

Cone topography and spectral sensitivity in two potentially trichromatic marsupials, the quokka (*Setonix brachyurus*) and quenda (*Isoodon obesulus*)

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The potential for trichromacy in mammals, thought to be unique to primates, was recently discovered in two Australian marsupials. Whether the presence of three cone types, sensitive to short- (SWS), medium- (MWS) and long- (LWS) wavelengths, occurs across all marsupials remains unknown. Here, we have investigated the presence, distribution and spectral sensitivity of cone types in two further species, the quokka (*Setonix brachyurus*) and quenda (*Isoodon obesulus*). Immunohistochemistry revealed that SWS cones in the quokka are concentrated in dorso-temporal retina, while in the quenda, two peaks were identified in naso-ventral and dorso-temporal retina. In both species, MWS/LWS cone spatial distributions matched those of retinal ganglion cells. Microspectrophotometry (MSP) confirmed that MWS and LWS cones are spectrally distinct, with mean wavelengths of maximum absorbance at 502 and 538 nm in the quokka, and at 509 and 551 nm, in the quenda. Although small SWS cone outer segments precluded MSP measurements, molecular analysis identified substitutions at key sites, accounting for a spectral shift from ultraviolet in the quenda to violet in the quokka.

The presence of three cone types, along with previous findings in the fat-tailed dunnart and honey possum, suggests that three spectrally distinct cone types are a feature spanning the marsupials.

Keywords: cone topography; spectral sensitivity; marsupial; opsin

1. INTRODUCTION

Until recently, dichromacy was thought to be the most prevalent system of mammalian colour vision, based on two cone pigments sensitive to short (SWS) and medium-to-long (MWS/LWS) wavelengths (Jacobs 1993; Szél *et al.* 1996; Ahnelt & Kolb 2000). The only exception was thought to be in primates where a third cone pigment had arisen by duplication of the MWS/LWS gene (Bowmaker 1991; Dulai *et al.* 1994; Yokoyama 2000; Ebrey & Koutalos 2001). The genetic division gave rise to two spectrally distinct cone types with sensitivities at approximately 530 nm (MWS/LWS green) and 560 nm (MWS/LWS red). Among the mammals, the trichromatic colour vision of primates was thought to be unique (Mollon 1991; Yokoyama 2000; Regan *et al.* 2001).

However, the discovery of three spectrally distinct cone types (SWS, MWS and LWS) in two Australian marsupials, the fat-tailed dunnart (*Sminthopsis crassicaudata*) and honey possum (*Tarsipes rostratus*), has provided evidence for the potential of trichromacy in mammals other than primates (Arrese *et al.* 2002).

To date, it is unknown whether other marsupials also possess the basis for a trichromatic colour system. A behavioural study (Hemmi 1999) and a molecular analysis

(Deeb *et al.* 2003) indicated dichromacy in the tamar wallaby, however, microspectrophotometric (MSP) data have not been obtained for this species. In addition, the presence, distribution and spectral characteristics of different cone types in marsupials remain poorly understood. The present study investigates spectral sensitivity in a small wallaby, the quokka (*Setonix brachyurus*) and a bandicoot, the quenda (*Isoodon obesulus*). These emblematic Western Australian marsupials are representative of the two major taxonomic divisions of marsupials, the diprotodonts and polyprotodonts, respectively.

Here, we provide evidence for the presence of three spectrally distinct cone types in both species, and describe the topography of the two immunohistochemically defined cone populations, SWS and M/LWS. As SWS cone outer segments in both species were unusually small for mammalian cones, we determined their opsin sequence to investigate and compare spectral tuning mechanisms.

