Spectral sensitivity of cone photoreceptors and opsin expression in two colour-divergent lineages of the lizard *Ctenophorus decresii*

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Abstract

Intraspecific differences in sensory perception are rarely reported but may occur when a species range extends across varying sensory environments, or there is coevolution between the sensory system and a varying signal. Examples in colour vision and colour signals are rare in terrestrial systems. The tawny dragon lizard *Ctenophorus decresii* is a promising candidate for such intraspecific variation, because the species comprises two geographically and genetically distinct lineages in which throat colour (a social signal used in intra- and inter-specific interactions) is locally adapted to the habitat and differs between lineages. Male lizards from the southern lineage have UV-blue throats, whereas males from the northern lineage are polymorphic with four discrete throat colours that all show minimal UV reflectance. Here we determine the cone photoreceptor spectral sensitivities and opsin expression of the two lineages, to test whether they differ, particularly in the UV wavelengths. Using microspectrophotometry on retinal cone photoreceptors, we identified a long wavelength sensitive visual pigment, a 'short' and 'long' medium wavelength sensitive pigment and a short wavelength sensitive pigment, all of which did not differ in λ_{max} between lineages. Through transcriptome analysis of opsin genes we found that both lineages express four cone opsin genes, including that SWS1 opsin with peak sensitivity in the UV range, and that amino acid sequences did not differ between lineages with the exception of a single leucine/valine substitution in the RH2 opsin. Counts of yellow and transparent oil droplets associated with LWS+MWS and SWS+UVS cones respectively showed no difference in relative cone proportions between lineages. Therefore, contrary to predictions, we find no evidence of differences between lineages in single cone photoreceptor spectral sensitivity or opsin expression; however, we confirm the presence of four single cones classes and thus likely tetrachromacy in *C. decresii*, and provide the first evidence of UV sensitivity in agamid lizards.

Key words: *Ctenophorus decresii*, lizard, microspectrophotometry, visual pigment, opsin

Symbols/abbreviations:

MSP: microspectrophotometry

λ_{max}: wavelength of peak absorbance spectra

RNA: Ribonucleic acid

CDS: coding DNA sequences

TPM: transcripts per million reads

KRT8: Keratin 8

LDHB: Lactate dehydrogenase B

CDC42: Cell Division Cycle 42

PURH: 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohyrolase

CNDP1: Carnosine Dipetidase 1

STAR5: StAR-related lipid transfer domain containing 5

LGALS1: Lectin, Glactoside-Binding, Soluble 1

KIRREL3: Kin of IRRE Like 3

ADE2: Adenine 2

BCO2: Beta-Carotene Oxygenase 2

The correlated evolution of communication signals and sensory perception in different environments can lead to reproductive isolation and the formation of new species (Boughman, 2001; Boughman, 2002; Safran et al., 2013; Seehausen et al., 2008). This hypothesis, termed sensory drive, predicts that signals will co-evolve with sensory perception by the optimisation of signal transmission through the habitat, the tuning of sensory perception to the local environment and by matching the sender's signal to the receiver's perception (Endler, 1991; Endler, 1992; Endler and McLellan, 1988; Endler and Basolo, 1998; Levine and MacNichol, 1979; Rodd et al., 2002; Smith C., 2004). Consequently, when the range of a species extends across different sensory environments, intraspecific differences in both signalling and sensory systems may evolve, and such co-evolution of signals and sensory perception has been demonstrated in numerous species (Maan et al., 2006; Ryan and Wilczynski, 1988; Schluter and Price, 1993; Tobias et al., 2010). The majority of evidence to date is restricted to visual signals in aquatic species for which the visual environment varies markedly between populations (Archer et al., 1987; Boughman, 2001; Endler, 1991; Fuller et al., 2003; Seehausen et al., 2008) and is often correlated to age dependent changes in the visual environment occupied by an animal (Bowmaker and Kunz, 1987; Temple et al., 2008). Terrestrial examples of intraspecific variation in visual physiology are rare. For example complex sex-specific variations in photoreceptor complement are known in New World monkeys, caused by genetic polymorphisms of the medium / longwave (M/L) opsin (Jacobs et al., 1981; Mollon et al., 1984; Tovée, 1995). Intraspecific variation also exists in humans where various forms of colour blindness and colour matching arise from loss, or changes in cone opsin genes via an X-linked autosomal recessive inheritance mode (Surridge et al., 2003; Winderickx et al., 1992) and individuals vary in the proportion and arrangement of medium wavelength-sensitive and long wavelength-sensitive cones (Roorda and Williams, 1999). In the butterfly *Pieris rapae*, there is sexual dimorphism both in the colour display of wings but also in the composition of short-wavelength photoreceptors with females having a single peak violet sensitive photoreceptor and the males have a double-peaked blue spectral sensitivity (Arikawa et al., 2005). The carotenoid-rich oil droplets found in the retinas of all birds, lungfish and many reptiles (Goldsmith et al., 1984; Vorobyev, 2003; Walls, 1942) are also a potential source of intraspecific variation in visual sensitivity, as the density of pigments in these droplets is known to been affected by external variables such as diet (Bowmaker et al., 1993; Knott et al., 2010) and ambient light intensity (Hart et al., 2006). However, comparisons of morphs and subspecies of varying plumage coloration (Eastwood et al., 2014) have so far not revealed systematic differences in visual sensitivity (Knott et al., 2012; Knott et al., 2013), even when

the species has intraspecific variation in acoustic and visual signals (Berg and Bennett, 2010; Ribot et al., 2009; Ribot et al., 2012).

