (Parry et al., 2005; Carleton et al., 2006; Jordan et al., 2006; Carleton et al., 2008; Carleton, 2009).

Visual pigments are the membrane-bound light sensitive molecules located in the discs of the photoreceptor outer segments. They consist of an opsin protein bound to a vitamin-A-based chromophore. In vertebrates, five classes of opsin have been identified, one rod opsin (*RH1*) and four classes of cone opsin, very short-wavelength sensitive (*SWS1*), short-wavelength sensitive (*SWS2*), rhodopsin-like (*RH2*) and long-wavelength sensitive (*LWS*) (Yokoyama, 2000). Each opsin produces a visual pigment with a unique spectral range (Yokoyama, 2000; Ebrey and Koutalos, 2001). All four classes of cone opsin have been found in cichlids and gene duplications have produced three additional genes, giving cichlids seven distinct opsin genes (*SWS1*, *SWS2b*, *SWS2a*, *RH2b*, *RH2aβ*, *RH2aα* and *LWS*), which all produce spectrally distinct visual pigments (Parry et al., 2005; Spady et al., 2006; Carleton et al., 2008). Interspecific variation in opsin gene expression means that different cichlid species express subsets of the seven cone opsin genes, giving each species a different complement of visual pigments and presumably a different spectral sensitivity (Parry et al., 2005; Jordan et al., 2006; Carleton et al., 2008; Carleton, 2009). Data from microspectrophotometric (MSP) measurements of the absorption properties of cone visual pigments in cichlids indicate that most species have three visual pigments (reviewed by Carleton, 2009). However, more detailed MSP studies on a small number of species have identified small numbers of cones expressing additional pigments (Parry et al., 2005; Carleton et al., 2008). These cones are low in number (less than 10% of the total cone population) and express pigments differing from the three main cone types found in a given individual, although the pigments are spectrally consistent with pigment types observed in other cichlid species (Parry et al., 2005; Carleton, 2009). The MSP data are supported by the results of quantitative reverse-transcription polymerase chain reaction (qRT-PCR) studies, which have found that more than three cone opsin genes are expressed in the retina of a number of cichlid species (Parry et al., 2005; Carleton et al., 2008; Carleton, 2009).

The presence of additional, rare cone types raises the question of whether or not they contribute to the spectral sensitivity of

cichlids. Limited electrophysiological evidence suggests that they do. Spectral sensitivity curves in cichlids, recorded using electroretinograms (ERGs), are modelled more adequately with four visual pigments as opposed to three (Garner et al., 2003). Unfortunately there have been few attempts to assess spectral sensitivity using electrophysiological or psychophysical techniques in cichlids (Bell, 1982; Allen and Fernald, 1985; Garner et al., 2003) and so our current predictions about the wavelength discrimination capabilities of cichlids are largely limited to MSP measurements of visual pigments in situ or in a reconstituted form (van der Meer and Bowmaker, 1995; Carleton et al., 2000; Parry et al., 2005; Jordan et al., 2006; Spady et al., 2006; Carleton et al., 2008). MSP is a powerful technique but, as indicated above, it is possible to overlook cells containing a particular visual pigment because of an inadequate number of sample scans, or because of the regionalization of a class of pigments within the retina, they may not be present in the area of retina examined (Bowmaker et al., 1991; Shand, 1993). MSP also provides no information about chromatic opponent interactions between different cone mechanisms, which can alter the sensitivity curves and maxima substantially, compared with those predicted by MSP alone, and can vary in nature between species (Neumeyer, 1984; Hughes et al., 1998; Parkyn and Hawryshyn, 2000; Hawryshyn et al., 2003).

We have begun to investigate whether the rare cone types found in cichlid retinas contribute to the spectral sensitivity of cichlids, using Nile tilapia *Oreochromis niloticus* (L.). This species represents an ancestral outgroup to the cichlid radiations of the East African Great Lakes (Kocher et al., 1995) and is of growing significance as a vertebrate model for biological research. Owing to its high growth rate, adaptability to a wide range of environmental conditions, ability to grow and breed in captivity and to feed at low trophic levels on a wide variety of organisms, the Nile tilapia is also one of the world's most important and widely cultured fish species (El-Sayed, 2006; Lim and Webster, 2006). The Nile tilapia has been shown to express all seven cichlid cone opsin genes, although not at the same time (Spady et al., 2006; Carleton et al., 2008), resulting in this species displaying an ontogenetic shift in cone opsin expression and visual pigment sensitivity. As adults, Nile tilapia predominantly expresses three cone opsins, *SWS2a*, *RH2aβ* and *LWS* (which is expressed at very high levels) and have three cone visual pigments with wavelengths of maximum absorbance (λ_{max}) of 449 nm (short-wavelength sensitive or SWS cones), 542 nm (medium-wavelength sensitive or MWS cones) and 596 nm (long-wavelength sensitive or LWS cones) (Spady et al., 2006; Carleton et al., 2008). However, there is evidence that small amounts of *SWS2b* opsin are also expressed and that adult Nile tilapia have violet-sensitive (VS) cones in the retina (Carleton et al., 2008). We have assessed spectral sensitivity in adult Nile tilapia using two electrophysiological techniques, ERGs and optic nerve compound action potentials (CAPs). Using a white (broad spectrum) background and long-wavelength chromatic adapting backgrounds we tested the hypothesis that Nile tilapia have violet sensitivity based on the presence of a VS cone mechanism. Here we provide evidence that adult Nile tilapia express four cone types including the VS cones.

MATERIALS AND METHODS

Animals

Nile Tilapia (*Oreochromis niloticus*) were obtained from a local fish farm (Northern Tilapia Inc., Lindsay, Ontario, Canada). Fish were kept in aquariums with cycled and filtered water at 20–24°C and fed daily with trout pellets (3PT Regular; Martin Mills Inc., Elmira, Ontario, Canada). The photic conditions within the aquarium room were provided by full spectrum fluorescent lamps featuring BlueMax TM color technology (Full Spectrum Solutions, Inc., Jackson, MI, USA). The photoperiod was set to 12 h:12 h L:D. Prior to use, fish were killed by immersion in a solution of 40–80 mg l^{-1} of clove oil followed by severing of the spinal cord. Fish ranging from 19.5 to 26.4 cm total length (TL) (140.0–333.5g) were used. All fish were at least 150 dpf (days postfertilisation) and, as Nile tilapia reach maturation at about 30 g under aquaculture conditions (El-Sayed, 2006), all the fish used were considered to be adults. All experimental procedures and animal care were approved by the Queen's University, Kingston, Ontario, Animal Care Committee under the auspices of the Canadian Council for Animal Care.

Eyecup preparation for electrophysiology

ERGs and CAPs were recorded from isolated eyecup preparations. An eye was excised from freshly killed fish and the anterior segment (the cornea, iris and lens) was removed under dim red light. The resultant eyecup was then placed in a superfusion chamber and superfused continuously (1.5 ml min^{-1}) with Ringer's solution. The Ringer's solution contained (in mmol l^{-1}) 102.0 NaCl, 2.6 KCl, 1.0 $MgCl_2$, 1.0 $CaCl_2$, 28.0 $NaHCO_3$ and 5.0 glucose (Kraaij et al., 1998), and was continuously bubbled with a mix of approximately 5% CO_2 and 95% O_2, yielding a pH of 7.4–7.8 at a temperature of 20±1°C.