2. MATERIALS AND METHODS

(a) Animals

Quokkas ($n=6$) were obtained from a breeding colony established at the University of Western Australia (Animal Service Division, Shenton Park). Quendas ($n=6$) were collected in the Harry Waring Marsupial Reserve using cage traps under licence from the Department of Conservation and Land Management. Owing to the threatened status of the

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Table 1. Sequences of oligonucleotide primers.

primer no.	sequence (5' to 3')
SWS1F63	SCCYCAGTACCACMTYGC
SWS1R905	GCAGTAGATGATGGGRTTGTA
SWS1F1	ATGTCAGGGGAYGAGGAGTT
SWS1R209	ATGTAGTTGAGTGGCTGGCG
SWS1F777	CTATGTGCCCTATGCTGCC
3' adapter primer	GACTCGAGTCGACATCGATA

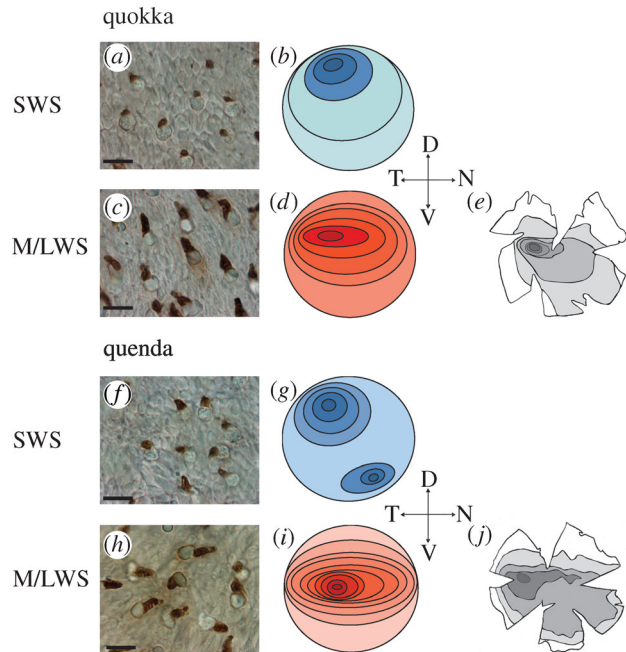


Figure 1. Immunolabelling of quokka (*a, c*) and quenda (*f, h*) retinæ. Photomicrographs of wholemounts treated with JH455 (*a, f*) and JH492 (*c, h*). Scale bar, 8 μ m. Contour maps for each cone type in quokka (*b, d*) and quenda (*g, i*): darker shades indicate highest densities, lighter shades, lowest densities. Retinal ganglion cell topographies in quokka (*e*; modified from [Beazley & Dunlop 1983](#)) and quenda (*j*; modified from [Tancred 1981](#)), showing distributions similar to M/LWS cones.

species (2000 IUCN Red List of Threatened Species), access to material was limited. Animals were terminally anaesthetized with Saffan (alphaxalone and alphadolone acetate, 0.1 ml per 10 g body weight, intraperitoneally). For MSP measurements, animals were dark adapted overnight before euthanasia. The study was approved by the Animal Ethics and Experimentation Committee of the University of Western Australia.

(b) Immunohistochemistry

(i) Tissue preparation

Eyes were removed and immersed in 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS) at pH 7.4 for 60 min. A dorsal cut was placed in the cornea for orientation. Wholemounts were prepared by dissecting the retina from the eye cup after removal of the lens and vitreous. The sclera and pigment epithelium were removed and the retina flattened, photoreceptor layer uppermost, on a gelatinized slide. The wholemounted retina was viewed in a light microscope and an outline, with salient features, was drawn to allow identification of sample areas.

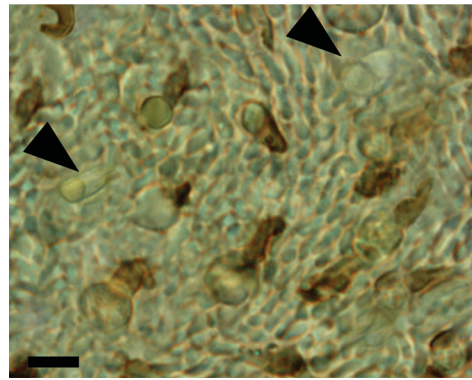


Figure 2. Wholemounted retina of quenda incubated with JH455 and JH492 revealing the presence of unlabelled outer segments (arrow head). Scale bar, 10 μ m.

(ii) Immunolabelling

Affinity purified rabbit antisera, JH455 (raised against human 'blue-cone' opsin) and JH492 (raised against human green- and red-cone opsin) were kindly supplied by Dr J. Nathans (Johns Hopkins University School of Medicine, Baltimore, MD, USA). Immunolabelling was performed on wholemounts, as previously described ([Arrese *et al.* 2003](#)).

