Supplementary Information

Multiple rod-cone and cone-rod photoreceptor transmutations in snakes – evidence from visual opsin gene expression

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METHODS

mRNA and gDNA extraction and cDNA synthesis

Single specimens of each sampled snake species were obtained through fieldwork or commercially. Snakes were euthanized using approved (UK Home Office Schedule 1; University of Adelaide Animal Ethics Committee approval S-2014_033) procedures and the eyes were extracted, lenses discarded and the remainder coarsely macerated and stored in RNAlater (Ambion) at -80°C until RNA extraction. Total RNA was extracted using TRIzol® followed by purification with PureLink™ RNA Mini Kit (Life Technologies/Ambion) following the manufacturer's protocol. First-strand complementary DNA (cDNA) was synthesized with a Transcriptor First Strand cDNA Synthesis Kit (Roche) with 500ng of total RNA according to manufacturer's instructions. RNA complementary to the cDNA was removed using 2 units of *E. coli* RNase H (Ambion) followed by incubation at 37°C for 20 minutes to leave pure cDNA. Genomic DNA was extracted from each sample using the DNA layer in Trizol of the RNA extraction following the Trizol manufacturer's instructions, and/or from muscle tissue stored in ethanol using the Qiagen blood and tissue kit.

Opsin sequence generation

We amplified visual opsin genes *sws1, lws* and *rh1* using universal primers previously used to amplify these genes in snakes [1]. All fragments were amplified in 25 µl polymerase chain reactions (PCRs) containing: 1x PCR buffer (Invitrogen), 1.5 mmol (mM) of $MgCl_2$ (Invitrogen), 50 µmol/L of deoxynucleotides (Bioline), 0.4 µmol/L of each primer and 1 unit Platinum Taq Polymerase (Invitrogen) and 100ng of cDNA. Amplification was by touchdown PCR with the following cycling parameters: initial denaturation at 95°C for 5 minutes; 20 cycles of 1 minute at 95°C (denaturation), 30 seconds at 60°C (annealing), and 1 minute at 72°C (extension) with a decrease of 0.5°C per cycle; 15 cycles of 1 minute at 95°C (denaturation), 30 seconds at 50°C (annealing), and 1 minute at 72°C (extension) followed by a final extension at 72°C for 5 minutes. PCR products were run on a 1% agarose gel, excised in a Blue Light Transilluminator (Safe Imager, Invitrogen) and purified with a PureLink Quick Gel Extraction Kit (Invitrogen). PCR fragments were cloned with a StrataClone PCR Cloning Kit (Agilent) and corresponding chemically competent cells following the manufacturer's protocol. Transformed cells were grown overnight on agar media treated with 100 mg/ml of Ampicilin (Bioline) and 1ml of 2% X-GAL at 37°C. Sixteen white colonies were picked and used as DNA template in 25µl PCR reactions: 1x PCR buffer (Bioline), 1 mmol (mM) of $MgCl_2$ (Bioline), 80 µmol/L of deoxynucleotides (Bioline), 0.2 µmol/L of M13F and M13R vector primers and 1 unit of BioTAQ Polymerase (Bioline) and 2µl of DNA (1 colony twirled in 50µl of ultra-pure water). The PCR had the following cycling parameters: initial denaturation at 95°C for 10 minutes; 30 cycles of 15 seconds at 95°C (denaturation), 30 seconds at 58°C (annealing), and 1 minute and 30 seconds at 72°C (extension) and a final extension at 72°C for 1.5 minutes. Between four and eight positive clones per gene per species were sequenced in both directions with M13 universal primers in an automated DNA sequencer. Vector regions were trimmed and sequences were assembled in Geneious R8.

Voucher barcodes

We generated mitochondrial *16s rRNA* 'barcodes' for each specimen using universal primers [2] in 25 µl PCR reactions: 1x PCR buffer (Invitrogen), 1 mmol (mM) of $MgCl_2$ (Invitrogen), 50 µmol/L of deoxynucleotides (Bioline), 0.4 µmol/L of each primer and 1 unit Platinum Taq Polymerase (Invitrogen) and 100ng of gDNA. The PCR cycling parameters were: initial denaturation at 95°C for 10 minutes; 30 cycles of 15 seconds at 95°C (denaturation), 30 seconds at 55°C (annealing), and 1 minute at 72°C (extension) and a final extension at 72°C for 1 minute. All successfully amplified products were sequenced in both directions using the same primers used for PCR, in an automated DNA sequencer. The barcodes were assembled in Geneious R8.