Here, we investigate the visual sensitivities of two geographically structured genetic lineages of the tawny dragon lizard, *Ctenophorus decresii*, that differ markedly in their visual sexual signals along a north-south axis (McLean et al., 2014b). *C. decresii* is a small sexually dichromatic agamid lizard endemic to South Australia. Adult males have brightly coloured throats which are emphasised during territorial and courtship head-bobbing and push-up displays (Gibbons, 1979; Osborne, 2005; Stuart-Fox and Johnston, 2005; Umbers et al., 2012). Male throat coloration differs between the two lineages whereas female throats are cream in both lineages (McLean et al., 2014a). All populations from the northern lineage are polymorphic with four, co-occurring, discrete male throat colours (orange, yellow, grey and a central orange patch surrounded by yellow) (Teasdale et al., 2013). Populations from the southern lineage are monomorphic with males having blue throats with an ultraviolet reflectance peak (McLean et al., 2013; Fig 1). Northern lineage populations occupy semi-arid, sparsely vegetated habitats, whereas the southern lineage occupies wetter, temperate, more vegetated habitats (Houston, 1974; McLean et al., 2013). Furthermore, male throat coloration is locally adapted to increase its conspicuousness to the visual system of lizards in the native habitat of each lineage (McLean et al., 2014b) . The two lineages of *C. decresii* meet at a narrow contact zone (<20km) at which there is asymmetric genetic introgression and very limited phenotypic admixture, suggesting potential barriers to gene flow and incipient speciation (McLean et al., 2013). Intraspecific colouration forms the basis for other studies into potential intraspecific variation (Knott et al., 2012; Knott et al., 2010; Knott et al., 2013) and, due to the marked difference in the spectral properties of the throat coloration between the two lineages (Fig. 1), and evidence of local adaptation of the throat signals to differing habitats, *C. decresii* is a promising species in which to test for correlated divergence of visual signals and visual perception.

To date, our knowledge of the photoreceptor spectral sensitivities of squamate reptiles comes only from a few species in each of the families Iguanidae (Bowmaker et al., 2005; Loew et al., 2002; Macedonia et al., 2009), Gekkonidae (Loew, 1994; Loew et al., 1996) and single species in the families Cordylidae (Fleishman et al., 2011) and Agamidae (Barbour et al., 2002) and several species of snakes (Davies et al., 2009; Hart et al., 2012; Sillman et al., 1999). These studies have described the visual sensitivities of single species or compared sensitivities

between closely related species but, to our knowledge, no study has investigated systematic differences in visual sensitivities within a lizard species. Current evidence suggests that all diurnal lizards share a highly conserved, ancestral pattern of four spectrally distinct cone classes, and likely tetrachromatic vision with ultraviolet sensitive (UVS) (364-383 nm), short wavelength sensitive (SWS) (440-467 nm), medium wavelength sensitive (MWS) (483-501 nm) and long wavelength sensitive (LWS) (560-625 nm) visual pigments as displayed by all studied species of Iguanids and the sole studied species of Cordylid *Platysaurus broadleyi* (Bowmaker et al., 2005; Loew et al., 2002; Macedonia et al., 2009). Due to their nocturnal ancestry, geckos and snakes have only three classes of photoreceptor (Davies et al., 2009; Ellingson et al., 1995; Fleishman et al., 2011; Hart et al., 2012; Loew et al., 1996; Olsson et al., 2013; Sillman et al., 1999). *Ctenophorus decresii* belongs to the family Agamidae for which spectral sensitivity data is only available for one species, the congeneric ornate dragon lizard *Ctenophorus ornatus*. In this species, microspectrophotometry (MSP) revealed yellow coloured and transparent oil droplets as well the presence of at least three classes of visual pigment (LWS, MWS, SWS) providing the basis for colour vision (Barbour et al., 2002). Although a UVS visual pigment was not found in *C. ornatus*, possibly due to the non-random nature of photoreceptor assessment using MSP, and previously reported difficulties in preparing lizard retina (Bowmaker et al., 2005; Loew et al., 2002), tetrachromatic colour vision is likely in the Agamidae, all of which are diurnal, visually-foraging species and many of which have conspicuous visual social signals (Manthey and Schuster, 1996).

We assessed differences in photoreceptor visual pigment spectral sensitivity, oil droplets and retinal opsin gene expression between two lineages of *C. decresii*. Firstly using MSP, we took *in situ* measurements of visual pigment absorbance spectra. From the study of the congeneric *C. ornatus* (Barbour et al 2002), we predict *C. decresii* will possess four single cone photoreceptors (LWS, MWS, SWS, UVS) and an LWS double cone. Secondly, as ultraviolet-sensitive (UVS) cells and short wavelength-sensitive (SWS) cells are always associated with transparent oil droplets in lizards (Bowmaker et al., 2005; Loew et al., 2002), we estimated the relative abundance of different classes of photoreceptor oil droplets. Due to the difficulty of investigating visual sensitivities using MSP in lizards, and particularly in identifying UVS photoreceptors, we sequenced the retinal transcriptomes from one southern and one northern individual to identify photoreceptor opsin genes and estimate relative opsin gene expression. If *C. decresii* possesses the cone photoreceptors that we predict above, then we expect to find corresponding opsins LWS, RH2, SWS2 and SWS1 respectively. We also expect to find the

RH1 opsin which, while usually associated with rod photoreceptors, has been identified multiple times in the pure cone retinas of other lizard species (Bennis et al., 2005; Kawamura and Yokoyama, 1998; New et al., 2012). We hypothesised that the southern lineage, which has an ultraviolet-blue coloured throat, would have a corresponding ultraviolet-sensitive visual pigment and a larger proportion of UVS and SWS than MWS and LWS visual pigments compared to the northern lineage which does not have an ultraviolet-blue visual signal.