(iii) Topography

Immunolabelled cones were counted using transmitted light microscopy at 1000 \times with a 100 \times oil immersion objective. Single cones were counted separately from double cones (considered one cone) but data were combined for density estimates. For each wholemount, 5% of retinal area was sampled at regular intervals using an eyepiece graticule ([Beazley & Dunlop 1983](#)). Sampling was increased in regions where cone density was high. Shrinkage owing to processing (less than 10%) was taken into account when constructing cell density maps.

(iv) Microspectrophotometry

Following enucleation, eyes were immersed in PBS and retinal tissue was dissected for examination by microspectrophotometry, following procedures described by [Shand *et al.* \(2002\)](#). Briefly, dissections were conducted in darkness under infrared (IR) illumination and visualized using a microscope fitted with a Find-R-Scope (IR Viewer, Electrophysics, Fairfield, NJ, USA). Retinal preparations were mounted in a solution of PBS containing 10% dextran (Sigma 250k RMM), on a rectangular 50 mm \times 22 mm No. 1 cover-slip, covered with a smaller cover-slip (19 mm \times 19 mm), and sealed with nail varnish to prevent dehydration.

(v) Measurements of absorbance spectra

A single-beam wavelength scanning microspectrophotometer was used to measure the spectral absorbance of the outer segments of individual photoreceptors and of the associated oil droplets of cones. The equipment has been described previously by [Partridge *et al.* \(1992\)](#), with recent modifications improving the optics and, hence, the transmission delivery of short wavelengths to the specimen ([Shand *et al.* 2002](#)). Sample and baseline scans were made from cellular and tissue-free samples, respectively. Measurements were made by placing the outer segment in the path of the measuring beam (2 \times 2 μ m²) and scanning over the wavelength range 350–750 nm. All cone inner segments

