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CORRECTION

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There was an error published in *J. Exp. Biol.* **218**, 1556-1563.

The corresponding author's email address was incorrect. The correct address is: myewers@gmail.com. This has now been corrected in the online full-text and PDF versions.

We apologise for any inconvenience this may have caused.

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RESEARCH ARTICLE

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 (LWS) visual pigment, a 'short' and 'long' medium-wavelength-sensitive (MWS) pigment and a short-wavelength-sensitive (SWS) 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 the *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 to 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 cone classes, suggesting tetrachromacy in *C. decresii*, and we also provide the first evidence of UV sensitivity in agamid lizards.

KEY WORDS: *Ctenophorus decresii*, Agamidae, Microspectrophotometry, Visual pigment, Opsin

INTRODUCTION

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, 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, 1992; Endler and McLellan, 1988; Endler and Basolo, 1998; Levine and MacNichol, 1979; Rodd et al., 2002; Smith et al., 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, 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 with 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, because the density of pigments in these droplets is known to be 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, 2013), even when the species has intraspecific variation in acoustic and visual signals (Berg and Bennett, 2010; Ribot et al., 2009, 2012).

Here, we investigate the visual sensitivities of two geographically structured genetic lineages of the tawny dragon lizard *Ctenophorus decresii*, which 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.

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The Journal of Experimental Biology

The Journal of Experimental Biology (2015) 218, 1556-1563 doi:10.1242/jeb.119404

Adult males have brightly coloured throats that 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 (<20 km) 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 variation in integument coloration forms the basis for other studies into potential intraspecific variation in colour vision (Knott et al., 2012, 2010, 2013). Because of the marked difference in the spectral properties of throat coloration between the two lineages in *C. decresii* (Fig. 1), as well as 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) and Gekkonidae (Loew, 1994; Loew et al., 1996), a 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, suggesting tetrachromatic colour 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). Because of 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 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 as a result of 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 UVS

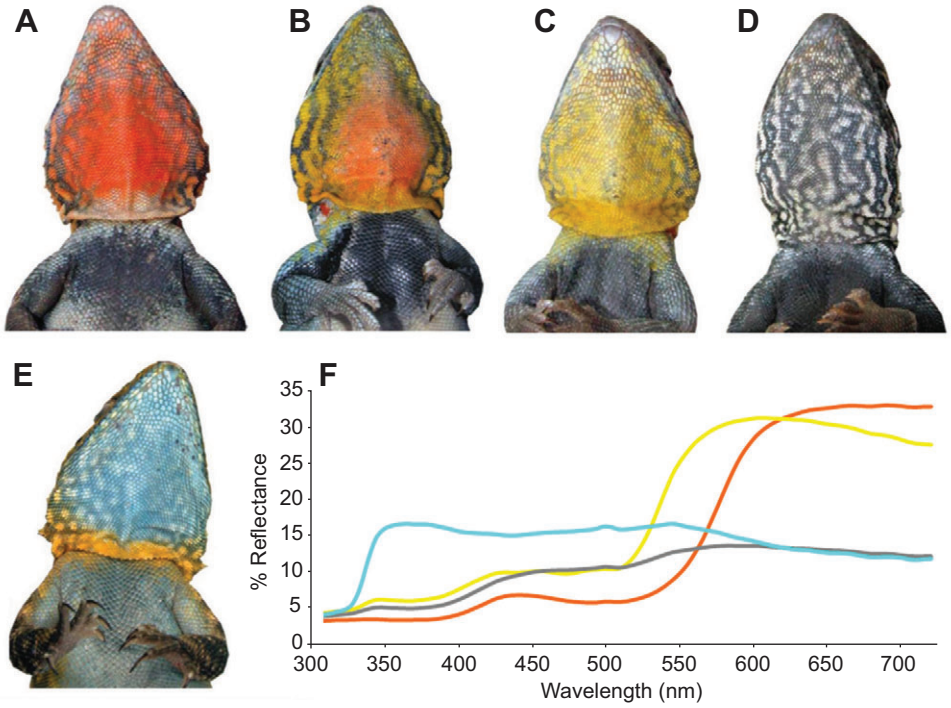

Fig. 1. Male throat colour of northern and southern lineages of the lizard *Ctenophorus decresii*. (A–D) Northern lineage male lizard throat colour morphs: (A) orange, (B) orange–yellow, (C) yellow, (D) grey. (E) Southern lineage lizard with blue throat colour. (F) The average reflectance of *C. decresii* throat colours. Lines are coloured orange, yellow, grey and blue to represent throat colours shown in A,C,D and E, respectively.