RESULTS

Consistent with results of MSP on *C. ornatus* (Barbour et al., 2002), spectra of three visual pigments were recorded in single cones of *C. decresii*: an LWS pigment with λ_{max} at 569 nm; an MWS pigment with λ_{max} at 495 nm; and an SWS pigment with λ_{max} at 436 nm (Fig 2). MSP assessment did not discover a VS (violet-sensitive) or UVS cone, though the prevalence of four spectral classes of single cones among diurnal lizards, and the SWS λ_{max} of 436 nm in *C. decresii*, and 440 nm in *C. ornatus* (Barbour et al., 2002), suggests that a VS/UVS cone is most likely present in *Ctenophorus* species, but is difficult to detect using the MSP methods. All three recorded visual pigment types were found in both northern and southern lineages. However, consistent with previous MSP on lizard retina (Barbour et al., 2002; Bowmaker et al., 2005; Loew et al., 2002), the photoreceptors were fragile and difficult to separate from the pigmented epithelium, and intact outer segments were scarce (Table 1). As such, we were unable to visually identify examples of double cones on the MSP preparations. However double cones were found in the congeneric *C. ornatus* through macroscopic analysis of retinae (Barbour et al., 2002), and we suggest similar methods would most likely identify double cones in *C decresii*. We did not find any rods, consistent with *C. ornatus* retina morphology (Barbour et al., 2002) and other studies of diurnal lizard retinal tissue (Bowmaker et al., 2005; Loew et al., 2002; Röll, 2001; Walls, 1942). Moreover, the sample size for each photoreceptor type was too low to facilitate statistical comparison of individual cell λ_{max} between lineages. Average curves were created for each lineage to calculate spectral sensitivity (Fig. 2, Table 1). To the nearest nanometre, LWS λ_{max} were identical for each lineage. SWS photoreceptors showed a λ_{max} difference of 2 nm, but this could only be calculated for one cell per lineage, so cannot be suggested as a difference between lineages. The λ_{max} from the average curve for MWS visual pigments shows a difference of approximately 13 nm between lineages. While this may initially suggest a notable difference in λ_{max} between lineages, further examination of the MWS individual cone λ_{max} suggests that these cones may occur in two discrete populations of 'long-

medium wave sensitive' with λ_{max} at 507 nm and 'short-medium wave sensitive' with λ_{max} at 491 nm (Fig. 3). Such discrete populations of cones are known in other lizard species (Bowmaker et al., 2005; Loew et al., 2002; Provencio et al., 1992). Although both discrete cone populations are found in each lineage, the 16 nm difference found here may have resulted from differential measurement of the two MWS cone types between lineages (Table 2), and our sample size is not large enough to suggest whether the proportions of the two cone types could differ between lineages.

Retinal photographs revealed a diffuse yellow pigment was present through the retinal preparations, which showed properties consistent with the yellow pigment observed in *C. ornatus* (Barbour et al., 2002). Lizard oil droplet types are not as easy to identify as birds oil droplet types. Although through MSP we identified 3 oil droplet types (Fig. 4), using light microscopy we were able to identify two categories of oil droplet; pigmented 'yellow' droplets in the LWS and MWS cones, and transparent droplets in shorter wavelength cones, such that a comparison could only be made between 'LWS+MWS' vs. 'SWS+UVS'. Using the proportion of SWS+UVS cones as the analysed variable, no difference in the relative cone proportions were found between lineages ($F_{1, 6} < 0.01$, $p = 0.105$, $n = 8$).

Transcriptome sequencing, opsin gene identification and relative expression estimates

We detected one rod opsin (RH1) and four cone opsin (LWS, RH2, SWS2, SWS1) genes in *C. decresii* retinal tissue. With the exception of a single leucine/valine substitution (north: leucine, south: valine) due to a single C/G nucleotide change in the RH2 gene, amino acid sequences of the five opsin genes did not differ between the northern and southern sample (Supplementary Info-fasta file of sequences). Examination of amino acid sites known to be involved in spectral tuning in the SWS1 gene indicated peak sensitivity in the ultraviolet range (355–380 nm; Hauser et al., 2014; Shi and Yokoyama, 2003). Within the southern lineage individual, expression estimates of the four cone opsin genes were at least 100-fold higher in the retina than in the other tissue types (skin, heart and pituitary gland). This was not due to differences in sample quality as other genes examined were similarly highly expressed in all tissue types (e.g. LDHB, CDC42), or were more highly expressed in non-retinal tissue types (e.g. LGALS1, KRT8; Table 3) The relative abundances of the four cone opsin genes were approximately UVS 1.0: SWS 2.4: MWS 2.5: LWS 31 for the northern retina and UVS 1.1: SWS 2.1: MWS 1: LWS 19 for the southern retina (Table 3). Given that our experimental design did not include replicates within lineages, we were unable to make statistical inferences

with regards to differential expression of opsin genes between lineages; however, in all cases there was less than a 2-fold difference between the two lineages.

DISCUSSION

Using MSP on retinal photoreceptors and opsin sequence data we have characterised the visual system of *C. decresii*. Across two lineages of *C. decresii*, using MSP, we found a short wavelength sensitive class of cone photoreceptor, a 'short' and 'long' medium wavelength sensitive class, and a long wavelength sensitive class. We also confirm the expression of LWS, RH1, RH2, SWS1 and SWS2 opsin genes in the retina of both *C. decresii* lineages, which most likely serve tetrachromatic colour vision, extending into the UV range, consistent with other lizard species (Bowmaker et al., 2005; Loew et al., 2002; Macedonia et al., 2009). The lineages did not differ in amino acid sequences of opsin genes, with the exception of a single leucine/valine substitution in the RH2 opsin. Furthermore, counts of yellow and transparent oil droplets associated with LWS+MWS and SWS+UVS cones respectively showed no difference in relative cone proportions between lineages. Therefore, our data do not suggest differences between lineages in cone photorecptor sensitivity or opsin expression, even though male *C. decresii* show marked differences in the colour of their throat signals.

We hypothesised that the southern lineage of *C. decresii* with a UV colour signal would have greater UV visual sensitivity compared to the northern lineage without a UV colour signal, either due to a higher proportion of UVS and/or SWS visual pigments, or to a shift of peak sensitivity to shorter wavelengths. Although we did not find a UVS visual pigment by MSP, we found that both lineages have an SWS1 opsin gene (which is most likely to be UVS) with no differences in the amino acid composition. The λ_{max} of the single SWS visual pigment isolated from each lineage was within 2 nm, a difference most likely attributable to the low signal to noise ratio within the individual cell spectra. Furthermore, the relative expression levels of SWS1 and SWS2 genes in the retina of the northern and southern individuals were identical and expression levels were within 2-fold variation, suggesting no obvious differences in either λ_{max} or relative photoreceptor abundance between lineages. We also found no differences in the peak spectral sensitivities or amino acid sequence of the LWS visual pigment/ opsin gene between lineages. We did not identify enough 'short' and 'long' MWS visual pigments via MSP to be able to statistically compare lineages; however, the rod-like

cone opsin, RH2 was present in both lineages but differed in amino acid sequence by a single leucine/valine substitution. Both MWS cone populations were found in each lineage through MSP, so the RH2 amino acid difference between lineages could simply be because we analysed retinal opsin expression in a single individual per lineage.