Microspectrophotometry

All procedures were performed under dim red light. Eyes were enucleated from dark-adapted snakes killed using approved procedures. Retinas were mounted in saline containing 10% (w/v) dextran and compressed between two coverslips sealed with wax. A single beam microspectrophotometer was used following the methods reported by Sillman *et al*. [3]. MSP data from the visual pigments were recorded every 1nm from 350 to 750nm. Selection criteria followed [4]. The data were normalized by estimating the spectral maximum by eye and fitting a Gaussian function to the data points 20nm either side of the wavelength. The peak absorbance (λ_{max}) of each pigment was estimated by methods developed by [5] and [6] with the templates from [7].

Table S1. GenBank accession codes for the sequences used in this study. Taxa highlighted in bold are those for which data were newly generated in this study. Voucher specimen details for these samples as follows: *Telescopus fallax*: Natural History Museum of Crete, Greece, NHMC 80.3.38.116; *Hypsiglena jani*: University of Texas, Arlington, USA, UTA R 62966; *Phyllorhynchus decurtatus*: Los Angeles County Museum of Natural History LACM187402; *Hydrophis peronii*: Western Australian Museum, Perth, Australia WAMR174263; *Notechis scutatus*: no voucher.

Higher Taxa	Family	Species	Accession Numbers					
			16S	*rh1*	*sws1*	*lws*	*rh2*	*sws2*
Squamata - Serpentes	Typhlopidae	*Amerotyphlops brongersmianus*	KR815889	KR336737	-	-	-	-
	Leptotyphlopidae	*Epictia collaris*	KR815892	KR336735	-	-	-	-
	Anomalepididae	*Liotyphlops beui*	KR815891	KR336734	-	-	-	-
	Anomalepididae	*Typhlophis squamosus*	KR815890	KR336733	-	-	-	-
	Aniliidae	*Anilius scytale*	KR815894	KR336736	-	-	-	-
	Tropidophiidae	*Tropidophis feicki*	KR815893	KR336738	KR336723	KR336709	-	-
	Xenopeltidae	*Xenopeltis unicolor*	NA	J49723	FJ497234	FJ497235	-	-
	Pythonidae	*Python regius*	NA	FJ497236	FJ4977237	FJ4977238	-	-
	Pythonidae	*Python bivittatus*	NA		PRJNA238085			
	Lamprophiidae	*Polemon collaris*	KR815896	KR336739	KR336724	KR336710	-	-
	Elapidae	*Ophiophagus hannah*	NA		PRJNA201683		-	-
		Hydrophis peronii	KU323976	KU324001	KU323991	KU323990	-	-
		Notechis scutatus	KU323981	KU324000	KU323999	KU323989	-	-
	Colubridae	*Pseustes poecilonotus*	KR815895	KR336741	KR336725	KR336711	-	-
		Actractus flamigerus	KR815897	KR336740	KR336726	KR336712	-	-
		Lampropeltis californiae	KU323980	KU324004	KU323992	KU323987	-	-
		Hypsiglena jani	KU323975	KU324007	KU323998	KU323988	-	-
		Telescopus fallax	KU323974	KU324005	KU323995	KU323984	-	-
		Phyllorhynchus decurtatus	KU323979	-	KU323996	KU323985	-	-
		Arizona elegans	KU323973	KU324006	KU323997	KU323986	-	-
		Thamnophis sirtalis	KU323978	KU324003	KU323994	KU323983	-	-
		Natrix maura	KU323977	KU324002	KU323993	KU323982	-	-
Other Squamata	Amphisbaenidae	*Amphisbaena infraorbitale*	KR815886	KR336730	KR336719	KR336704	KR336755	KR336746
	Amphisbaenidae	*Amphisbaena alba*	KR815887	KR336729	KR336720	KR336705	KR336756	KR336745
	Amphisbaenidae	*Amphisbaena* sp.	KR815888	KR336728	KR336721	KR336706	KR336756	KR336747
	Lacertidae	*Takydromus sexlineatus*	KR815885	KR336727	KR336722	KR336707	KR336757	KR336744
	Gymnophthalmidae	*Bachia* cf. *flavescens*	KR815884	KR336731	KR336715	KR336703	-	KR336748
	Scincidae	*Melanoseps occidentalis*	KR815882	KR336743	KR336718	KR336713	KR336753	KR336750
	Scincidae	*Feylinia* sp.	KR815883	KR336742	KR336717	KR336714	KR336754	KR336751
	Diploglossidae	*Ophiodes striatus*	KR815881	KR336732	KR336716	KR336708	KR336752	KR336749
	Dactyloidae	*Anolis carolinensis*	NA		Ensembl v75			
	Phrynosomatidae	*Uta stansburiana*	DQ100323	DQ100325	DQ129869	DQ100324	DQ100326	DQ100323
Testudines	Trionychidae	*Pelodiscus sinensis*		Ensembl v75				
	Emydidae	*Chrysemys picta*	NA	Ensembl v75				

