Supplementary Material

Visual pigments in a palaeognath bird, the emu Dromaius novaehollandiae: implications for spectral sensitivity and the origin of ultraviolet vision

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Running title: Emu photoreceptors and visual pigments

Key words: Spectral tuning, opsin evolution, microspectrophotometry, oil droplets, Casuariiformes

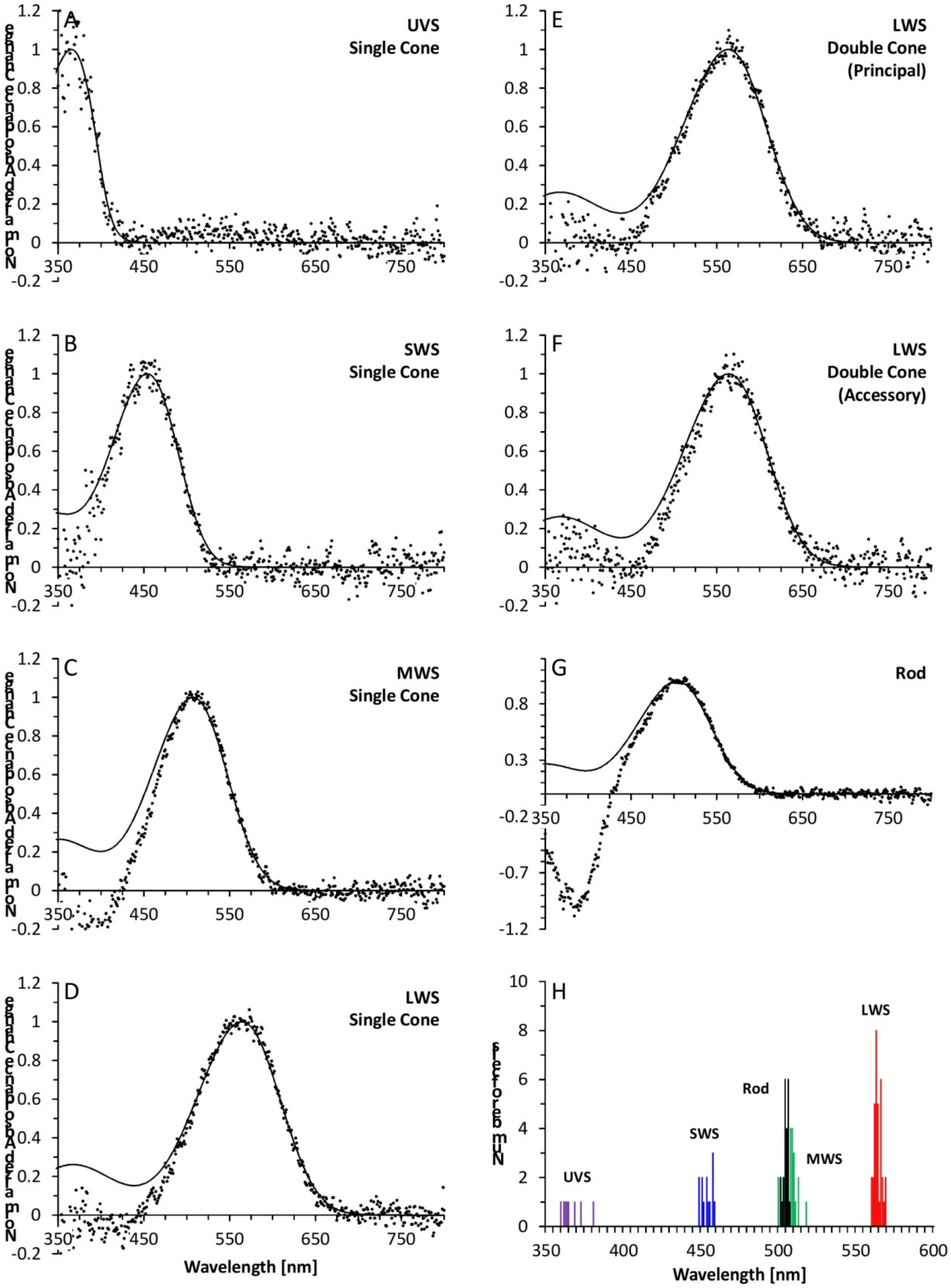

Figure S1. *(a–g)* Normalised mean bleaching difference spectra (black circles) of visual pigments measured using microspectrophotometry from emu photoreceptor outer segments. Difference spectra are overlayed with best-fit rhodopsin (vitamin A1) templates (black line). *(h)* Histogram shows the spectral distribution of the