Previous studies that have found intraspecific variation in visual sensitivities are largely restricted to aquatic species, due perhaps to the highly variable visual environment where habitat features can radically alter visual signal transmission (Endler, 1991; Levring and Fish, 1956; Reimchen, 1989). Although habitats of the northern and southern lineage of *C. decresii* differ in aridity and vegetation cover, with throat coloration locally adapted to enhance conspicuousness to conspecifics in local habitat, irradiance spectra from northern and southern populations are similar (McLean et al., 2014b). In fact, variation in irradiance within lineages due to changing light conditions over the course of a day as well as due to weather conditions (e.g. cloud cover) is very large (Endler, 1993) and so likely to be greater than any variation between lineages. Furthermore, although the reflectance spectra of backgrounds against which lizards display (rock with patchy lichen) differ between lineages, there is little reflectance (or variation) in the UV spectrum (McLean et al., 2014b). The species composition of predators and prey is also similar between lineages. Thus, similar selection imposed by both the visual environment and inter-specific interactions may account for the similarities in cone photoreceptor sensitivities and opsin expression between lineages, despite substantial differences in spectral characteristics of the throat colour signal.

In many species of fish, intraspecific differences in visual sensitivity are age-dependent, with age-classes varying in habitat and diet preferences (Bowmaker and Kunz, 1987; Shand et al., 2002; Shand et al., 1988). Differences in diet, particularly concentrations of carotenoids can also affect the density of carotenoid-rich oil droplets leading to intraspecific variation in visual sensitivity (Bowmaker et al., 1993; Knott et al., 2010) . Prey is unlikely to vary substantially either between lineages or between age groups of *C. decresii* due to their generalist insect diet high in carotenoids (Kayser, 1982; pers. obs.). Therefore it is not surprising that there are no differences between the proportions of transparent oil droplets versus yellow oil droplets between lineages. Although we only tested the cone photoreceptor sensitivities and opsin expression of adult *C. decresii*, there is no strong *a priori* reason to expect juveniles of this species to differ within or between lineages.

Here we have shown that *C. decresii* most probably has tetrachromatic vision, due to the presence of three cone photoreceptor classes (LWS, MWS and SWS) and the presence of the LWS, RH1, RH2, SWS2 and notably the SWS1 opsins, implying ultraviolet spectral sensitivity in Agamidae. The estimated peak spectral sensitivities in *C. decresii* (LWS λ_{max} 569 nm, MWS λ_{max} 491 nm, MWS λ_{max} 507 nm, SWS λ_{max} of 436 nm) closely matches those found in the congeneric *C. ornatus* (LWS λ_{max} 571 nm, MWS λ_{max} 493 nm, SWS λ_{max} 440 nm; Barbour et al., 2002). Furthermore, we confirmed the presence of a UVS sensitive visual pigment with estimated peak sensitivity within the range of 355-380 nm based on SWS1 opsin expression in the retina. Overall, therefore, the cone photoreceptor spectral sensitivities of *C. decresii* are consistent with those of all previously studied diurnal lizards (LWS: 560-625 nm; MWS: 483-501 nm; SWS: 440-467 nm; UVS: 364-383 nm) (Bowmaker et al., 2005; Loew et al., 2002; Macedonia et al., 2009) . Within MWS pigments of *C. decresii*, we identified two potential discrete photoreceptor populations comprising a 'long-medium wave sensitive' visual pigment with λ_{max} at 507 nm and 'short-medium wave sensitive' visual pigment with λ_{max} at 491 nm, a difference of 16 nm. Indeed discrete populations of MWS pigments are found in other diurnal lizards although the physiological basis varies between species. In the chameleon *Chamaeleo dilepis*, two distinct cone populations of MWS cones may be separated by porphyropsin or rhodopsin dominance, with a λ_{max} difference of 15 nm (Bowmaker et al., 2005). In the anole *Anolis carolinensis*, two MWS cone populations differ in λ_{max} by 6 nm and are likely due to morphologically similar MWS cones either containing the RH2 cone opsin or the RH1 rod opsin (Loew et al., 2002) . RH1 rod opsin is orthologous to rhodopsin in other vertebrates but is found in the pure-cone retina of some lizards (Bennis et al., 2005; Kawamura and Yokoyama, 1998; New et al., 2012). RH1 is detected in the retina of both the southern and northern lineages of *C. decresii* but its expression levels are approximately 16-fold lower than the expression levels of the cone opsins (SWS1, SWS2, RH2, and LWS; Table 3). Furthermore due to the non-random nature of MSP and difficulty of retinal tissue preparations in lizards, it is unlikely that we measured MWS cones containing RH1. Rather, the different MWS cone populations may be more likely due to differences in rhodopsin and porphyropsion dominance and related levels of vitamin A1 and A2 (Bowmaker et al., 2005) though this would require further investigation.

In summary, our results suggest that although lineages of *C. decresii* differ in the UV reflectance of their throat signal, contrary to predictions, they do not differ in their UV cone photoreceptor sensitivity. The lack of evidence for correlated divergence between visual

signals and visual sensitivity in this species is consistent with the view that daily variation in visual environments within terrestrial systems is so large (Lythgoe, 1979), and the nature of tasks for which visual systems have evolved are so general across species, that it precludes evolution of intraspecific variation in spectral tuning in response to coloration of social signals. By combining MSP and analysis of retinal opsin expression from transcriptomes we have characterised the visual system of *C. decresii* including the presence of the SWS1 opsin, most likely indicating UV sensitivity in the Agamidae. Our results give further weight to the view that diurnal lizards share highly conserved, ancestral, tetrachromatic vision, which is seen across lizard clades regardless of their phylogenetic position (Fleishman et al., 2011; i de Lanuza and Font, 2014; Olsson et al., 2013).