The Journal of Experimental Biology (2015) 218, 1556-1563 doi:10.1242/jeb.119404

and 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. Owing 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 with the northern lineage, which does not have an ultraviolet-blue visual signal.

RESULTS

Microspectrophotometry

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 show 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 that 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.

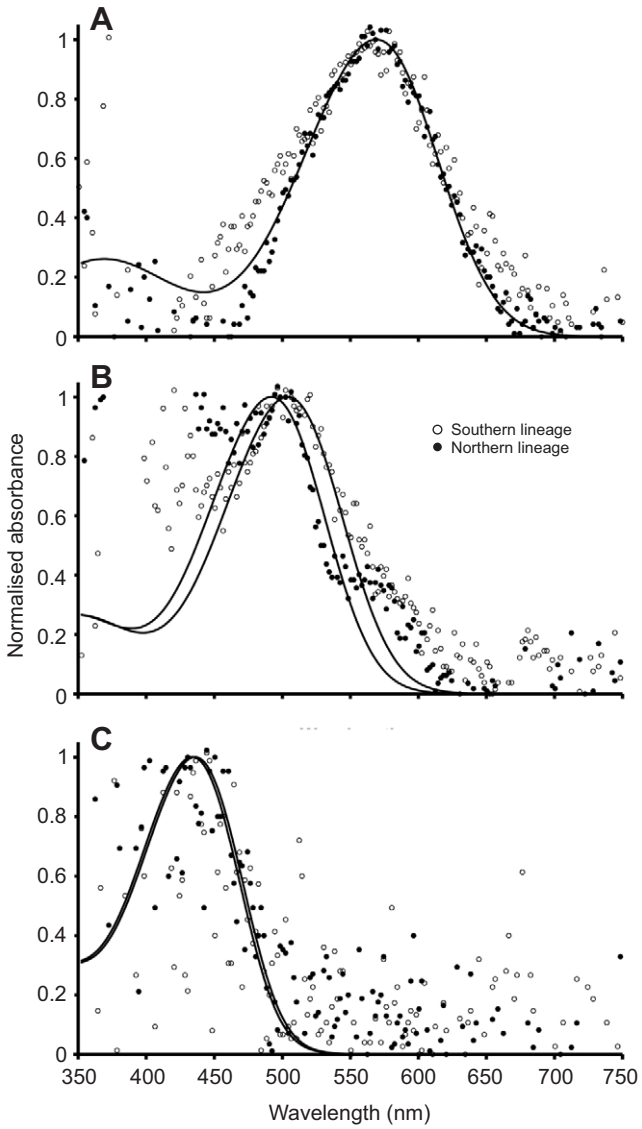

Fig. 2. Spectral sensitivity of averaged difference spectra of the visual pigments for northern and southern lineages of *Ctenophorus decresii*. (A) LWS, (B) MWS and (C) SWS visual pigments for each lineage. λ_{max} values for all pigments are provided in Table 1. Solid lines are Govardovskii visual pigment templates (Govardovskii et al., 2000) modelled using the calculated λ_{max} of the averaged difference spectra: LWS: 569 nm for both lineages; MWS: 492 nm and 504 nm for northern and southern lineages, respectively; SWS: 436 nm and 434 nm for northern and southern lineages, respectively.

Table 1. λ_{max} for average spectral sensitivity calculated from the averaged difference spectra from all recorded visual pigments for *Ctenophorus decresii*

	Visual pigment λ_{max} (nm)		
Lineage	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)

Numbers in parentheses represent the numbers of cones for each cell in each lineage.