Experimental apparatus

Aspects of the optical system and recording apparatus have been described previously (Hawryshyn et al., 2003; Ramsden et al., 2008). Two background channels using 250 W quartz-halogen lamps (Ushio, Cypress, CA, USA) provided constant background illumination and chromatic adaptation conditions (Fig. 1). The intensity and spectral content of the background light was controlled by neutral-density (ND) filters and long wavelength-pass (LP) interference filters (Coherent Inc., Santa Clara, CA, USA). A bifurcated fibre optic (fused silica, NA=0.22; Fiberoptic Systems Inc., Simi Valley, CA, USA) with a quartz diffuser placed at the terminal end of the fibre optic, projected uniform background illumination to the eyecup. The stimulus channel used a xenon arc lamp system (Thermo Oriel, Stratford, CT, USA) with a 300 W lamp (Ushio) and a calibrated, computer-controlled stimulus delivery system that was used to manipulate stimulus spectral, temporal and intensity characteristics *via* a monochrometer (10 nm half maximum bandwidth; Instruments SA Inc., Edison, NJ, USA), Inconel quartz ND wedge (0–4.0ND; Melles-Griot, Rochester, NY, USA), shutter (Uniblitz, Vincent Associates, Rochester, NY, USA), optical filters to block spectral sidebands, and UV optics to match the numerical aperture of the fibre optic (NA=0.22, Fiberoptic Systems Inc.), which projected the stimulus on to the eyecup.

Electrodes and recording techniques

Retinal responses to light stimuli were recorded using glass microelectrodes with a 100–200 μm diameter, fire polished tip. The electrodes were filled with a NaCl solution (40 p.p.t.) and inserted into a half-cell (A-M Systems, Inc., Carlsborg, WA, USA) filled with the same solution. For ERGs the electrode was placed in the vitreous humor of the eyecup, close to the retina, and for CAPs, the electrode tip was placed against or slightly inserted into the optic nerve head. A second glass microelectrode was used as a reference and it was placed in the Ringer's solution surrounding the eyecup. The glass microelectrodes were pulled from borosilicate glass [1 mm i.d., 1.5 mm o.d. (inner and outer diameter, respectively) World Precision Instruments (WPI; Sarasota, FL, USA)] with a Sutter P-97 Flaming-Brown micropipette puller (Sutter Instruments Company,

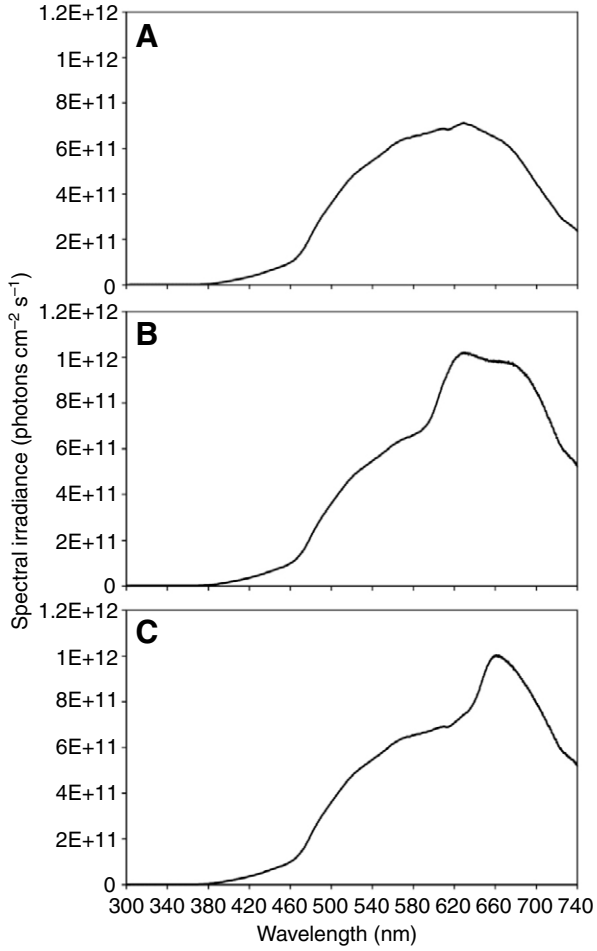

Fig. 1. Spectral energy distribution of the adapting backgrounds. (A) 'White' broad-spectrum quartz-halogen adapting background. (B) Long-wavelength adapting background created using a 600LP filter in a quartz-halogen background channel. (C) Long-wavelength adapting background created using a 650LP filter.

Novato, CA, USA). The electrodes were connected to an ISO-80 bioamplifer (WPI) and signals were digitized with Cambridge Electronic Design (CED; Cambridge, UK) 1401+ data acquisition hardware and Signal 4 software (also by CED). The electrode position was manipulated using micromanipulators until the signal:noise ratio was optimized for every fish. Retinal responses were recorded differentially, amplified 1,000 times with a cut-off bandwidth of 5–100 Hz and digitally filtered using Infinite Impulse Response (IIR) filters in the Signal 4 software to remove 60 Hz, 180 Hz and high-frequency noise.

Each eyecup was allowed to adapt to a background condition for 25–30 min prior to the onset of recording. Sensitivity was determined by measuring the amplitude of the ERG *b*-wave response and the CAP ON and OFF responses for wavelengths between 360 nm and 720 nm, using custom designed scripts written for the Signal 4 software. ERG *b*-wave amplitude was calculated measuring the change in potential from the *a*-wave trough to the *b*-wave peak during the period 200 ms post-stimulus onset, while for CAP ON and OFF responses the positive deflection was measured during the period 200 ms post-stimulus onset and offset, respectively. Stimulus wavelengths were presented in 20 nm intervals, using a staggered wavelength approach to prevent adaptation to a certain region of the spectrum. Stimulus intensity was gradually increased from 4.0 to 0.0 optical density in 0.1 or 0.25 steps and stimuli were presented as 500-ms flashes, with an interstimulus interval of 4.5 s. Example waveforms are shown in Fig. 2A,B. Response was plotted against the stimulus intensity (log photon irradiance) to produce response *versus* intensity (RI) curves for each wavelength (Fig. 2C,D). These were fitted to a Hill relation (Naka and Rushton, 1966a; Naka and Rushton, 1966b; Naka and Rushton, 1966c) using the least-squares method. Sensitivity was defined as $-\log_{10}$ of the photon irradiance that produced the half maximal response amplitude for each test wavelength. These values were normalized and plotted on a linear scale and spectral sensitivity curves where expressed on a 0–1 scale for each fish (where 1 indicates maximum sensitivity) to remove differences in absolute sensitivity between individuals. To obtain the final spectral sensitivity curves for each recording technique and background condition, the sensitivity curves of all individuals were averaged and normalized to the maximum so that in each curve the maximum sensitivity equalled 1.

Two different background conditions were used (Fig. 1A–C); a white (broad spectrum) background condition, where only one of the background channels (containing 2.0 ND plus a 0.5 ND filter and no interference) was in operation and a long-wavelength adapting background condition, where in addition to the white background, the second background channel contained either a 600 LP (Fig. 1B) or a 650 LP (Fig. 1C) interference filter. Visual pigment absorption spectra consist of a main absorbance band or 'α-band', the peak of which is referred to as the λ_{max}, and a secondary, shorter wavelength band, the 'β-band', with its own absorbance peak (Govardovskii et al., 2000; Deutschlander et al., 2001). An LWS visual pigment with a λ_{max} in the region of 580–640 nm will have a β-band peak in the ultraviolet-violet (380–400 nm) region of the spectrum. Therefore the long-wavelength adapting background was used to chromatically adapt the LWS cone mechanism in order to assess whether sensitivity in the violet region was due to the β-band of the LWS cone mechanism, or due to the α-band of the VS cone mechanism. Long-wavelength adaptation of an LWS visual pigment causes a proportional reduction in both the α- and β-bands (Deutschlander and Phillips, 1995; Deutschlander et al., 2001).