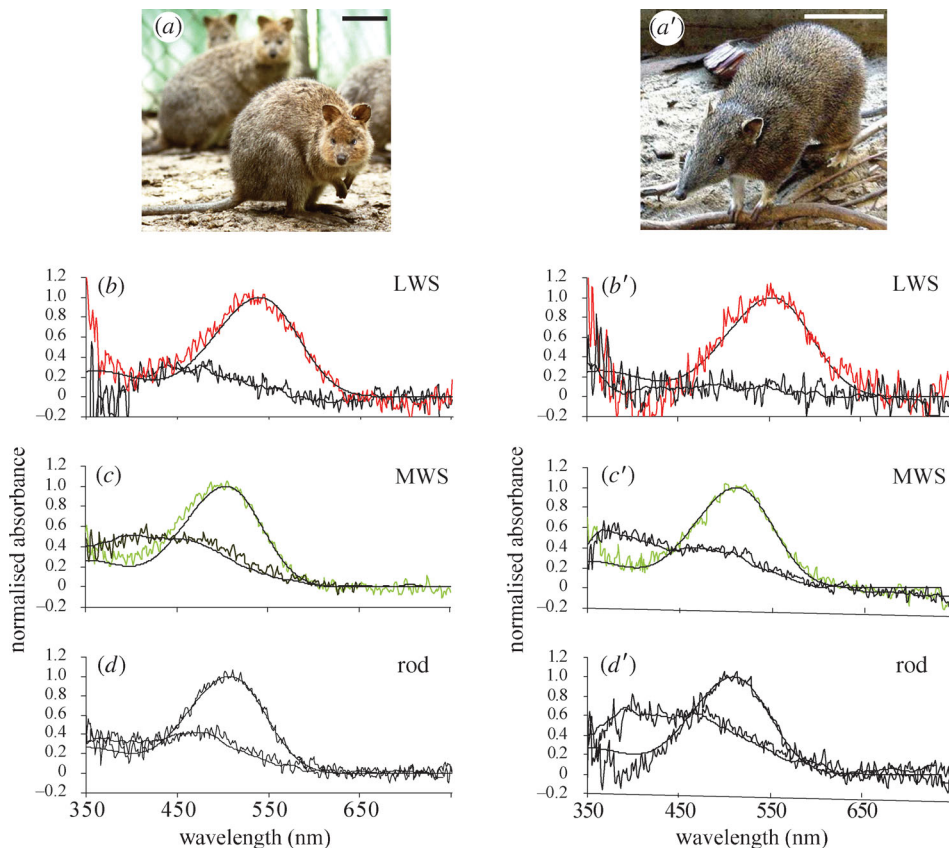


Figure 3. The diprotodont quokka (*a*) and polyprotodont quenda (*a'*). Normalized mean averaged absorbance spectra of visual pigments in the quokka (*b–d*) and quenda (*b'–d'*), showing pre-bleach spectra (upper traces) with best-fitted visual pigment templates (solid lines) and post-bleach spectra (lower traces) with running averages (solid lines). Scale bar, 10 cm.

possessed an oil droplet. For each outer segment or oil droplet, one 'pre-bleach' sample scan was combined with two separate baseline scans from an adjacent area, and the two absorbance spectra obtained were averaged. Following the pre-bleach scans, outer segments were bleached for 2 min with white light from the monochromator, and an identical number of sample and baseline scans were made to confirm that the molecules were photolabile (i.e. indicated a visual pigment).

(vi) Analysis

Baseline and sample data were converted to absorbance values at 1 nm intervals. Upward and downward scans were averaged together by fitting a weighed three-point running average to the absorbance data (Hart *et al.* 2000). Absorbance spectra were normalized to the peak and long-wavelength offset absorbances were obtained by fitting a variable-point unweighted running average. Following the method of MacNichol (1986), a regression line was fitted to the long wavelength limb of the normalized absorbance spectrum between 30 and 70% of the normalized maximum absorbance. The regression equation was used to predict the wavelength of maximum sensitivity (λ_{\max}) and fit the visual pigment template following the methods of Govardovskii *et al.* (2000). Acceptable pre-bleach spectra showing a clear alpha peak and that were fitted well by the A_1 template were averaged together and reanalysed (Levine & MacNichol 1985).

(vii) Molecular biology

Total RNA was extracted from freshly dissected retinae using the EpiCentre MasterPure RNA Purification Kit.

Purification of mRNA was performed using Qiagen Oligotex Spin Columns. Single stranded cDNA was synthesized using an oligo-d(T) anchor primer and Invitrogen Superscript III RT Polymerase.

For both species, primers SWS1F63 and SWS1R905 (table 1) were used to generate a 843 bp fragment. The 5' sequence was completed with primers by SWS1F1 and SWS1R209 and the 3' sequence with primers SWS1F777 and 3' adapter primer. PCR products were visualized by agarose gel electrophoresis and cloned into a Promega pGEM T-Easy plasmid. Positive colonies were sequenced using T7 and SP6 primers. Sequencing was carried out on both strands using Big Dye Terminator Version 3.1 and an ABI 3730 sequencer.

3. RESULTS

(a) Immunohistochemistry

The presence of unlabelled cones in retinae incubated with JH455 or JH492 confirmed the specificity of the antisera. The two cone types were strongly immunopositive to their respective antisera. In both species, the outer segments of SWS cones were considerably smaller than those of M/LWS cones (figure 1*a,c,f,h*). As reported for the fat-tailed dunnart and honey possum (Arrese *et al.* 2003), SWS cones were far outnumbered by M/LWS cones. All cone populations appeared to contain an oil droplet.

In the quokka, SWS cone density was highest in the dorso-temporal retina (2900 mm^{-2} , s.d. ± 128 , figure 1*b*), with the lowest density located in far-ventral periphery (2100 mm^{-2} , s.d. ± 89 , figure 1*b*). Densities elsewhere were uniform (between 2400 and 2500 mm^{-2} ,