wavelength of maximum absorbance change (λ_{max}) values for individual photoreceptor cell outer segments that were used to generate the mean spectra. The λ_{max} value distribution of long-wavelength-sensitive (LWS) pigments includes measurements from LWS single cones as well as both the principal and accessory members of double cones. UVS, ultraviolet-sensitive; SWS, short-wavelength-sensitive; MWS, medium-wavelength-sensitive.

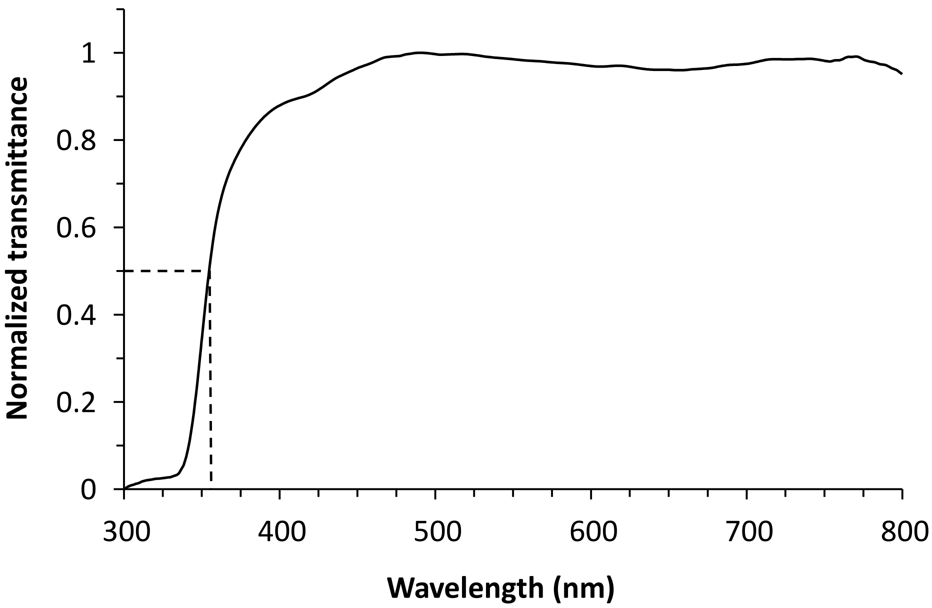

Figure S2. Normalised mean spectral transmittance of the combined ocular media (cornea, aqueous humour and lens) of the emu (n = 2 eyes from different birds). The wavelength at 0.5 normalised transmittance ($\lambda_{T0.5}$) is 355 nm and is indicated by the dashed line.