Fitting visual pigment templates and modelling cone contributions

The spectral sensitivity curves (white background and chromatic adaptations) were modelled using a two-step process. Initially, visual pigment templates (Govardovskii et al., 2000) were fitted to the sensitivity curves using a least-squares fit. It was assumed that the sensitivity curves receive inputs from four cone mechanisms; VS, SWS, MWS and LWS cone mechanisms. Nile tilapia have mixed chromophores consisting of retinal derived from both vitamin A1 (11-cis retinal) and vitamin A2 (3,4-didehydroretinal) and the A1/A2 ratio varies between individuals (Carleton et al., 2008). Shifting from A1- to A2-based chromophores in the same opsin has the effect of shifting the λ_{max} of the visual pigment to a longer wavelength, broadening spectral bandwidth of absorbance and decreasing the molar extinction coefficient (Bridges, 1972). The magnitude of the shift in λ_{max} caused by shifting from an A1-based chromophore to an A2-based chromophore in the same opsin is wavelength dependent (Harosi, 1994), with visual pigments with λ_{max} values at longer wavelengths the most affected. In the majority of freshwater fish species, including riverine cichlids, the retina is dominated by A2-based chromophores (Bowmaker, 1995; Carleton et al., 2008) and, in the absence of specific information on the A1/A2 ratios in the fish used in this study, the data where modelled

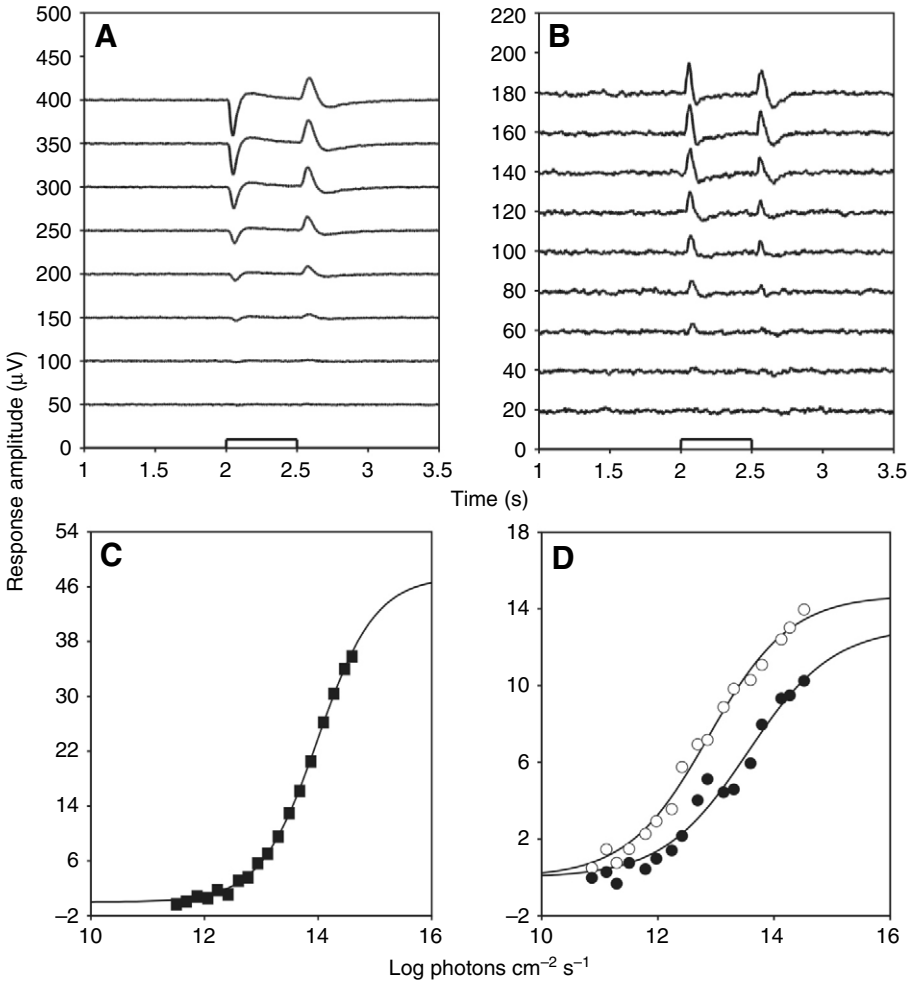

Fig. 2. Waveforms (A,B) and response *versus* intensity (RI) curves (C,D) for electroretinogram (ERG) and compound action potential (CAP) recordings. The stimulus wavelength was 640 nm. (A) ERG waveforms at increasing intensities. (B) CAP waveforms at increasing intensities. Note the 'ON' response at stimulus onset and 'OFF' response at stimulus offset. The individual traces in A and B are vertically displaced for clarity of presentation. (C) RI curve based on ERG data taken from A. (D) RI curve based on CAP ON response (open circles) and OFF response (filled circles) data taken from B.

assuming that all visual pigments have an A2-chromophore composition. The template for each visual pigment was fitted to a given region of a spectral sensitivity curve but was restricted to the λ_{max} range reported for that particular visual pigment in adult Nile tilapia ('restricted fit'), as determined using MSP (Carleton et al., 2008) (K. Carleton, personal communication). The ranges were: VS cone 410–445 nm; SWS cone 443.7–470 nm; MWS cone 527–556.1 nm; LWS cone 569–608.5 nm. Carleton et al. (Carleton et al., 2008) only found one VS cone in an adult fish, which had a λ_{max} of 438.7 nm, so for this pigment type the range of λ_{max} values reported for larval, juvenile and adult fish was used. For comparison, unrestricted, 'best fit' templates were also used and allowed to find the best fit to the curve.

A multiple-mechanisms model (Sperling and Harwerth, 1971; Kalloniatis and Harwerth, 1990; Hughes et al., 1998) was then used to determine the relative input weight from each cone type to each of the spectral sensitivity curves by assigning weights, which can be positive (excitatory) or negative (inhibitory) for each cone mechanism. The model that consistently provided the best fits takes the form:

$$S_{LWS} = k_1 (A_{LWS}) + k_2 (A_{MWS}) \quad (1)$$

$$S_{MWS} = k_3 (A_{LWS}) + k_4 (A_{MWS}) + k_5 (A_{SWS}) \quad (2)$$

$$S_{SWS} = k_6 (A_{MWS}) + k_7 (A_{SWS}) + k_8 (A_{VS}) \quad (3)$$

$$S_{VS} = k_9 (A_{SWS}) + k_{10} (A_{VS}) , \quad (4)$$

where S_{LWS}, S_{MWS}, S_{SWS} and S_{VS} refer to the sensitivity of the long-wavelength, medium-wavelength, short-wavelength and violet portions of the spectral sensitivity curve, respectively. The portion of the spectral sensitivity curve used in each equation was varied slightly between background conditions and recording methods, and was determined by the location of depressions and notches in each sensitivity curve (Hughes et al., 1998). A_{LWS}, A_{MWS}, A_{SWS} and A_{VS} refer to the sensitivity of the LWS, MWS, SWS and VS cone mechanisms, respectively, and k_1–k_{10} are the weight coefficients that represent the contribution of a particular class of cone. The sensitivity of each cone type was determined using the restricted fit visual pigment templates whose λ_{max} values were restricted to the ranges previously reported in adult Nile tilapia. The model was fitted using a least-squares fit. For all modelling, coefficients of determination or R^2 values were derived in order to assess the amount of variance accounted for by a model (Hughes et al., 1998). All analyses were performed using Microsoft Excel.