Crocodylia	Alligatoridae	*Alligator mississippiensis*	NA	UCS genome				
Aves	Columbidae	*Columba livia*	NA			Ensembl v75		
Aves	Falconidae	*Falco cherug*	NA			PRJNA168071		
Aves	Falconidae	*Falco peregrinus*	NA			PRJNA159791		
Aves	Muscicapidae	*Ficedula albicolabris*	NA			Ensembl v75		
Aves	Estrildidae	*Taeniopygia guttata*	NA			Ensembl v75		
Mammalia	Bovidae	*Bos taurus*	NA	NM_001014890	NM_174567	NM_174566	-	-
	Ornithorhynchidae	*Ornithorhynchus anatinus*	NA	EF050076	-	EU624413	EU624412	-

Table S2. Known amino acid spectral tuning sites for *rh1* ([8,9]) and predicted peak absorbance (λ_{max}) of RH1-based visual pigment for snakes. Site values in first row represent amino acid positions numbered with respect to bovine rhodopsin. Underline indicates amino acids with stronger effects on spectral tuning [16-19]. All λ_{max} values are predicted based on amino acid sequences (for a review see [8]) except those in parentheses (measured using MSP or *in vitro* expression).

	Amino acid residues													
Species	**83**	**90**	**113**	**118**	**122**	**164**	**180**	**261**	**265**	**269**	**285**	**292**	**308**	**λmax**
Anolis carolinensis	N	G	E	T	E	A	P	F	W	A	P	A	L	493 (491[1])
Amerotyphlops brongersianus	N	G	E	T	E	A	P	F	W	A	P	A	L	493
Typhlophis squamosus	N	G	E	T	E	A	P	F	W	A	P	A	L	493
Liotyphops beui	N	G	E	T	E	A	P	F	W	A	P	A	L	493
Epictia collaris	N	G	E	T	E	A	P	F	W	A	P	A	L	493
Anilius scytale	N	G	E	T	E	A	P	F	W	A	P	A	L	493 (493[2])
Python regius	N	G	E	T	E	A	P	F	W	A	P	A	V	493 (494[3])
Python bivittatus	N	G	E	T	E	A	P	F	W	A	P	A	V	493
Xenopeltis unicolor	N	G	E	T	E	A	P	F	W	A	P	A	V	493 (497[4])
Ophiophagus hannah	N	G	E	T	E	A	P	F	W	A	P	S	V	?
Tropidophis feickii	N	G	E	T	E	A	P	F	W	A	P	A	L	493
Polemon collaris	N	G	E	T	E	A	P	F	W	A	P	S	V	?
Pseustes poecilonotus.	N	G	E	T	E	A	P	F	W	A	P	S	V	?
Atractus flammigerus	N	G	E	T	E	A	P	F	W	A	P	A	V	493
Hydrophis peronii	N	G	E	T	E	A	P	F	W	A	P	A	V	493(496[5])
Notechis sculatus	N	G	E	T	E	A	P	F	W	A	P	A	V	493
Thamnophis sirtalis	N	G	E	T	E	A	P	F	W	A	P	S	V	? (485[6])
Natrix maura	N	G	E	T	E	A	P	F	W	A	P	S	V	?
Lampropeltis californiae	D	G	E	T	E	A	P	F	W	A	P	A	V	499
Telescopus fallax	D	G	E	T	E	A	P	F	W	A	P	A	V	499
Hypsiglena jani	N	G	E	T	E	A	P	F	W	A	P	A	V	493
Arizona elegans	N	G	E	T	E	A	P	F	W	A	P	S	V	? (484)