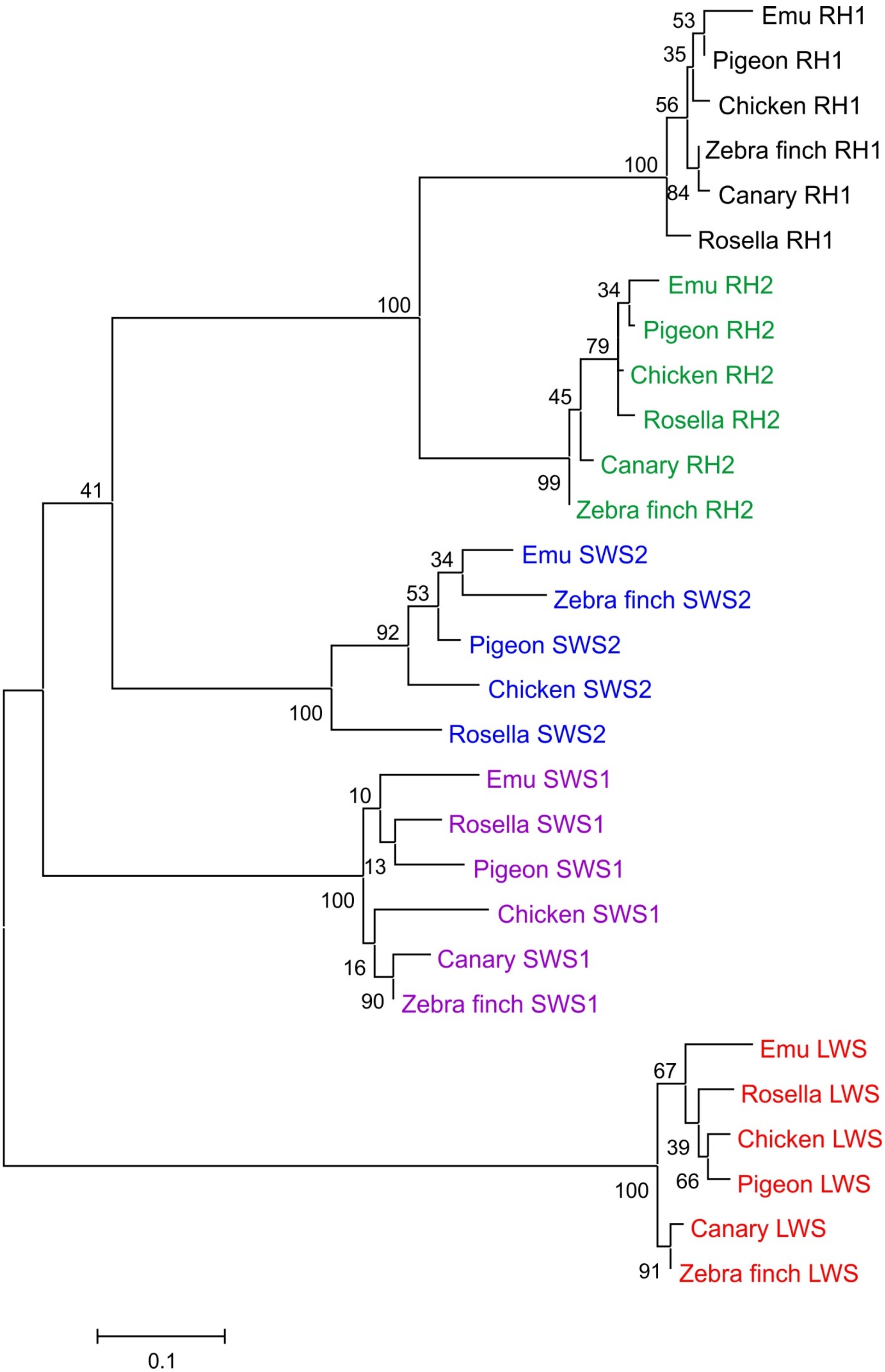

Figure S3. Maximum Likelihood phylogenetic analysis of emu (*Dromaius novaehollandiae*) LWS, SWS1, SWS2, RH2 and RH1 visual opsins compared to orthologues present in other representative birds with *Drosophila* RH1 opsin (GenBank accession no. X65877) as an outgroup (not shown). The degree of support for internal branching is expressed as a percentage with the scale bar indicating the number of amino acid substitutions per site. The sequences used for generating the tree are as follows: (1) RH1 opsin class: emu (*Dromaius novaehollandiae*), KU568456; pigeon (*Columba livia*), AH007730; chicken (*Gallus gallus*), NM001030606; zebra finch (*Taeniopygia guttata*), NM001076695; canary (*Serinus canaria*), AJ277926; crimson rosella (*Platycercus elegans*), KF134487; (2) RH2 opsin class: emu (*Dromaius novaehollandiae*), KU568455; pigeon (*Columba livia*), AH007731; chicken (*Gallus gallus*), M92038; crimson rosella (*Platycercus elegans*), KF134489; canary (*Serinus canaria*), AJ277924; zebra finch (*Taeniopygia guttata*), NM001076696; (3) SWS2 opsin class: emu (*Dromaius novaehollandiae*), KU568454; zebra finch (*Taeniopygia guttata*), NM001076697; pigeon (*Columba livia*), AH007799; chicken (*Gallus gallus*), NM205517; crimson rosella (*Platycercus elegans*), KF134491; (4) SWS1 opsin class: emu (*Dromaius novaehollandiae*), KU568453; crimson rosella (*Platycercus elegans*), KF134492; pigeon (*Columba livia*), AH007798; chicken (*Gallus gallus*), NM205438; canary (*Serinus canaria*), AJ277922; zebra finch (*Taeniopygia guttata*), NM001076704; and (5) LWS opsin class: emu (*Dromaius novaehollandiae*), KU568452; crimson rosella (*Platycercus elegans*), KF134493; chicken (*Gallus gallus*), NM205440; pigeon (*Columba livia*), AH007800; canary (*Serinus canaria*), AJ277925; zebra finch (*Taeniopygia guttata*), NM001076702.