Lens transmittance

Spectral transmittance measurements (300–800 nm) of eleven lenses were made using a Cary 300 UV-Vis spectrophotometer (Varian, Inc, Palo Alto, CA, USA), following methods similar to those used by Douglas and McGuigan (Douglas and McGuigan, 1989). Lenses were mounted in a hole drilled in a block of black plastic, which fitted inside a standard sample cuvette. All transmission curves were normalized so that the transmission at 700 nm equalled 100% (Douglas and McGuigan, 1989).

RESULTS

ERG and CAP responses to 500 ms flashes of light were determined for a number of wavelengths and intensities (Fig. 2). RI curves were constructed and the intensities required to produce a half maximal response were determined as previously described. Fig. 2A,B shows the ERG and CAP responses to 640 nm light at various intensities. ERGs are characterized by a negative *a*-wave at stimulus onset, quickly followed by a positive *b*-wave, and then a positive *d*-wave at stimulus offset. Optic nerve recording waveforms exhibited positive responses at stimulus onset (CAP ON response) and offset (CAP OFF response).

Spectral sensitivity under white background conditions

The spectral sensitivity curves generated from both ERG and CAP recordings made under a white background are generally similar (Fig. 3). Adult Nile tilapia have visual capability from at least 360–720 nm (pre-retinal spectral filters notwithstanding), and show increased sensitivity to longer wavelengths (600–680 nm). At least three sensitivity peaks are present in all of the curves, although in comparison to the ERG data (sensitivity peaks at 380, 500–520 and 600–640 nm; Fig. 3A), the peaks in the CAP data are long-wavelength shifted (380–440, 560–580 and 640–680 nm; Fig. 3B,C). Evidence of a forth peak at around 460 nm is also seen in the CAP OFF data. The maximum sensitivity in the ERG and CAP curves is around 600–680 nm. Two distinct 'notches' or 'troughs' in sensitivity are apparent in the ERG sensitivity curve, at approximately 440–460 and 540 nm. Similar notches are not present in the CAP ON curve, but there is evidence of a notch at 620 nm in the CAP OFF curve.

In Figs 3 and 4, both restricted and best fit VS, SWS, MWS and LWS visual pigment templates are shown in combination with the spectral sensitivity curves. Table 1 shows the results of fitting restricted and best fit visual pigment templates to the spectral sensitivity curves. The peaks in the spectral sensitivity curve generated using ERG generally had a good fit with the restricted SWS and LWS cone visual pigment templates, but there was not such a good match between the violet- and medium-wavelength peaks and the VS and MWS cone templates, as indicated by the R^2 values and the differences in nanometres between the λ_{max} values of the restricted and best fit templates (Fig. 3A; Table 1). For the CAP sensitivity curves the restricted MWS and LWS cone templates did not fit the medium- and long-wavelength peaks well, and neither did the restricted SWS cone template fit the short-wavelength peak in the CAP ON curve, or the restricted VS cone template fit the violet wavelength peak in the CAP OFF curve (Fig. 3B,C; Table 1).

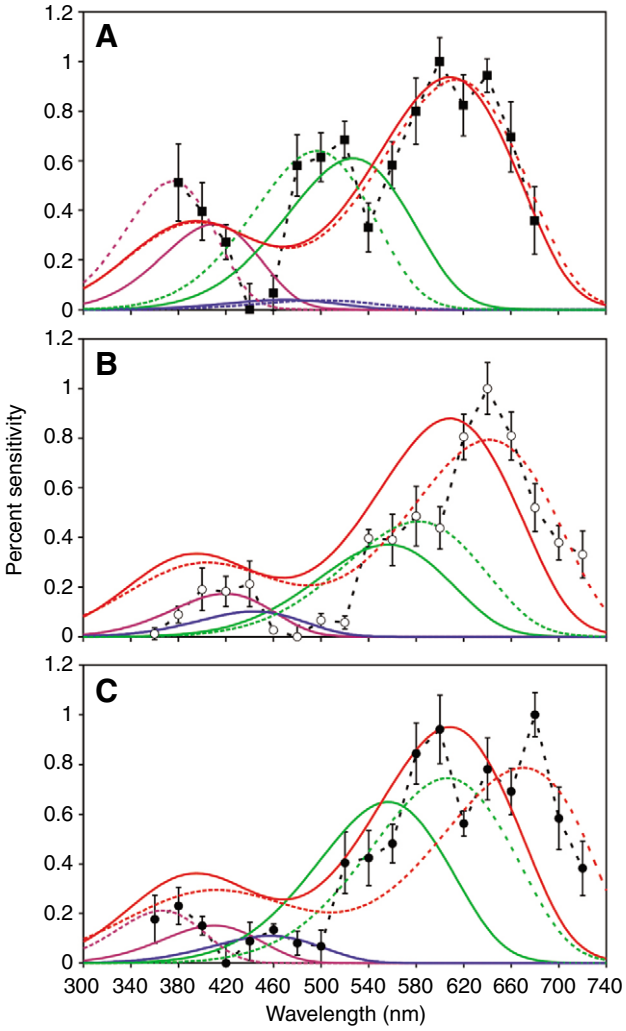

Fig. 3. Spectral sensitivity curves (black dashed lines) recorded under a white, broad spectrum adapting background using (A) electroretinograms and (B,C) optic nerve compound action potentials (CAPs; B, ON responses and C, OFF responses, respectively) in adult Nile tilapia. Error bars show standard error of the mean (*N*=6 or 7 except, A: 380 nm, *N*=5; 700 nm, *N*=3; B: 360 nm, *N*=4). Coloured solid and dashed lines (red, LWS cones; green, MWS cones; blue, SWS cones; violet; VS cones) represent restricted visual pigment templates the λ_{max} values of which have been restricted to the range previously reported for adults of this species (solid lines), and unrestricted, best fit visual pigment templates (dashed lines).

Chromatic adaptations

Under a long wavelength chromatic adapting background (Fig. 4) the sensitivity at longer, and to a lesser extent medium wavelengths is depressed, while sensitivity at violet and short wavelengths is increased, relative to the spectral sensitivity curves recorded under white background conditions. In particular, there is an increase in sensitivity in the 400–500 nm range, with the maximum sensitivity being between 380–420 nm. In the ERG curve (Fig. 4A), the sensitivity peak in the medium-wavelength range also becomes long-wavelength shifted relative to that seen under white background conditions, while in the CAP curves (Fig. 4B,C) this peak becomes somewhat short-wavelength shifted (as does the long-wavelength sensitivity peak in the CAP OFF curve). In the ERG curve there are two distinct notches at 500 nm and 580–600 nm, while in the CAP ON curve a notch is present at 420 nm and the sensitivity peak in the violet range was short-wavelength shifted to 380 nm. There is a notch at 480 nm in the CAP OFF curve. The peaks in the ERG spectral sensitivity curve fitted relatively well with restricted visual pigment templates, especially the templates for VS, SWS and MWS cone visual pigments (Fig. 4A; Table 1). Other than the VS cone template the restricted visual pigment templates fitted the CAP ON sensitivity curves reasonably well, especially at longer wavelengths (Fig. 4B; Table 1). A similar situation is seen with the CAP OFF curve, although in this case the restricted VS cone template is a better fit with the violet peak in the sensitivity curve (Fig. 4C; Table 1).