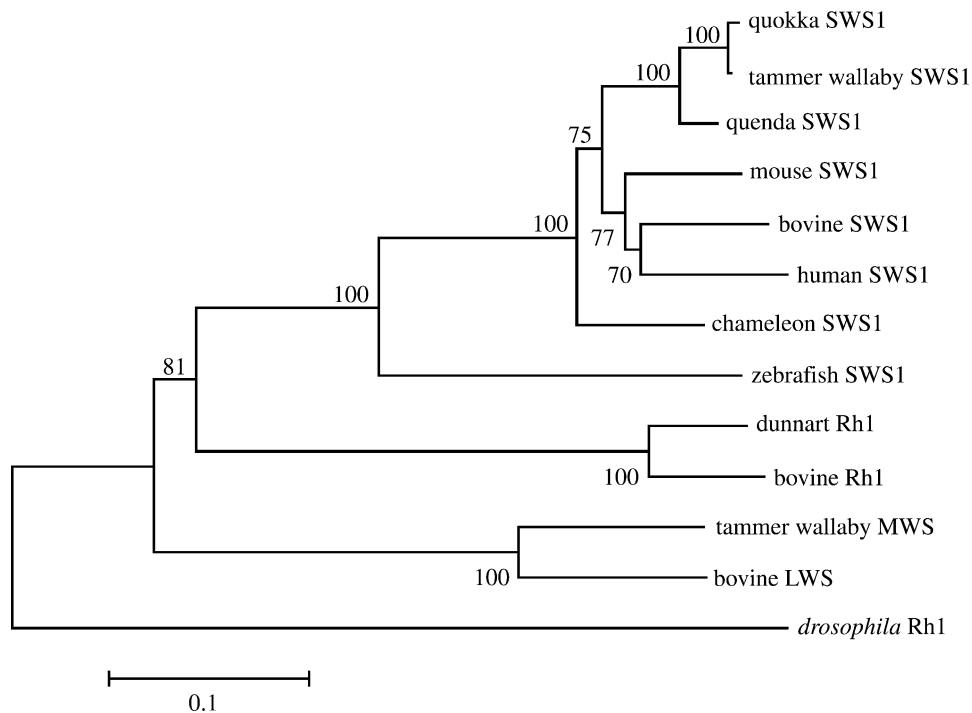


Figure 4. Phylogenetic tree of marsupial SWS1 and other opsins generated by the neighbour-joining method (Saitou & Nei 1987) with 1000 bootstrap replications. The calibration bar shows substitutions per site. Amino acid sequences were aligned by Clustal X (Higgins *et al.* 1996).

s.d. ± 113 , figure 1*b*). In the quenda, the topography of the SWS cones revealed two regions of peak density located in dorso-temporal (2300 mm^{-2} , s.d. ± 121 , figure 1*g*) and naso-ventral retina (2200 mm^{-2} , s.d. ± 117 , figure 1*g*). SWS cone densities decreased concentrically with the lowest concentration of SWS found in far-nasal and -temporal retina (1500 mm^{-2} , s.d. ± 63 , figure 1*g*).

M/LWS cone topography of the quokka revealed a horizontal streak with a peak density in mid-dorso-temporal retina ($27\,000 \text{ mm}^{-2}$, s.d. ± 1100 , figure 1*d*) decreasing to values of $18\,000 \text{ mm}^{-2}$ (s.d. ± 650) in ventral periphery (figure 1*d*). M/LWS cone distribution was similar to that of retinal ganglion cells, which form an *area centralis* in mid-temporal retina embedded in a visual streak (figure 1*e*; Beazley & Dunlop 1983). In the quenda, M/LWS density decreased concentrically from a horizontal streak in mid-temporal retina ($21\,000 \text{ mm}^{-2}$, s.d. ± 760 , figure 1*i*), to the dorsal and ventral periphery ($12\,000 \text{ mm}^{-2}$, s.d. ± 530 , in figure 1*i*). Except for a more centrally located peak density, M/LWS cone distribution resembled that of retinal ganglion cells (figure 1*j*; Tancred 1981).

In both species, as in the fat-tailed dunnart and honey possum (Arrese *et al.* 2003), consecutive incubations of a same wholemout with the two antisera revealed the presence of a small minority of unlabelled cones (figure 2).