Figure S4. Alignment of the amino acid sequences of four cone opsins (SWS1, SWS2, RH2 and LWS) expressed in the emu (*Dromaius novaehollandiae*) compared to orthologues found in other bird species, including zebra finch (*Taeniopygia guttata*), canary (*Serinus canaria*), chicken (*Gallus gallus*), pigeon (*Columba livia*), and the crimson rosella (*Platycercus elegans*). Gaps inserted to maintain a high degree of sequence identity and unsequenced regions are indicated by dashes (–). Spectral tuning sites mentioned in the text are highlighted in red.

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Zebra finch     MNGTEGQDFYVPMSNKTGVVRSPFEYPQYYLAEPWKFSALAAYMFMLILLGFPINFLTLY
Canary          ----------------------PFEYPQYYLAEPWKFSALAAYMFMLILLGFPINFLTLY
Chicken         MNGTEGQDFYVPMSNKTGVVRSPFEYPQYYLAEPWKFSALAAYMFMLILLGFPVNFLTLY
Pigeon          MNGTEGQDFYVPMSNKTGVVRSPFEYPQYYLAEPWKFSALAAYMFMLILLGFPVNFLTLY
Rosella         ---------------------------------------------------------TLY
Emu             -----------PISNKTGVVRSPFEYPQYYLAEPWKFSALAAYMFMLILLGFPINFLTLY
Bovine          MNGTEGPNFYVPFSNKTGVVRSPFEAPQYYLAEPWQFSMLAAYMFLLIMLGFPINFLTLY

Zebra finch     VTIQHKKLRTPLNYILLNLAVADLFMVFGGFTTTMYTSMNGYFVFGVTGCYIEGFFATLG
Canary          VTIQHKKLRTPLNYILLNLAVADLFMVFGGFTTTMYTSMNGYFVFGVTGCYIEGFFATLG
Chicken         VTIQHKKLRTPLNYILLNLVVADLFMVFGGFTTTMYTSMNGYFVFGVTGCYIEGFFATLG
Pigeon          VTIQHKKLRTPLNYILLNLAIADLFMVFGGFTTTMYTSMNGYFVFGVTGCYIEGFFATLG
Rosella         VTIQHKKLRTPLNYILLNLAVADLFMVFGGFTTTMYTSMNGYFVFGVTGCYIEGFFATLG
Emu             VTIQHKKLRTPLNYILLNLAVANLFMVFGGFTTTLYTSMHGYFVFGVTGCYIEGFFATLG
Bovine          VTVQHKKLRTPLNYILLNLAVADLFMVFGGFTTTLYTSLHGYFVFGPTGCNLEGFFATLG