[1] [10]; [2] [1]; [3] [11]; [4] [12]; [5] [13] [6] [14]

Table S3. Known amino acid spectral tuning sites for *sws1* [15] and predicted peak absorbance (λ_{max}) of SWS1-based visual pigment for snakes. Site values in first row represent amino acid positions numbered with respect to bovine opsin. Underline indicates amino acids with stronger effects on spectral tuning [16-19]. All λ_{max} values are predicted based on amino acid sequences (for a review see [20]) except those in parentheses (measured using MSP or *in vitro* expression).

	Amino acid residues												
Species	**46**	**49**	**52**	**86**	**90**	**93**	**97**	**113**	**114**	**116**	**118**	**265**	**λmax**
Anolis carolinensis	F	F	T	F	S	T	A	E	A	L	S	Y	360 (359[1])
Tropidophis feickii	L	F	T	F	A	A	S	E	A	L	S	Y	360
Python regius	L	F	T	F	A	T	A	E	A	L	S	Y	360 (361[2])
Xenopeltis unicolor	L	F	T	F	A	T	A	E	A	L	S	Y	360 (360[3])
Python bivittatus	L	F	T	F	A	T	A	E	A	L	S	Y	360
Ophiophagus hannah	L	F	T	F	A	V	S	E	A	L	T	Y	?
Atractus flammigerus	L	F	T	F	A	T	S	E	A	L	T	Y	358
Polemon collaris	L	F	T	F	A	V	S	E	A	L	S	Y	?
Pseustes poecilonotus	L	F	T	F	A	V	S	E	A	L	T	Y	?
Hydrophis peronii	L	F	T	Y	A	V	S	E	A	L	T	Y	426 (430[4])
Notechis sculatus	L	F	T	F	R	V	S	E	A	L	T	Y	?
Thamnophis sirtalis	L	F	T	F	A	T	S	E	A	L	S	Y	360 (360[5])
Natrix maura	L	F	T	L	A	T	S	E	A	L	S	Y	?
Lampropeltis californiae	L	F	T	F	A	V	S	E	A	L	T	Y	?
Telescopus fallax	L	F	T	F	A	V	C	E	A	L	T	Y	?
Phyllorhynchus decurtatus	L	F	T	F	A	V	S	E	A	L	S	Y	?
Hypsiglena jani	L	F	T	F	A	T	S	E	A	L	T	Y	358
Arizona elegans	L	F	T	F	A	V	S	E	A	L	T	Y	? (366)