(i) Microspectrophotometry

The mean wavelengths of maximum absorbance (λ_{max}) of the cone visual pigments were 502 nm ($n=17$, s.d. = 5.4) and 538 nm ($n=12$, s.d. = 9.8) in the quokka, and 509 nm ($n=10$, s.d. = 6.9) and 551 nm ($n=5$, s.d. = 10.3) in the quenda, representing sensitivity to MWS and LWS, respectively (figure 3). The MWS- and LWS-visual pigments were found in either single or double cones,

but not as MWS/LWS pairings. A minority of cones with small outer segments (presumed SWS cones) were measured, but they did not provide useable spectra. The λ_{max} of the rod visual pigment was determined at 505 nm ($n=12$, s.d. = 5.7) in the quokka and 508 nm ($n=9$, s.d. = 5.1) in the quenda (figure 3*d,d'*). Oil droplets were found to be transparent in both species, as indicated by their negligible absorbance (<0.002) between 350 and 750 nm.

(ii) Molecular biology

Coding sequences of the SWS1 opsin for the quokka and quenda, derived from retinal cDNA, have been deposited in GenBank (accession numbers AY726545 and AY726544, respectively). A phylogenetic tree was generated by neighbour-joining (Saitou & Nei 1987) from amino acid sequence data of the two marsupial opsins together with other mammalian and non-mammalian opsins (figure 4). The cladding of the quokka and quenda SWS1 with the SWS1 sequences of placental mammals demonstrates that the genes are orthologous.

Sequence comparison between the two species reveals a total of 17 amino acid differences, including site 86, which is critical in determining ultraviolet (UV) or violet sensitivity. In the quokka, Tyr86 implies that a violet sensitive (VS) SWS1 pigment is present, whereas Phe86 in the quenda indicates a UVS pigment. Tyr86 is present in ungulate (Cowling *et al.* 2002; Fasick *et al.* 2002) and tammar wallaby (Deeb *et al.* 2003) VS pigments, whereas Phe86 is retained in mouse and rat UVS pigments (Yokoyama 2000). In addition, the two SWS1 sequences differed at site 114, with Gly in the quokka and Ala in the quenda.

4. DISCUSSION

Our results provide evidence for the presence of three spectrally distinct cone photoreceptor types in the quokka

Table 2. Variation at five key amino acid sites of vertebrate SWS1 pigments.

species	λ_{\max} (nm)	46	52	86	114	118
bovine	438	Phe	Thr	Tyr	Ala	Cys
human	424	Thr	Phe	Leu	Gly	Thr
tammar wallaby	424	Phe	Thr	Tyr	Gly	Ser
quokka	(VS)	Phe	Thr	Tyr	Gly	Ser
quenda	(UVS)	Phe	Thr	Phe	Ala	Ser
mouse	358	Phe	Thr	Phe	Ala	Ser
chameleon	358	Phe	Thr	Phe	Ala	Ser
zebrafish	362	Phe	Thr	Phe	Ala	Ser

and quenda. The three cone types, SWS, MWS and LWS, forming the basis for trichromacy, were also reported in the fat-tailed dunnart and honey possum (Arrese *et al.* 2002). The findings are consistent in the four phylogenetically distant species, indicating that the presence of three spectrally distinct cone types is a common feature of marsupials.

Cone topographies suggesting regional specializations related to visual tasks (Hemmi & Grünert 1999; Ahnelt & Kolb 2000; Peichl *et al.* 2000; Arrese *et al.* 2003) varied between the quokka and quenda.

(a) Cone topographies

Cone topographies in the two marsupials reveal three distinct regions of distribution, with species-specific differences. In both species, the combination of a high SWS cone distribution with a moderate to high M/LWS cone density in dorsal retina, would optimize spectral discrimination in the inferior field of view, as reported for the tammar wallaby (Hemmi & Grünert 1999) and fat-tailed dunnart (Arrese *et al.* 2003). In the mid-temporal retina, the high density of M/LWS cones coincides with the ganglion cell *area centralis* embedded in a visual streak (quokka; Beazley & Dunlop 1983, quenda; Tancred 1981). Visual acuity would be enhanced in these regions of highest densities of M/LWS cones and ganglion cells. In the ventral retina of the quokka, the high convergence of M/LWS cones to a low density of ganglion cells would enhance contrast sensitivity in the superior field of view (Hemmi & Grünert 1999). By contrast, spectral discrimination in the superior field of view of the quenda would be optimized by the higher density in naso-ventral retina of SWS cones and their combination with a moderate M/LWS cone distribution in naso-ventral retina. Spectral discrimination and visual acuity in the inferior and frontal fields would enhance food detection and acquisition in both species. However, different capabilities in the superior fields of the quokka and quenda may be related to their different habitats. Although contrast sensitivity is compatible with the detection of airborne predators in the open habitat of the quokka, enhanced spectral discrimination may further increase predator visibility against the background of *Banksia* woodland foliage.