Zebra finch     GEIALWSLVVLAIERYVVVCKPMSNFRFGENHAIMGVAFSWIMALACAAPPLFGWSRYIP
Canary          GEIALWSLVVLAIERYVVVCKPMSNFRFGENHAIMGVAFSWIMALACAAPPLFGWSRYIP
Chicken         GEIALWSLVVLAVERYVVVCKPMSNFRFGENHAIMGVAFSWIMAMACAAPPLFGWSRYIP
Pigeon          GEIALWSLVVLAIERYVVVCKPMSNFRFGENHAIMGVAFSWVMALACAAPPLFGWSRYIP
Rosella         GEIALWSLVVLAIERYVVVCKPMSNFRFGENHAIMGVAFSWIMALACAAPPLFGWSRYIP
Emu             GEIALWSLVVLAIERYVVVCKPVSNFRFGENHAIMGLALTWVMALACAAPPLFGWSRYIP
Bovine          GEIALWSLVVLAIERYVVVCKPMSNFRFGENHAIMGVAFTWVMALACAAPPLVGWSRYIP

Zebra finch     EGMQCSCGIDYYTLKPEVNNESFVIYMFVVHFMIPLSIIFFCYGNLVCTVKEAAAQQQES
Canary          EGMQCSCGIDYYTLKPEVNNESFVIYMFVVHFMIPLLIIFFCYGNLVCTVKEAAAQQQES
Chicken         EGMQCSCGIDYYTLKPEINNESFVIYMFVVHFMIPLAVIFFCYGNLVCTVKEAAAQQQES
Pigeon          EGMQCSCGIDYYTLKPEINNESFVIYMFVVHFMIPLMVIFFCYGNLVCTVKEAAAQQQES
Rosella         EGMQCSCGIDYYTLKPEINNESFVIYMFVVHFMIPLMIIFFCYGNLVCTVKEAAAQQQES
Emu             EGMQCSCGIDYYTLKPEVNNESFVIYMFVVHFTIPLMVIFFCYGNLVCTVKEAAAQQQES
Bovine          EGMQCSCGIDYYTPHEETNNESFVIYMFVVHFIIPLIVIFFCYGQLVFTVKEAAAQQQES

Zebra finch     ATTQKAEKEVTRMVIIMVIAFLICWVPYASVAFYIFTNQGSDFGPIFMTIPAFFAKSSAI
Canary          ATTQKAEKEVTRMVIIMVISFLICWVPYASVAFYIFTNQGSDFGPIFMTIPAFFAKSSAI
Chicken         ATTQKAEKEVTRMVIIMVIAFLICWVPYASVAFYIFTNQGSDFGPIFMTIPAFFAKSSAI
Pigeon          ATTQKAEKEVTRMVIIMVIAFLICWLPYASVAFYIFTNQGSDFGPIFMTIPAFFAKSSAI
Rosella         ATTQKAEKEVTRMVIIMVIAFLICWVPYASVAFYIFTNQGSDFGPIFMTIPAFFAKSSSI
Emu             ATTQKAEKEVTRMVVIMVIAFLICWLPYASVAFYIFTNQGSDFGPIFMTIPAFFAKSSAI
Bovine          ATTQKAEKEVTRMVIIMVIAFLICWLPYAGVAFYIFTHQGSDFGPIFMTIPAFFAKTSAV