METHODS AND MATERIALS

Seven northern lineage individuals were caught in South Australia at Caroona Creek Conservation Park (Longitude: 139.103, Latitude: -33.443) or at Telowie Gorge Conservation Park (Longitude: 138.106, Latitude: -33.023), (120 km and 200 km north of the contact zone respectively) and eight southern lineage individuals were caught 40 km south of the contact zone in the Barossa Valley (Longitude: 139.212, Latitude: -34.563) between 9th and 16th December 2013. The subjects were housed in individual plastic tubs (55L x 34W x38D) containing a layer of sand and 2 terracotta tiles providing rock shelter at The University of Melbourne, Melbourne and Deakin University, Geelong, Australia. The photoperiod of the room approximately matched natural conditions at time of capture (10h:14h light dark cycle). Each tub had a suspended heat lamp creating a thermal gradient allowing the lizard to thermoregulate to their preferred body temperature (approx. 36°C; Walker, unpublished data) Lizards were misted, and crickets were provided every second day. Lizards were kept in captivity for 3 months at most.

Microspectrophotometry

The spectral sensitivities of retinal cone photoreceptors of the 15 subjects were assessed using the microspectrophotometer at Deakin University. For this work, subjects were dark adapted for at least 1 hour before humane sacrifice using an injection of sodium pentabarbitone (150mg/kg) into the caudal tail vein. The left eye was enucleated, and retinal tissue samples were prepared for MSP under infrared light using methods reported previously (Bowmaker et al., 1997; Knott et al., 2010). Spectral analysis of photoreceptors was undertaken on a single beam computer-controlled microspectrophotometer. The measuring beam was aligned to pass

transversely through the photoreceptor outer segment and run in 2 nm intervals from 750 nm to 350 nm, then back from 351 nm to 749 nm. After each measurement, the pigment was bleached with white light and the outer segment was rescanned to confirm the post-bleaching disappearance of the pigment and appearance of short-wavelength absorbing photoproducts. The peak spectral sensitivity (λ_{max}) of each cone type was calculated from the averaged spectrum for each individual, and for each lineage using a standardised computer program as described previously (Bowmaker et al., 1997). The pigment spectra were analysed using a vitamin-A1 visual pigment template (Knowles, 1977), which provided a better fit to the data than an A2 template. Further tissue samples were similarly prepared from dorsal and ventral sections of the right eye and photographed under a light microscope. From these, oil droplets counts were taken for comparison of photoreceptor ratios.

Transcriptome sequencing, opsin gene identification and relative expression estimates

To identify the opsins expressed in the retina of *C. decresii*, we sequenced the retinal transcriptomes (RNA transcribed from the protein-coding regions of the genome) of one northern and one southern lineage individual. We also sequenced the skin, heart and pituitary gland of the southern individual so that we could compare gene expression estimates among different tissue types. All tissues were stored in RNAlater (Ambion, Inc. Austin, TX) at 4°C for 24 hours, and then subsequently at -20°C until total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Library preparation and transcriptome sequencing were done by the Georgia Genomics Facility (GGF; Athens, GA). RNA libraries were prepared using an Illumina TruSeq RNA Sample Prep Kit (Illumina, Inc., San Diego, CA), including a poly A selection step, and were checked for quality with Bioanalyzer runs (Agilent Technologies, Inc., Clara, CA). Paired ends were then sequenced on either a single lane of the Illumina HiSeq2000 (southern retina, skin, heart and pituitary gland) or NextSeq500 (northern retina) sequencing platform (Illumina, Inc., San Diego, CA). In both cases, samples were multiplexed and included a total of 12 and 29 samples on a lane respectively.

The quality of the raw sequence reads were assessed using FastQC ver.0.11.2 (Andrews, 2010). We used Trimmomatic ver. 0.32 (Bolger et al., 2014) to remove adaptor sequences and low quality reads with a minimum quality (Phred) score of 25 per 15 bp sliding window and a minimum sequence length of 50 bp. The trimmed reads for each transcriptome were *de novo* assembled using Trinity (release 2014-04-13; Grabherr et al., 2011; Haas et al., 2013) using default setting and a minimum contig length of 151 bp.

Opsin genes in *C. decresii*, were identified using the blastx algorithm (with a minimum E-value threshold of 10-10; Altschul et al., 1990) to compare the assembled transcriptomes to a database constructed from 19,407 protein coding regions of the Australian central bearded dragon (*Pogona vitticeps*). This dataset has been annotated with the aid of transcriptome data as part of the *Pogona* Genome Project, a collaboration between the University of Canberra and the Beijing Genomics Institute (A. Georges and G. Zhang, in prep). We constructed a target gene set for the expression analysis consisting of five opsin genes (four cone opsins and one rod opsin) and ten other genes which may or may not be present in the retina. Given that untranslated regions may affect gene expression estimates, we restricted the expression analysis to the coding DNA sequence (CDS). We calculated relative expression levels (in transcripts per million reads; TPM) by mapping the RNA-seq data for each transcriptome to the target gene set using RSEM (Li and Dewey, 2011) and Bowtie 2 ver. 2.2.2 (Langmead and Salzberg, 2012), as implemented in the Trinity pipeline.

Ethics statement

Animals were caught under permit from the Department of Environment and Natural Resources, South Australia (permit no.: E25861-4), and with approval by The University of Melbourne Animals Ethics Committee (approval no.: 1312927.1) and the Wildlife Ethics Committee, South Australia (approval no.: 35/2013). Animals were held at Deakin University, and microspectrophometry performed there, under permit from the Department of Environment and Primary Industries, Victoria, Australia (permit no.: 10007000), and with approval from the Deakin Animal Ethics Committee (project no.: G39-2013).

Author Contributions

M.S.Y.: MSP data collection, assisted with data analysis, prepared manuscript. C.M.: fieldwork, analysis and interpretation of opsin sequences, assisted with manuscript preparation. A.M.: analysis and interpretation of opsin sequences. D.S-F.: conceived and designed project, funded fieldwork and transcriptome sequencing and assisted with manuscript preparation. A.T.D.B.: conceived and designed project, conceived and funded the MSP suite at Deakin University, assisted with manuscript preparation. B.K.: MSP specimen dissection and preparation, oversaw MSP training and supervision, conducted MSP data analysis and interpretation, assisted with manuscript interpretation.