The Journal of Experimental Biology (2015) 218, 1556-1563 doi:10.1242/jeb.119404

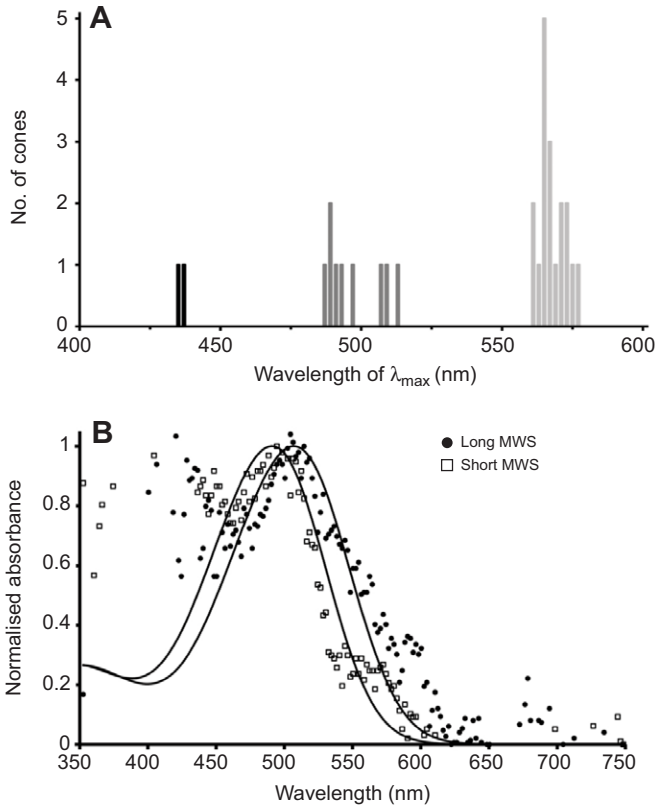

Fig. 3. Cone types in all *Ctenophorus decresii* subjects. (A) Frequency histogram of cones types from all animals assessed. (B) Spectral sensitivity of the averaged difference spectra for the two MWS cone populations. Solid lines are Govardovskii visual pigment templates modelled using the calculated λ_{max} of the averaged spectra: long MWS, 507 nm; short MWS, 492 nm.

Retinal photographs revealed the presence of a diffuse yellow pigment throughout 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 three oil droplet types (Fig. 4), using light microscopy, we were able to identify only 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' versus '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 to 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 samples (supplementary material Fig. S1). 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 because 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: 1.0:2.4:2.5:31 (UVS:SWS:MWS:LWS) for the northern retina and 1.1:2.1:1:19 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.

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)

MWS cone type	Visual pigment λ_{max} (nm)
Short (2 southern, 4 northern)	491.4
Long (1 southern, 2 northern)	506.6

Numbers in parentheses represent the numbers of cones examined for each cell for each lineage.

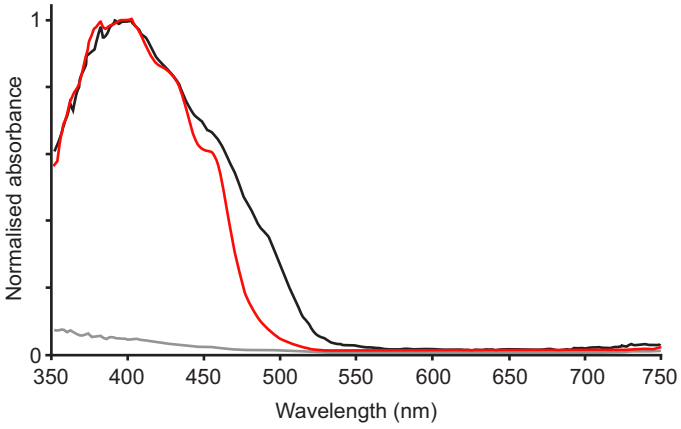

Fig. 4. Retinal oil droplet absorbance spectra from *Ctenophorus decresii*. λ_{cut} occurs at 481.7 nm (black line) and 459.3 nm (red) in droplets associated with both LWS and MWS photoreceptors. No detectable absorbance was observed in the droplets associated with SWS photoreceptors (grey).