Modelling cone contributions

A multiple-mechanisms model was used to assess the relative input weight from each cone type to spectral sensitivity curves generated using ERG and CAP recordings. The weights assigned to each cone mechanism resulting in the best fit are presented in Table 2,

Table 1. Results of fitting visual pigment templates (Govardovskii et al., 2000) to spectral sensitivity curves generated using two different recording methods, ERG and CAP, using a least-squares approach

Background condition	Recording method	Visual pigment template	Wavelengths fitted to (nm)	Restricted fit		Best fit		Difference (nm)
				λ_{max} (nm)	R^2	λ_{max} (nm)	R^2	
White	ERG	VS	380–440	410.0	0.843	375.5	0.991	–34.5
		SWS	440–460	470.0	0.971	470.0	0.971	0.0
		MWS	480–540	527.0	0.897	496.8	0.979	–30.2
		LWS	560–680	608.5	0.956	614.2	0.966	+5.7
	CAP:	VS	360–460	419.6	0.975	419.6	0.975	0.0
	ON responses	SWS	440–480	443.7	0.900	443.7	0.900	0.0
		MWS	500–600	556.1	0.883	581.7	0.997	+25.6
		LWS	600–720	608.5	0.731	641.5	0.926	+33.0
	CAP:	VS	360–420	410.0	0.910	366.4	0.989	–43.6
	OFF	SWS	440–500	457.0	0.997	457.0	0.997	0.0
	responses	MWS	500–620	556.1	0.665	606.4	0.912	+50.3
		LWS	620–720	608.5	0.416	670.7	0.935	+62.2
600LP/650LP	ERG	VS	380–420	424.1	0.968	424.1	0.968	0.0
		SWS	440–500	443.7	0.831	440.7	0.835	–3.0
		MWS	520–580	539.7	0.804	539.7	0.804	0.0
		LWS	600–680	608.5	0.644	616.3	0.648	+7.8
	CAP:	VS	360–420	410.0	0.261	364.2	0.939	–45.8
	ON responses	SWS	440–480	470.0	0.935	480.1	0.939	+10.0
		MWS	480–600	527.0	0.942	510.3	0.984	–16.7
		LWS	620–720	608.5	0.976	613.4	0.977	+4.9
	CAP:	VS	360–420	410.0	0.613	384.3	0.831	–25.7
	OFF	SWS	420–460	443.7	0.981	423.7	0.998	–20.0
	responses	MWS	480–580	540.1	0.949	540.1	0.949	0.0
		LWS	600–720	608.5	0.977	618.2	0.987	+9.7

The wavelengths of maximum absorbance (λ_{max}) of the 'restricted fit' templates were restricted to the range reported for that particular visual pigment by Carleton et al. (Carleton et al., 2008) and K. Carleton, personal communication, whereas the 'best fit' visual pigment templates were unrestricted and were allowed to find the best fit to the curve. The difference between the best fit λ_{max} and the restricted fit λ_{max} is also shown.

along with the R^2 values, which indicate the total amount of variance accounted for by the model. The total amount of variance accounted for by the model was ≥0.947 for all of the spectral sensitivity curves. Figs 5 and 6 show the ERG and CAP spectral sensitivity curves for all background conditions plotted with the model based on Eqns 1–4. Across all the sensitivity curves, the sensitivity of the long-wavelength and violet regions was consistently fit by inhibitory interactions between the LWS and

Table 2. Results of modelling cone contributions to spectral sensitivity curves generated using two different recording methods, ERG and CAP

Background condition	Recording method	Cone mechanism	Wavelengths fitted to (nm)	Weight notation	Final weights				
					VS	SWS	MWS	LWS	R^2
White	ERG	VS	380–440	k_{10}, k_9	+2.218	–17.672	–	–	0.992
		SWS	440–460	k_8, k_7, k_6	–0.331	+1.211	+0.212	–	
		MWS	460–540	k_5, k_4, k_3	–	+4.314	+3.061	–2.594	
		LWS	560–680	k_2, k_1	–	–	–0.340	+1.073	
	CAP:	VS	360–460	k_{10}, k_9	+1.166	–0.309	–	–	0.983
	ON	SWS	460–480	k_8, k_7, k_6	–0.380	+1.098	+0.342	–	
	responses	MWS	500–600	k_5, k_4, k_3	–	–4.415	+0.215	+0.474	
		LWS	600–720	k_2, k_1		–	–3.970	+1.695	
	CAP:	VS	360–420	k_{10}, k_9	+3.892	–7.032	–	–	0.947
	OFF	SWS	420–500	k_8, k_7, k_6	–0.237	+0.626	+0.063	–	
	responses	MWS	500–620	k_5, k_4, k_3	–	–3.168	+0.196	+0.715	
		LWS	620–720	k_2, k_1	–	–	–4.426	+1.883	
600LP/	ERG	VS	380–420	k_{10}, k_9	+0.983	+0.020	–	–	0.957
650LP		SWS	440–500	k_8, k_7, k_6	–1.881	+2.875	–1.951	–	
		MWS	520–580	k_5, k_4, k_3	–	–5.205	+2.891	–2.419	
		LWS	600–680	k_2, k_1	–	–	–2.219	+1.662	
	CAP:	VS	360–420	k_{10}, k_9	+3.282	–5.610	–	–	0.982
	ON	SWS	460–480	k_8, k_7, k_6	–2.293	+5.008	–4.700	–	
	responses	MWS	480–600	k_5, k_4, k_3	–	+0.427	+0.862	+0.432	
		LWS	620–720	k_2, k_1	–	–	–0.425	+1.119	
	CAP:	VS	360–420	k_{10}, k_9	+2.563	–4.964	–	–	0.979
	OFF	SWS	420–460	k_8, k_7, k_6	–0.125	+1.941	_1.112	–	
	responses	MWS	480–580	k_5, k_4, k_3	–	+0.075	+0.963	+0.051	
		LWS	600–720	k_2, k_1	–	–	–0.283	+1.075	

The relative input weight from each cone mechanism is determined by assigning weights to the different cone mechanisms, using a least-squares approach, where a positive weight indicates an excitatory response and a negative weight indicates an inhibitory response. *k*, weight coefficient.

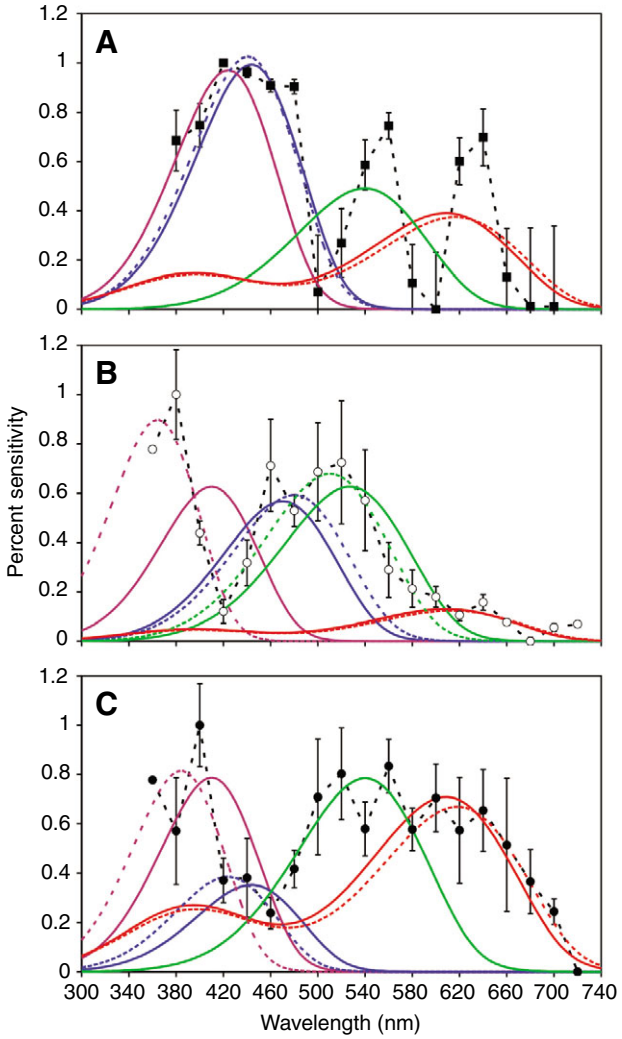

Fig. 4. Spectral sensitivity curves (black dashed lines) recorded under a long-wavelength adapting background using electroretinograms (A) and optic nerve compound action potentials (CAPs; B, ON responses and C, OFF responses, respectively) in adult Nile tilapia. Error bars show standard error of the mean (N=4 except, A: 360, 380, 620, 640, 680 and 700 nm, N=3; B: 380, 520 and 680 nm, N=3; C: 380, 540-680 nm, N=3). Coloured solid and dashed lines (red, LWS cones; green, MWS cones; blue, SWS cones; violet, VS cones) represent restricted visual pigment templates (solid lines) the λ_{max} values of which have been restricted to the range previously reported for adults of this species, and unrestricted, best fit visual pigment templates (dashed lines).