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Figures

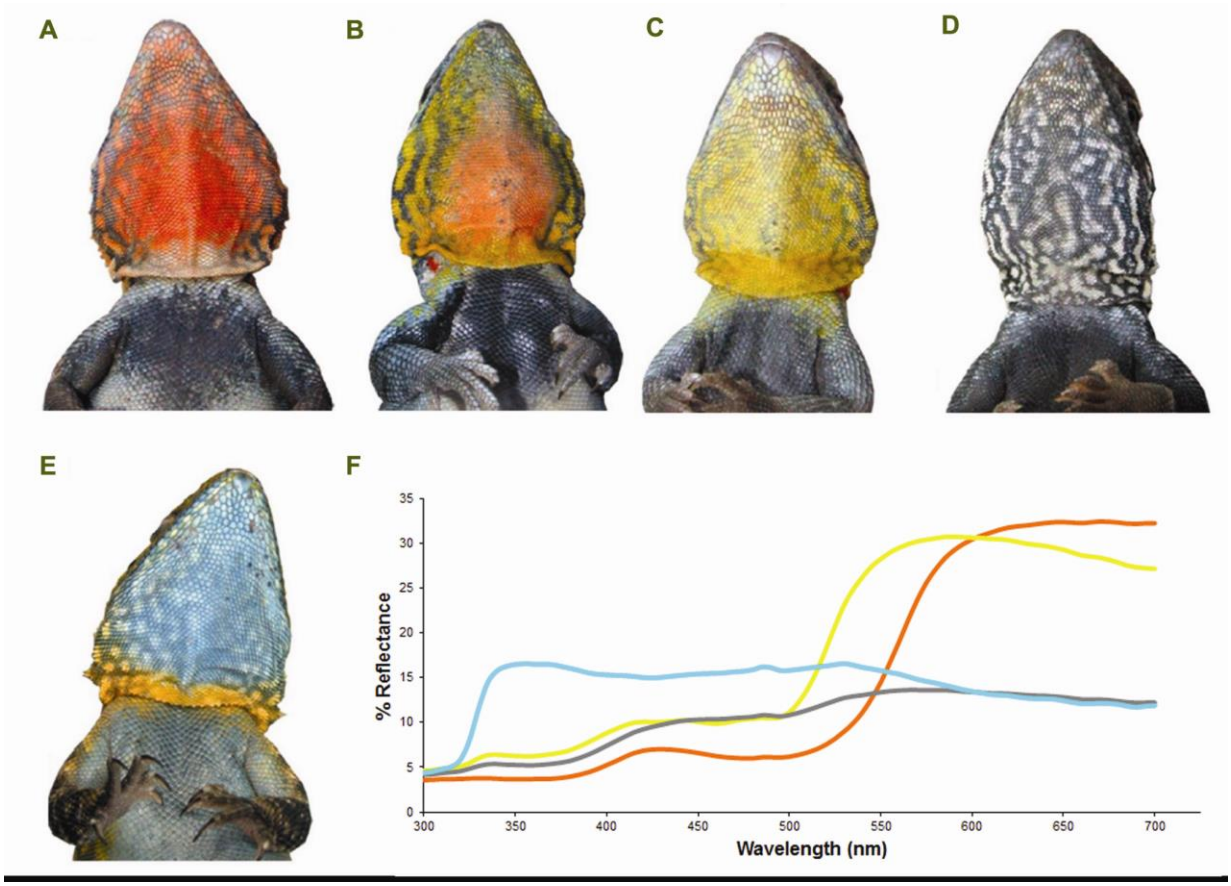

Figure 1: **Male throat colour of the northern lineage (A-D) and southern lineage (E).** Northern lineage male throat colour morphs. B: Orange, C: Orange-yellow, D: Yellow, E: Grey. **The average reflectance of *C. decresii* throat colours (F).** Orange (—), Yellow (—), Grey (—) and Blue (—)