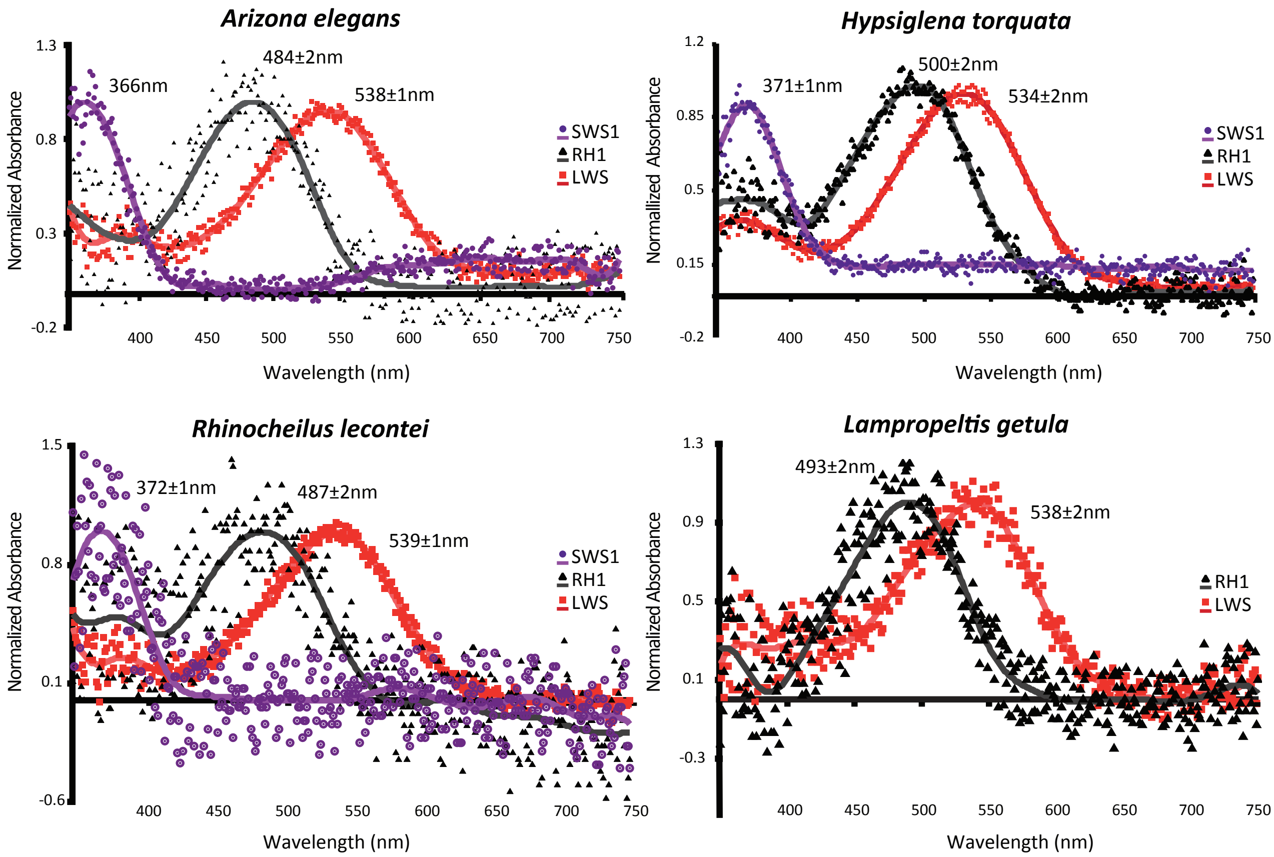
[1][10]; [2][11]; [3][12]; [4][13]; [5][14]

Table S4. Known amino acid spectral tuning sites for *lws* [21] and predicted peak absorbance (λ_{max}) of LWS-based visual pigment for snakes. Site values in first row represent amino acid positions numbered with respect to bovine rhodopsin. Underline indicates amino acids with stronger effects on spectral tuning [16-19]. All λ_{max} values are predicted based on amino acid sequences (for a review see [20]) except those in parentheses (measured using MSP or *in vitro* expression).

	Amino acid residues					
Species	**180**	**197**	**277**	**<u>285</u>**	**<u>308</u>**	**λmax**
Anolis carolinensis	S	H	Y	T	A	560 (560[1])
Python bivittatus	S	H	Y	T	A	560
Python regius	S	H	Y	T	A	560 (551[2])
Xenopeltis unicolor	S	H	Y	T	A	560 (560[3])
Polemon collaris	S	H	Y	T	A	560
Tropidophis feickii	A	H	Y	T	A	555
Pseustes poecilonotus	S	H	Y	T	A	560
Atractus flammigerus	A	H	Y	A	A	543
Ophiophagus hannah	S	H	Y	T	A	560
Hydrophis peronii	S	H	Y	T	A	560 (559[4])
Notechis sculatus	S	H	Y	T	A	560
Thamnophis sirtalis	A	H	Y	T	A	555(554[5])
Natrix maura	A	H	Y	T	A	555
Lampropeltis californiae	A	H	Y	T	A	555
Telescopus fallax	A	H	Y	A	A	536
Phyllorhynchus decurtatus	A	H	Y	T	A	536
Hypsiglena jani	S	H	Y	A	A	536
Arizona elegans	A	H	Y	A	A	536

[1][10]; [2][11]; [3][12]; [4][13]; [5][14]

FIGURE S1. Microspectrophotometry data showing pre-bleach absorbance spectra for visual pigments for four species of colubrid snakes. Each spectrum (circles, squares, triangles) is overlaid with a vitamin-A_1 (rhodopsin) visual pigment template (lines). Peak absorbance (λ_{max}) values (and standard deviations) are shown for each spectrum. See Table 3 for number of cells measured, and main text for further details. An SWS1 pigment was detected for *Lampropeltis getula* (λ_{max} = c. 370 nm) but none of the six readings passed selection criteria so the spectrum is not shown here.



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