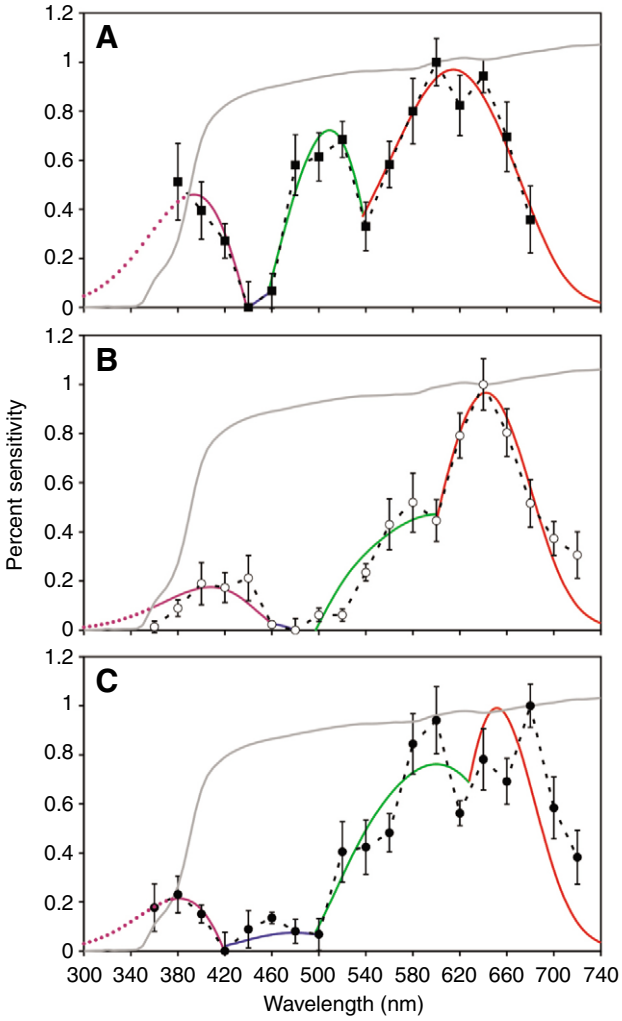

Fig. 5. Results of fitting a multiple mechanisms model to spectral sensitivity curves (black dashed lines) recorded under a white, broad spectrum adapting background using electroretinograms (A) and optic nerve compound action potentials (CAPs; B, ON responses and C, OFF responses, respectively) in adult Nile tilapia. Error bars show standard error of the mean (N=6 or 7 except, A: 380 nm, N=5; B: 360 nm, N=4; 520 nm, N=5; C: 360 nm, N=4). The multi-coloured line is the multiple mechanisms model that best described the data (see Eqns 1–4 and Table 2), and the different coloured sections represent the contributions of the LWS (red), MWS (green), SWS (blue) and VS (violet) cones. The grey line is the lens transmission curve (normalized to 100% at the highest sensitivity peak), and serves to illustrate how the VS cone mechanism is influenced by the spectral filtering of the lens (dotted violet line).

MWS cones (LWS-MWS) and the SWS and VS cones (VS-SWS), respectively, although the relative weights did vary (Table 2). The exception was the violet region of the ERG-generated curve recorded under a long-wavelength adapting background, which was fit by an excitatory interaction between the SWS and VS cones (VS+SWS). The nature of the interactions between cones contributing to the medium- and short-wavelength-sensitive regions varied depending on method (ERG or CAP) and the background condition (Table 2). The sensitivity of the medium-wavelength region was fit by interactions between the LWS, MWS and SWS cones (MWS+/–LWS+/–SWS), while the short-wavelength region was fit by interactions between the MWS, SWS and VS cones (SWS+/–MWS+/–VS).

Lens transmittance

The transmission spectra were essentially the same in the eleven lenses (4.2–5.0 mm diameter) measured and so lens spectral transmittance measurements were averaged (Fig. 7). Transmittance declined steeply from approximately 410 to 380 nm, followed by a slightly less steep decline to approximately 345 nm, below which no light was transmitted. The wavelength at which 50% of the maximal transmittance was reached (the $T_{0.5}$ value) was 393 nm. The effect of lens transmission on spectral sensitivity would be to truncate the short-wavelength limb of the VS cone visual pigment, so reducing the sensitivity to wavelengths below 400 nm as assessed using electrophysiology (Figs 5 and 6), especially under background conditions where the VS cone appears to be short-wavelength shifted.

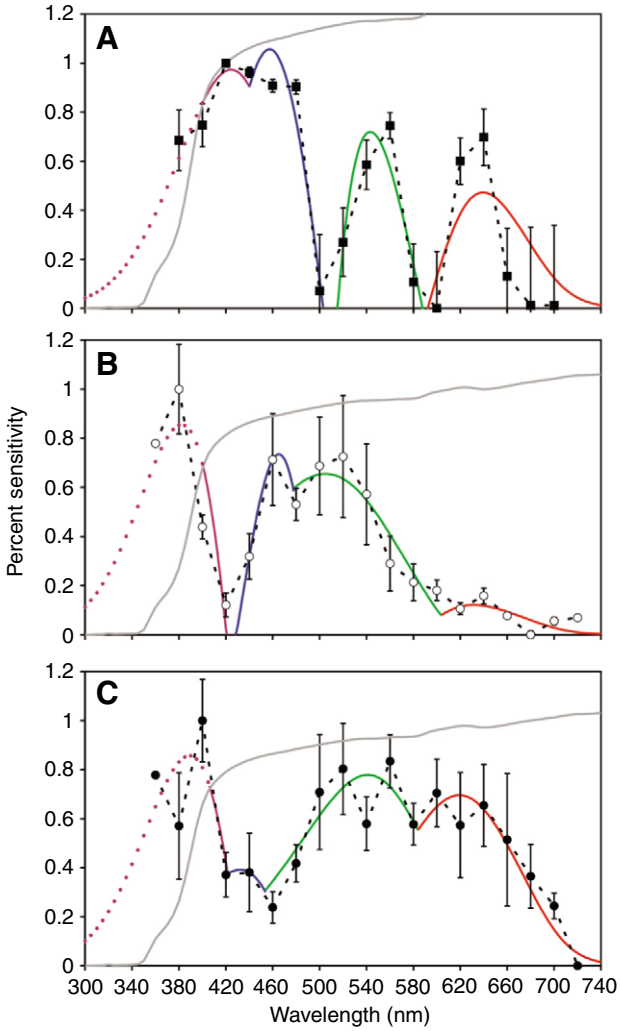

Fig. 6. Results of fitting a multiple mechanisms model to spectral sensitivity curves (black dashed lines) recorded under long-wavelength adapting backgrounds using electroretinograms (A) and optic nerve compound action potentials (CAPs; B, ON responses and C, OFF responses, respectively) in adult Nile tilapia. Error bars show standard error of the mean (N=4 except, A: 380, 620, 640 and 680 nm, N=3; B, 380 and 680 nm, N=3; C, 500 and 680 nm, N=3). The multi-coloured line is the multiple mechanisms model that best described the data (see Eqns 1–4 and Table 2), and the different coloured sections represent the contributions of the LWS (red), MWS (green), SWS (blue) and VS (violet) cones. The grey line is the lens transmission curve (normalized to 100% at the highest sensitivity peak except in B and C where it is normalized to 100% at the highest sensitivities of the CAP ON and OFF white curves; 640 nm and 680 nm, respectively), and serves to illustrate how the VS cone mechanism is influenced by the spectral filtering of the lens (dotted violet line).