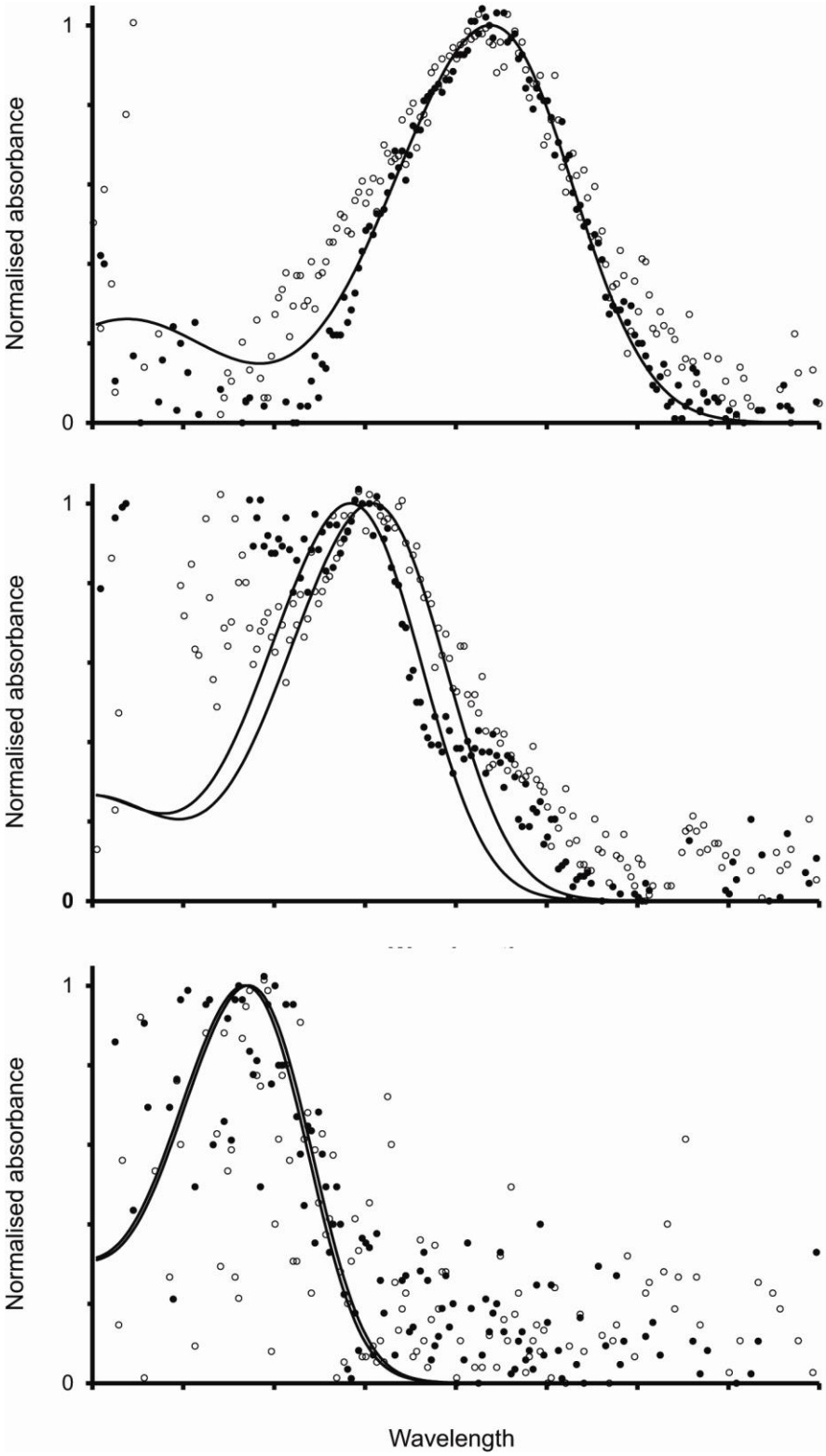

Figure 2: **Spectral sensitivity of averaged difference spectra of the visual pigments for northern and southern lineages of *Ctenophorus decresii*.** Top-bottom: LWS; MWS; and SWS visual pigments respectively for each lineage. Open circles: PB lineage; filled circles PO

lineage. λ_{max} for all pigments are provided in Table 1. Solid line: Govardovskii visual pigment template (Govardovskii et al., 2000) modelled using the calculated λ_{max} of the averaged difference spectra: LWS: 569nm for both lineages; MWS: 492nm and 504nm for northern and southern lineages respectively; SWS: 436nm and 434nm for northern and southern lineages respectively.

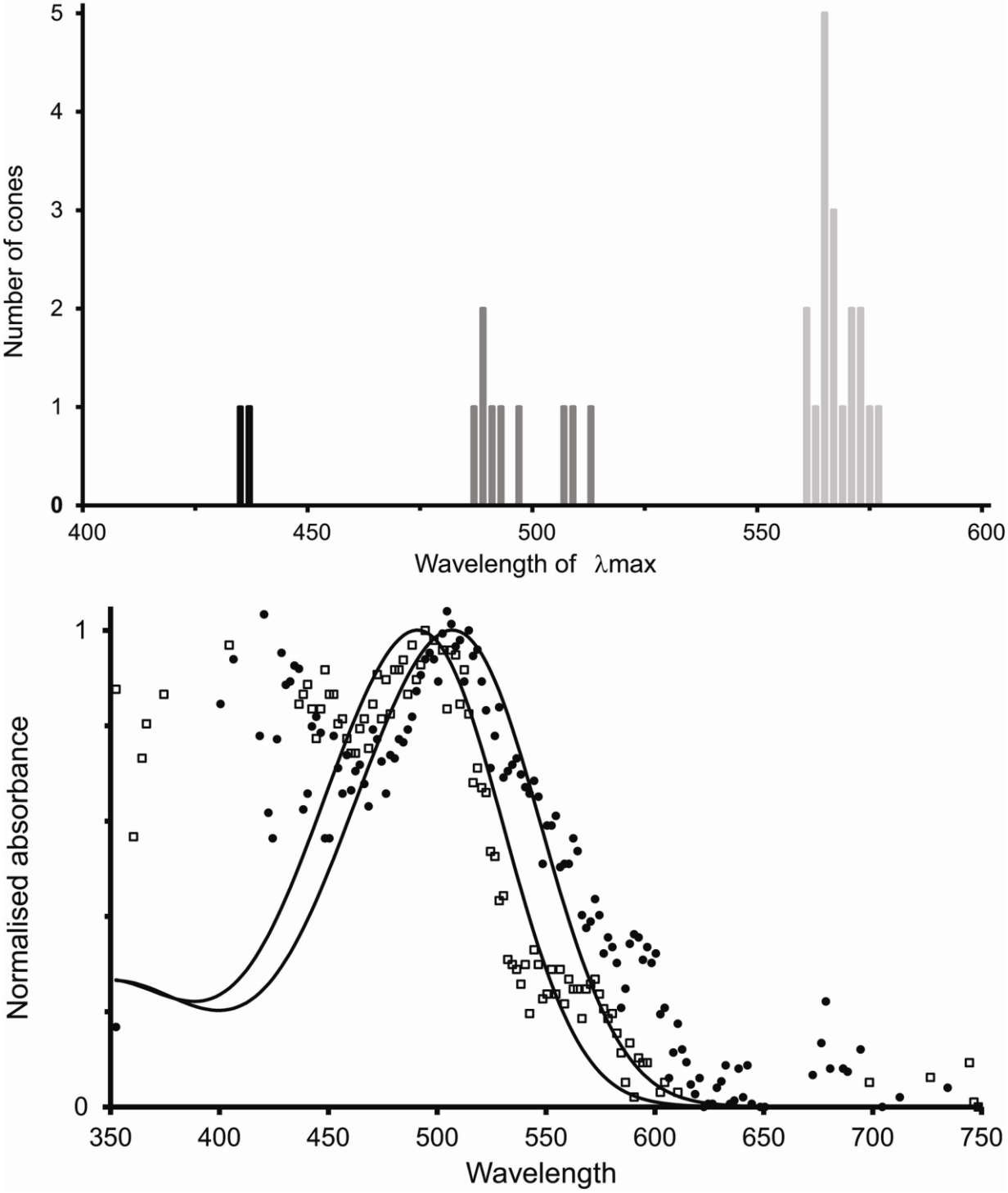

Figure 3: **Cone types in all *Ctenophorus decresii* subjects.** Top: frequency histogram of all cones types from all animals assessed. Bottom: Spectral sensitivity of the averaged difference spectra for the two MWS cone populations: filled circles: MWS-long; open squares: MWS-short. Solid line: Govardovskii visual pigment template modelled using the calculated λ_{max} of the averaged spectra: MWS-long: 507nm; MWS-short: 492nm.