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* we found an SWS class of cone photoreceptor, a 'short' and 'long' MWS class, and an LWS 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 to 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 with the northern lineage without a UV colour signal, either due to a

The Journal of Experimental Biology (2015) 218, 1556-1563 doi:10.1242/jeb.119404

Table 3. Relative expression estimates of 15 target genes among lineages and tissue types

	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	58,465.71	45,922.54	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	13,746.47	13,856.07	2922.09	10,093.16	7144.97
CDC42	3742.39	5601.57	4267.71	22,715.15	9880.05
PURH	156.63	206.07	537.11	3289.54	795.85
CNDP1	0	0	1.73	4.81	22,119.28
STAR5	334.37	206.87	7306.55	191.48	1557.11
LGALS1	395.90	150.37	136,906.10	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

These include the rod opsin (*RH1*) and four cone opsin genes (*SWS1*, *SWS2*, *RH2* and *LWS*). Estimates are in units of transcripts per million reads (TPM). *ADE2*, adenine 2; *BCO2*, beta-carotene oxygenase 2; *CDC42*, cell division cycle 42; *CNDP1*, carnosine dipetidase 1; *KIRREL3*, kin of IRRE like 3; *KRT8*, keratin 8; *LDHB*, lactate dehydrogenase B; *LGALS1*, lectin, galactoside-binding, soluble 1; *PURH*, 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohyrolase; *STAR5*, StAR-related lipid transfer domain containing 5.

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 or 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 to 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, perhaps because of 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 the local habitat, irradiance spectra from northern and southern populations are similar (McLean et al., 2014b). In fact, the variation in irradiance within lineages due to changing light conditions as well as weather conditions (e.g. cloud cover) over the course of a day, 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, 1988). Differences in diet, particularly in 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* as a result of their generalist insect diet, which is high in carotenoids (Kayser, 1982; our personal observations). 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* probably has tetrachromatic vision, as a result of 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}, 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 visual pigment with estimated peak sensitivity within the range of 355 to 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' MWS visual pigment with λ_{max} at 507 nm and 'short' MWS 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 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 because of 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 could more likely be due to differences in rhodopsin and

The Journal of Experimental Biology (2015) 218, 1556-1563 doi:10.1242/jeb.119404

porphyropsion dominance and related levels of vitamin A1 and A2 (Bowmaker et al., 2005), although 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, indicating UV sensitivity in the Agamidae. Our results add 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; Perez i de Lanuza and Font, 2014; Olsson et al., 2013).

MATERIALS AND METHODS

Animals

Seven northern lineage *Ctenophorus decresii* (Duméril and Bibron 1837) 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 [55×34×38 cm (L×W×D)] containing a layer of sand and with two 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 (10 h:14 h, light:dark cycle). Each tub had a suspended heat lamp creating a thermal gradient allowing the lizard to thermoregulate to their preferred body temperature (~36°C; S. Walker, unpublished results). Lizards were misted and crickets were provided every second day. Lizards were kept in captivity for 3 months at most. 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).

Microspectrophotometry

The spectral sensitivities of retinal cone photoreceptors of the 15 subjects were assessed using a microspectrophotometer at Deakin University. For this work, subjects were dark adapted for at least 1 h before humane killing using an injection of sodium pentabarbitone (150 mg kg^{-1}) 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 and Dartnall, 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 droplet 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, USA) at 4°C for 24 h, 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, USA). RNA libraries were prepared using an Illumina TruSeq RNA Sample Prep Kit (Illumina, Inc., San Diego, CA, USA), including a polyA selection step, and were checked for quality with Bioanalyzer runs (Agilent Technologies, Inc., Clara, CA, USA). 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). 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 v0.11.2 (Andrews, 2010). We used Trimmomatic v0.32 (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, unpublished results). 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 v2.2.2 (Langmead and Salzberg, 2012), as implemented in the Trinity pipeline.

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Competing interests

The authors declare no competing or financial interests.

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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Supplementary material

Supplementary material available online at
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