DISCUSSION

Cichlid fishes predominately expresses three cone pigments (reviewed by Carleton, 2009). However, rare populations of spectrally distinct cones have been found in a number of species (Parry et al., 2005; Carleton et al., 2008), including the Nile tilapia. Adults of this species have SWS, MWS and LWS cones, based on *SWS2a*, *RH2aβ* and *LWS* cone opsins, respectively (Spady et al., 2006; Carleton et al., 2008). They also express low levels of *SWS2b* opsin and there is MSP evidence of VS cones in the retinas of adults. The aim of this study was to assess spectral sensitivity in adult fish using electrophysiology, and to look for evidence of a VS cone mechanism.

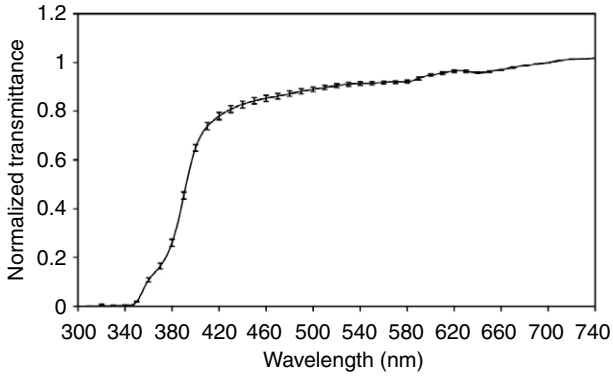

Fig. 7. Mean transmittance spectrum (normalized to 1 at 700 nm) of the lens in adult Nile tilapia. Error bars (only presented for every 10 nm for clarity) show standard error of the mean (N=11). The wavelength at 0.5 of the normalized transmittance ($T_{0.5}$) is 393 nm.

Spectral sensitivity curves and violet sensitivity

The spectral sensitivity curves presented in this paper indicate that adult Nile tilapia have four sensitivity peaks in the ranges 380–420, 440–480, 500–600 and 600–680 nm. The last three of these four sensitivity maxima correspond approximately to the visual pigment λ_{max} values reported for adults by Carleton et al. (Carleton et al., 2008), which are 449±5 nm (SWS cone), 542±6 nm (MWS cone) and 596±6 nm (LWS cone; all λ_{max} values are presented ±1 s.d.), although the sensitivity peaks described here tend to be more long-wavelength shifted. The variability in the position of the sensitivity peaks between our study and the MSP data of Carleton et al. (Carleton et al., 2008) is probably due to intraspecific variation in the A1/A2 ratio found in the Nile tilapia (Carleton et al., 2008) as well as opponent interactions between cone mechanisms (Neumeyer, 1984). The finding that the peak sensitivity is at long wavelengths (600–680 nm) is not unexpected as *LWS* is the dominant cone opsin class expressed in the retinas of adult fish, accounting for 80% of total retinal cone opsin expression (Spady et al., 2006; Carleton et al., 2008).

A 380–420 nm peak was seen in the spectral sensitivity curves recorded under a white, broad spectrum background. The continued presence of this peak under long-wavelength chromatic adapting background indicates that this is due to a VS cone mechanism not β-band of the LWS cone mechanism (Deutschlander and Phillips, 1995; Deutschlander et al., 2001). Using MSP, Carleton et al. (Carleton et al., 2008) found cones containing a VS cone visual pigment with a λ_{max} of 427±8 nm (±1 s.d.) in larvae and juvenile fish, and one VS cone in adult fish, with a λ_{max} of 438.7 nm. In addition, the qRT-PCR assessment of opsin expression in adults has shown that *SWS2b* opsin is present at low levels (less than 10% of total opsin expressed) (Spady et al., 2006; Carleton et al., 2008). This provides supporting evidence for the presence of a VS cone mechanism in adults, but it also indicates that such a VS cone type may not be very abundant in the retina. It is possible to miss a particular cone type using MSP, especially if it is sparsely distributed or concentrated in a particular region of the retina (Bowmaker et al., 1991; Shand, 1993). The fish used in our study may have more VS cones than those used by Carleton et al. (Carleton et al., 2008) because they were kept under different lighting conditions. Changes in the frequency of different cone classes in the retina in fishes can

be influenced by the spectral composition of the lighting under laboratory (Kröger et al., 1999; Kröger et al., 2003; Shand et al., 2008) and natural (McDonald and Hawryshyn, 1995; Fuller et al., 2004) conditions. Carleton (Carleton, 2009) has also reported differences in opsin gene expression between lab-reared and wild-caught cichlids. Another potential factor could be that, as a result of thousands of years of 'domestication' and selective breeding (Kocher et al., 1998; El-Sayed, 2006), different strains or populations of Nile tilapia vary in their visual capabilities. In larval and juvenile Nile tilapia there is a greater proportion of VS and indeed ultraviolet-sensitive (UVS) cones in the retina, and correspondingly higher levels of *SWS2b* and *SWS1* opsins (Spady et al., 2006; Carleton et al., 2008). This apparent ontogenetic shift in spectral sensitivity, with a general loss in short wavelength sensitivity and increase in long wavelength sensitivity, is mirrored by changes in lens transmission (Thorpe and Douglas, 1993). In the adult lenses assessed in this study, transmittance declined steeply from approximately 410 to 380 nm, with a $T_{0.5}$ value of 393 nm. This would have the effect of truncating the short-wavelength limb of the VS cone visual pigment.

There is limited electrophysiological evidence from a small number of Lake Malawi cichlids that ERG-derived spectral sensitivity curves are modelled more adequately with four visual pigments as opposed to three (Garner et al., 2003), indicating that a small population of LWS cones is present in the retina and contributes to spectral sensitivity in addition to the UVS/VS, SWS and MWS cones commonly found in Lake Malawi species (Carleton et al., 2006; Carleton, 2009). Unfortunately, cone opsin expression studies or the characterization of cone visual pigments using MSP have not yet been performed on any of the species used by Garner et al. (Garner et al., 2003). Given that it is becoming increasingly apparent that cichlid visual communication is important for mate choice and speciation through sensory drive (Seehausen et al., 1997; Seehausen et al., 2008; Maan et al., 2006; Carleton, 2009), it is important to have a better appreciation of cichlid spectral sensitivities than we currently do. We are currently using electrophysiology to assess spectral sensitivity in Lake Malawi cichlids in our laboratory.