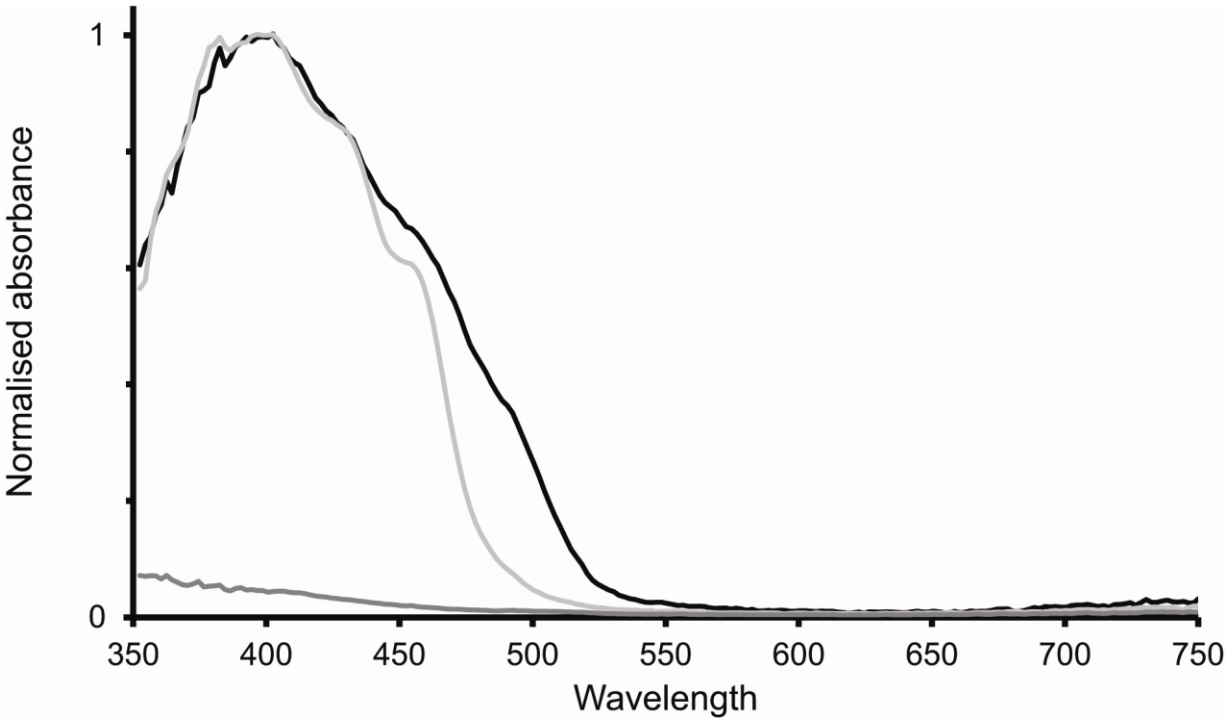

Figure 4: **Retinal oil droplet absorbance spectra from *Ctenophorus decresii*.** λ_{cut} occurs at 481.7nm (—) and 459.3nm (—) in droplets associated with both LWS and MWS photoreceptors. No detectable absorbance was observed in the droplets associated with SWS photoreceptors (—).

Table 1: λ_{max} for average spectral sensitivity calculated from the averaged difference spectra from all recorded visual pigments from each lineage, and for all recorded visual pigments from both lineages. Numbers in parentheses represent the numbers of cones for each cell in each lineage.

Lineage	Visual pigment λmax (nm)		
	LWS	MWS	SWS
North	568.7 (15)	491.9 (5)	436.3 (1)
South	569 (3)	504.2 (4)	434.8 (1)
All lizards	569 (18)	494.8 (9)	435.5 (2)

Table 2: λ_{max} for average spectral sensitivity calculated from the averaged difference spectra from each MWS cone type from all lizards (no lineage separation). Numbers in parentheses represent the numbers of cones for each cell for each lineage.

MWS cone type	Visual pigment λmax (nm)
Short (Southern: 2; Northern:4)	491.4
Long (Southern:1; Northern:2)	506.6

Table 3: Relative expression estimates of 15 target genes among lineages (northern and southern) and tissue types. These include the rod opsin (RH1) and 4 cone opsin genes (SWS1, SWS2, RH2 and LWS). Estimates are in units of transcripts per million reads (TPM).

	Northern	Southern			
Gene	Retina	Retina	Skin	Heart	Pituitary
SWS1	1885.58	2567.89	0	22.52	0
SWS2	4519.83	5108.56	0	45.51	0
RH2	4632.34	2423.98	0	0	27.2
LWS	58465.71	45922.54	0	24.75	15.04
RH1	277.09	151.27	0	73.81	7.85
KRT8	0	0	543.46	74.72	4901.76
LDHB	13746.47	13856.07	2922.09	10093.16	7144.97
CDC42	3742.39	5601.57	4267.71	22715.15	9880.05
PURH	156.63	206.07	537.11	3289.54	795.85
CNDP1	0	0	1.73	4.81	22119.28
STAR5	334.37	206.87	7306.55	191.48	1557.11
LGALS1	395.90	150.37	136906.10	2543.49	8382.40
KIRREL3	194.59	347.78	2.73	245.70	167.89
ADE2	391.43	347.29	383.34	2391.17	927.79
BCO2	2921.57	2393.44	1073.48	2743.63	606.91

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Fig. S1. Amino acid sequences of the five opsin genes.