(b) Spectral sensitivities

Measurements in the quokka and quenda provide evidence for the presence of MWS cones, which have been lost from placental mammals. In both species, MWS cones possess similar spectral sensitivities and photochemical properties to those of rods (figure 3*b,c,e,f*), with a post-bleach build-up of photoproduct

at wavelengths below 450 nm, as reported for the fat-tailed dunnart and honey possum (Arrese *et al.* 2002) and birds (Bowmaker *et al.* 1997; Das *et al.* 1999; Hart 2001). The λ_{\max} of MWS cones is identical in the polyprotodont quenda and fat-tailed dunnart (509 nm) and similar in the diprotodont quokka (502 nm) and honey possum (505 nm; Arrese *et al.* 2002). In contrast, the LWS cones absorb at longer wavelengths, with a wider range of λ_{\max} values at 535 nm (fat-tailed dunnart), 538 nm (quokka), 551 nm (quenda) and 557 nm (honey possum), respectively. The λ_{\max} of the rod visual pigment in the quokka (505 nm) and quenda (508 nm) is characteristic of mammalian rods.

(c) Molecular biology

The majority of amino acid differences in SWS1 sequences of the quokka and quenda are conservative changes that would not be expected to affect the spectral characteristics of the pigment (Yokoyama 2000; Cowing *et al.* 2002; Fasick *et al.* 2002; Hunt *et al.* 2004). Among the key tuning sites at which substitutions generate a spectral shift in mammalian SWS1 pigments (Yokoyama 2000; Cowing *et al.* 2002; Fasick *et al.* 2002), the amino acid at site 86 was found to differ between the two marsupial opsins (table 2). Phe86 is thought to be present in the ancestral, ultraviolet sensitive SWS1 pigment (Yokoyama & Shi 2000; Hunt *et al.* 2001; Shi *et al.* 2001). As demonstrated by Cowing *et al.* (2002) and Fasick *et al.* (2002), a single Phe/Tyr substitution at site 86 alone is responsible for the generation of the VS pigment in bovine from the ancestral UVS pigment. Having retained Phe at site 86, the SWS1 of the quenda is UVS, as found in other mammalian and non-mammalian UVS pigments (Hunt *et al.* 2001, 2004). In the quokka, a substitution of Phe86 by Tyr indicates a long-wave shift, generating a VS pigment, as found in the related tammar wallaby (Deeb *et al.* 2003). Although further long-wave shifting may result from the substitution of Ala114 by Gly in the quokka (table 2), the single Phe86Tyr substitution would account for the majority of the spectral shift between the two opsins (Yokoyama 2000). Because UVS pigments are found in other members of the order Diprotodontia (e.g. honey possum, Arrese *et al.* 2002), the evolution of VS pigments by a Phe86Tyr substitution may have occurred at the base of the Macropodidae lineage. A study of other diprotodonts is needed to confirm this inference.

In conclusion, our results add to the evidence presented previously for SWS, MWS and LWS cones in Australian marsupials, with SWS cones showing the greatest degree of variation in topography and spectral sensitivity between species. The presence of three spectrally distinct cone

types in four phylogenetically distant species indicates that the potential for trichromacy is a common feature among the Australian marsupials. Although no evidence was provided by molecular analysis for the presence of an MWS visual pigment in the tammar wallaby, it is probable that, to date, MSP affords the most reliable approach to determine the presence of a third cone type in marsupials. Consequently, an MSP investigation would be critical to verify the potential for trichromacy in the tammar wallaby.

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