The spectral sensitivity curves generated using ERGs and CAPs are generally in congruence with each other and both reveal increased sensitivity to longer wavelengths. However, the peaks in the CAP curves are generally longer-wavelength shifted than those in the ERG curves, and the position of notches in the spectral sensitivity curves varies between the recording techniques. As the ERGs and CAPs were performed at different times of the year for logistical reasons (ERGs in February to March, CAPs in August to October) and on different sized fish (average size of fish used for ERGs: 20.8±0.7 cm TL, 167.0±15.0 g; average size of fish used for CAPs: 24.5±1.0 cm TL, 267±28.6 g; all values ±1 s.e.m.) shifts in the A1/A2 ratio seem plausible. Shifts in the A1/A2 ratio are well documented in over 150 fish species (Toyama et al., 2008) and appear to be associated with the seasons and/or a migration or metamorphic event, and are regulated by hormones (Bridges, 1972). Carleton et al. (Carleton et al., 2008) reported A1/A2 shifts in Nile tilapia that were variable between individuals, but it is currently unknown what factors influence A1/A2 shifts in this species.

Differences between the ERG and CAP ON generated curves may also reflect differences between the inner retina (early stage processing) and outer retina (late stage processing). The ERG *b*-wave reflects the activity of ON bipolar cells and the CAPs reflect the activity of the ganglion cells (Cameron, 2002; Ramsden et al., 2008). Studies that compare ERGs and CAPs in the same species are rare, but Ramsden et al. (Ramsden et al., 2008) found differences in the polarization sensitivity curves of rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) using both techniques. These differences could be removed pharmacologically (using cobalt to block horizontal cell feedback), suggesting that the differences are due to outer retinal inhibition derived from feedback of horizontal cells onto cones.

The distinct notches in the spectral sensitivity curves indicates the presence of chromatic opponent interactions and further evidence for this is provided by using a multiple-mechanisms model to assess the spectral sensitivity curves. The sensitivity of the long-wavelength region was described by inhibitory interactions between the LWS and MWS cones (LWS-MWS). Such LWS-MWS opponency appears to be common in vertebrates (Sperling and Harwerth, 1971; Neumeyer, 1984; Kalloniatis and Harwerth, 1990). The sensitivity of the medium-wavelength region was modelled as being the result of interactions between three cone mechanisms, and the nature (i.e. excitatory or inhibitory) of the interaction varied. Neumeyer (Neumeyer, 1984) found that the medium-wavelength region of the behaviourally derived goldfish spectral sensitivity curve was best described by the MWS cone mechanism receiving inhibitory input from the LWS and SWS cones. The short-wavelength and violet regions of the sensitivity curves were fitted by interactions between the SWS and VS cones, with the MWS cone also contributing to the short-wavelength region. Opponent interactions between SWS and UVS cone mechanisms have been reported in fish (Hashimoto et al., 1988) and turtles (Ventura et al., 2001), but such descriptions are uncommon, perhaps because the discovery of widespread ultraviolet- and violet-sensitivity in vertebrates has been relatively recent. As a species with four cone mechanisms can have up to 16 (2^4) possible combinations of excitatory and inhibitory neurons in its retina (Ventura et al., 2001), the possibility that fish with four cone mechanisms exhibit a larger number of opponent interactions than have been previously described should not be ruled out and warrants further investigation. The strong evidence for chromatic opponent interactions indicates that adult Nile tilapia possess retinal mechanisms necessary for colour vision, which may potentially be tetrachromatic in this species, although this needs to be assessed and confirmed by behavioural discrimination measures (Neumeyer, 1992).

Spectral sensitivity curves generated from CAP OFF responses tend to be dominated the MWS cone mechanism in fishes (Novales Flamarique and Hawryshyn, 1996; Novales Flamarique and Hawryshyn, 1997; Novales Flamarique and Hawryshyn, 1998; Barry and Hawryshyn, 1999; Parkyn and Hawryshyn, 2000). This does not appear to be the case in adult Nile tilapia, although under the white background condition the MWS cone sensitivity peak is only slightly lower than the LWS cone sensitivity peak, and the MWS cone appears to have a substantial inhibitory effect on the LWS cone, pushing the sensitivity peak to longer wavelengths. A similar result has been found in rainbow trout (Parkyn and Hawryshyn, 2000). The ON response is important in detecting targets that are brighter than the background, while the OFF response is used to detect targets that are darker than the background, i.e. shadows (Wheeler, 1979; Barry and Hawryshyn, 1999; Parkyn and Hawryshyn, 2000). For species where the spectral properties of their natural habitats have been characterized, the peak of the MWS cone OFF response tends to fall within the most abundant wavelengths (Novales Flamarique and Hawryshyn, 1996; Barry and Hawryshyn, 1999). The spectral characteristics of the ambient underwater light in the natural habitats of Nile tilapia has not been determined, but if longer wavelength light is more abundant this may in part explain why the MWS cone mechanism does not appear to dominate the OFF response.

Spectral sensitivity and visual ecology

Nile tilapia evolved as a riverine fish living in marginal waters and floodplain pools in central and eastern Africa, feeding on benthic detritus, algae and invertebrates (El-Sayed, 2006; Lim and Webster, 2006). Like many freshwater habitats these are likely to have a restricted spectral range with maximum transmission levels in the medium- and long-wavelength regions of the spectrum (Bowmaker, 1995). The optical properties of the water will also vary considerably on a temporal scale, especially during the wet season when influxes of suspended particles from runoff change the water colour and increase levels of turbidity (Bowmaker, 1995). Nile tilapia have a major influence on their underwater light environment as they can contribute to sustaining eutrophication by enhancing nutrient cycling (Starling et al., 2002) through phosphorus excretion and phosphorus release from sediments disturbed during foraging and nest building. The sensitivity to long wavelengths is probably an adaptation to the predominant wavelengths of light in such habitats. Increased sensitivity to longer wavelengths may also be important during sexual selection. Males have conspicuous red breeding colours (Lim and Webster, 2006), which may be important signals for females during mate choice (Castro et al., 2009) and for the detection of rival males during male–male competition (Boulcott and Braithwaite, 2007). The VS and SWS cones are presumably offset from the prevailing background light and may function as contrast detectors of brightly reflecting targets, such as conspecifics or predators, against a darker background (Lythgoe, 1968; Loew and Lythgoe, 1978; McFarland and Munz, 1975). Visual communication is important for social interaction in Nile tilapia (Volpato et al., 2003; Lim and Webster, 2006; Castro et al., 2009), which in turn has implications for the successful aquaculture of this species (Fessehaye et al., 2006). As Nile tilapia have body patches that reflect in violet wavelengths (S. M. Gray, M. Tremblay, F. Hart and C. W. Hawryshyn, unpublished data) the possibility that shorter wavelength signals are also used for visual communication should not be ruled out. Given the highly variable nature of their natural habitat, it would not be surprising if Nile tilapia had evolved a generalist visual system, as has been suggested for salmonids (Parkyn and Hawryshyn, 2000) and the capacity to respond to environmental changes by regulating cone opsin expression, A1/A2 ratio and therefore spectral sensitivity (Shand et al., 2002). This may play a role in the ‘ecological flexibility’ of this species and its ability to successfully adapt to a wide range of environmental conditions.

ACKNOWLEDGEMENTS

We would like to thank the Editor and two anonymous reviewers for their comments and feedback, which greatly improved this paper; Northern Tilapia Inc., Lindsay, Ontario, Canada for supplying the fish; and Corey Coffin, Matthew Gizzi, Suzanne Gray and Chengfeng Xiao for technical assistance and useful input at various stages of the project. Karen Carleton kindly allowed us to use her unpublished, raw MSP data and also contributed much helpful feedback on the manuscript. This research was supported by an NSERC Discovery Grant and the Canada Research Chair program (C.W.H.). The experiments described in this paper comply with the ‘Principles of animal care’, publication 86-23, revised 1985 of the National Institute of Health and also with the Queen’s University Animal Care Committee under the auspices of the Canadian Council on Animal Care. T.J.L. was supported by a post-doctoral stipend from the Carl Tryggers Foundation for Scientific Research during the analysis and write-up of this research.

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