Zebra finch     YNPVIYIVMNKQFRNCMITTLCCGKNPLGDEDTSAGKTETSSVSTSQVSPA
Canary          YNPVIYIVMNKQFRNCMITTLCCGKNPLGDEDTSAGKTETSS---------
Chicken         YNPVIYIVMNKQFRNCMITTLCCGKNPLGDEDTSAGKTETSSVSTSQVSPA
Pigeon          YNPVIYIVMNKQFRNCMITTLCCGKNPLGDEDTSAGKTETSSVSTSQVSPA
Rosella         FNPLIYVFRDKQFRNCMITTLCCGKNPLGDEDTSAGKTETSSVSTSQVSPA
Emu             YNPVIYIVMNKQFRNCMI---------------------------------
Bovine          YNPVIYIMMNKQFRNCMVTTLCCGKNPLGDDEAST---TVSKTETSQVAPA
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Figure S5. Alignment of the amino acid sequences of the emu (*Dromaius novaehollandiae*) rod opsin (RH1) compared to orthologues found in other bird species, including zebra finch (*Taeniopygia guttata*), canary (*Serinus canaria*), chicken (*Gallus gallus*), pigeon (*Columba livia*), and the crimson rosella (*Platycercus elegans*), as well as the common cow (*Bos taurus*). Gaps inserted to maintain a high degree of sequence identity and unsequenced regions are indicated by dashes (–). Spectral tuning sites mentioned in the text are highlighted in red.

Supplementary Tables:

Table S1. Primers used to isolate and amplify emu opsin gene sequences from retinal cDNA.

Primer	**Sequence**
LWS_F1	AAGCGTATTYAYTTAYACCRACASCAACAA
LWS_R1	CATCCTBGACACYTCCYTCTCVGCCTTCTG
LWS_F2	AGTGTCATCAACCAGWTCTYBGGSTAYTTC
LWS_R2	CATCATCCACYTTYTTSCCRAASAGCTGCA
SWS1_F1	TCCCATGTCCGGAGAVGAVGABTTYTACCT
SWS1_R1	CACCACSACCATSCGVGASACCTCCCGCTC
SWS1_F2	GGCCTTCGARCGHTACATYGTYATCTGCAA
SWS1_R2	TTAGCTGGGGCYGACYTGRCTGGAGGACAC
SWS2_F1	CAACATCACRRCSCTSAGCCCBTTCCTGGT
SWS2_R1	CAGGAAGCCCADSACCATSACYACYACCAT
SWS2_F2	CTGCAAGATAGAGGGNTTYDCBGCMACGCT
SWS2_R2	AAGAATTTTABGCBGGGGMSACBTGGCTGG
RH2_F1	ATCAACATCCTCACCYTVYTKGTSACCTTC
RH2_R1	CAAGGAGGAATCCMADCACCATSARRATCA
RH2_F2	CTTCTCTGCCACTCAYGCCWTRWTRGGCAT
RH2_R2	CACTTGGCTGGAAGARAYRGAVGAKACCTC
RH1_F1	GTCAAAATTTCTAYRTBCCCWTKTCCAACA
RH1_R1	ACAGTGCAGACAAGRYKYCCRTAGCAGAAG
RH1_F2	AATAGGATGCWRCWTYGARGGCTTCTTTGC
RH1_R2	ATTCTTTCCACARCARAGRGTBRTGATCAT

Table S2. Spectral absorption characteristics of retinal photoreceptors in the emu measured using MSP. λ_{max}, wavelength of maximum absorbance/absorbance change; λ_{cut}, cut-off wavelength; λ_{mid}, wavelength of half maximum absorptance. Values are ±1 standard deviation. Avian rods do not contain oil droplets and no oil droplets were observed in the accessory members of double cones in the emu (although a diffuse pigmentation was present). T-, C-, Y-, R- and P-type oil droplets are located in the UVS, SWS, MWS and LWS single cones, and the principal members of the LWS double cones, respectively. D, dorsal retina; V, ventral retina; N, number of cells used in the analysis. Subscripts 1 and 2 identify two different spectral types of P-type oil droplet located in the dorsal retina.

	Single Cones				**Double Cones**		**Rods**
Visual Pigments	**UVS**	**SWS**	**MWS**	**LWS**	**Principal**	**Accessory**	
Mean λ_{max} of prebleach spectra (nm)	367.0±2.5	453.2±2.3	502.3±2.8	562.0±2.0	562.8±2.6	563.4±2.0	501.0±1.0
λ_{max} of mean prebleach spectrum (nm)	366.8	453.1	501.2	562.0	562.9	562.1	500.7
Mean transverse absorbance at λ_{max}	0.018±0.008	0.012±0.003	0.016±0.005	0.018±0.004	0.013±0.004	0.012±0.003	0.023±0.004
Mean λ_{max} of difference spectra (nm)	366.6±6.8	453.7±3.5	507.2±4.0	564.2±1.9	562.8±2.8	563.5±2.3	504.0±1.7
λ_{max} of mean difference spectrum (nm)	366.5	454.0	507.0	564.0	563.0	563.7	503.5
N prebleach (difference)	9 (8)	9 (13)	21 (25)	10 (14)	12 (12)	7 (8)	21 (22)

Oil Droplets	**T-type**		**C-type**		**Y-type**		**R-type**		**P-type**			**A-type**		
	D	V	D	V	D	V	D	V	D_1	D_2	V	D	V	
Mean λ_{cut} of absorptance spectra (nm)	-	-	408.4±4.3	408.5±3.5	508.1±1.9	507.0±4.0	558.8±3.8	559.9±4.2	404.0±2.0	475.9±11.8	491.1±3.8	480.2±3.9	479.5±2.0	-
λ_{cut} of mean absorptance spectrum (nm)	-	-	409.3	407.5	507.6	506.8	559.2	560.6	403.5	479.2	492.3	479.2	480.1	-
Mean λ_{mid} of absorptance spectra (nm)	-	-	426.0±1.9	432.0±4.9	524.6±3.4	526.8±4.2	580.9±3.8	582.2±4.2	433.5±5.9	499.1±6.2	507.4±2.3	494.4±3.5	491.9±0.6	-
λ_{mid} of mean absorptance spectrum (nm)	-	-	426.2	430.9	524.3	527.3	581.4	582.8	432.9	500.6	508.1	494.6	492.1	-
Mean maximum absorptance	0.04±0.02	0.05±0.03	0.54±0.09	0.47±0.11	0.84±0.08	0.84±0.06	0.85±0.08	0.83±0.08	0.46±0.10	0.66±0.14	0.58±0.10	0.21±0.13	0.20±0.08	-
Mean diameter (µm)	2.4±0.2	3.0±0.4	3.3±0.8	3.7±0.6	3.3±0.6	3.6±0.4	3.2±0.3	3.5±0.5	2.8±0.3	3.1±0.3	3.1±0.4	-	-	-
N	5	4	12	11	18	22	13	19	13	11	20	9	7	-

Table S3. Amino acid differences between ultraviolet-sensitive (UVS) and violet-sensitive (VS) short-wavelength-sensitive-1 (SWS1) visual pigments at known or potential tuning sites, including residues 46, 49, 52, 114 and 118 [1]; 261, 269 and 292 [2]; 86 and 90 [3, 4]; and 93 [5]. Sequence data for avian SWS1 pigments are derived from this study and published articles [6-13], and are compared to residues found in the green anole (*Anolis carolinensis*) SWS1 pigment (GenBank Accession Number AH007736).

Pigment	**Tuning sites**											
	46	**49**	**52**	**86**	**90**	**93**	**114**	**118**	**164**	**261**	**269**	**292**
Avian UVS	Phe Leu Val	Leu Met Val	Thr	Ala Cys	Cys	Thr	Gly	Ala	Gly	Phe	Ala	Ala
Avian VS	Phe Ile	Ala Leu	Thr	Cys Ser	Ser	Thr Val	Ala Gly	Ala Thr	Gly	Phe	Ala	Ala
Anole UVS	Phe	Phe	Thr	Phe	Ser	Thr	Ala	Ser	Gly	Phe	Ala	Ala
Emu UVS	Phe	Phe	Thr	Phe	Cys	Met	Gly	Ser	Gly	Phe	Ala	Ala

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