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RESEARCH ARTICLE

Ultraviolet vision in lacertid lizards: evidence from retinal structure, eye transmittance, SWS1 visual pigment genes and behaviour

Guillem Pérez i de Lanuza[1,2,*] and Enrique Font[2]

ABSTRACT

Ultraviolet (UV) vision and UV colour patches have been reported in a wide range of taxa and are increasingly appreciated as an integral part of vertebrate visual perception and communication systems. Previous studies with Lacertidae, a lizard family with diverse and complex coloration, have revealed the existence of UV-reflecting patches that may function as social signals. However, confirmation of the signalling role of UV coloration requires demonstrating that the lizards are capable of vision in the UV waveband. Here we use a multidisciplinary approach to characterize the visual sensitivity of a diverse sample of lacertid species. Spectral transmission measurements of the ocular media show that wavelengths down to 300 nm are transmitted in all the species sampled. Four retinal oil droplet types can be identified in the lacertid retina. Two types are pigmented and two are colourless. Fluorescence microscopy reveals that a type of colourless droplet is UV-transmitting and may thus be associated with UV-sensitive cones. DNA sequencing shows that lacertids have a functional SWS1 opsin, very similar at 13 critical sites to that in the presumed ancestral vertebrate (which was UV sensitive) and other UV-sensitive lizards. Finally, males of *Podarcis muralis* are capable of discriminating between two views of the same stimulus that differ only in the presence/absence of UV radiance. Taken together, these results provide convergent evidence of UV vision in lacertids, very likely by means of an independent photopigment. Moreover, the presence of four oil droplet types suggests that lacertids have a four-cone colour vision system.

KEY WORDS: Behaviour, Colour vision, Ocular transmittance, Oil droplet, SWS1 opsin, UV

INTRODUCTION

Understanding the sensory worlds of different species is crucial for the study of animal behaviour (e.g. von Uexküll, 1957; Partan and Marler, 2002; Stevens, 2013). In species with colour vision, even small differences in spectral sensitivity often have dramatic consequences for the animals' behaviour and ecology (e.g. Burkhardt and Finger, 1991; Endler, 1991; Majerus et al., 2000; Fleishman and Persons, 2001; Vorobyev et al., 2001; Cummings et al., 2003; Gómez and Théry, 2007). These differences are also crucial for our (i.e. human) understanding of phenomena relating to animal coloration. The widespread and erroneous assumption that visual perception is similar in humans and non-humans has been an endless source of misunderstanding (e.g. Bennet et al., 1994; D'Eath, 1998; McGraw et al., 1999; Cuthill et al., 1999; Rivas and Burghardt, 2002).

The ability to perceive the ultraviolet (UV) light spectrum, to which humans are blind, is present in most animals with colour vision (Goldsmith, 1994; Pichaud et al., 1999; Briscoe and Chittka, 2001; Ödeen and Håstad, 2013). The ancestral condition of the visual system of vertebrates, which has been retained in many extant taxa, includes a cone photoreceptor whose visual pigment contains the short-wavelength-sensitive type 1 (SWS1) opsin. This photoreceptor is known as the UV-sensitive (UVS) cone (Yokoyama, 2002; Yokoyama, 2008; Shi and Yokoyama, 2003; Bowmaker, 2008; Jacobs and Rowe, 2004). Although considerable progress has been made in the study of the distribution and variability of UV photoreceptors in birds (e.g. Bennett and Cuthill, 1994; Hart and Hunt, 2007; Ödeen et al., 2009; Ödeen et al., 2010; Ödeen et al., 2011; Ödeen et al., 2012; Carvalho et al., 2012; Coyle et al., 2012; Ödeen and Håstad, 2013; van Hazel et al., 2013) and fish (e.g. Carleton et al., 2000; Siebeck and Marshall, 2001; Siebeck and Marshall, 2007; Siebeck et al., 2010), data on other vertebrate linages are relatively scant and encompass few species [e.g. amphibians (Govardovskiĭ and Zueva, 1974; Perry and McNaughton, 1991; Takahashi and Yokoyama, 2005), mammals (Jacobs et al., 1991; Winter et al., 2003; Wang et al., 2004; Palacios et al., 2010; Carvalho et al., 2012), turtles (Ventura et al., 1999; Loew and Govardovskiĭ, 2001), crocodiles (Sillman et al., 1991) and Squamata, i.e. lizards and snakes (Fleishman et al., 1997; Fleishman et al., 2011; Sillman et al., 1999; Sillman et al., 2001; Loew et al., 2002)].

Several methods have traditionally been used to characterize the spectral sensitivity of vertebrates: microspectrophotometry (MSP), electroretinography (ERG), immunocytochemical identification of photoreceptors (II), and behavioural experiments (Mollon et al., 1984; Bowmaker and Dartnall, 1980; Jacobs, 1993; Kelber et al., 2003). MSP involves recording intracellularly the response of single photoreceptors to monochromatic stimuli of known intensity and is considered the hallmark for establishing the spectral sensitivity of photoreceptors (e.g. Loew et al., 1996; Loew et al., 2002; Bowmaker et al., 2005; Hart and Hunt, 2007). However, MSP requires killing the experimental animals, and consequently has been applied to the study of relatively few species. Gene analyses provide an alternative to determine the phylogenetic distribution of UV vision that obviates the need to kill animals. The light sensitivity of a visual pigment is to a large extent determined by the amino-acid sequence of its opsin moiety (e.g. Wilkie et al., 2000; Yokoyama et al., 2000). Hence, it is possible to predict with reasonable accuracy the wavelength of maximal absorption of the SWS1 opsin of a given species simply by examining its amino-acid sequence. This method has been used to infer the existence of UV vision in fish (Carleton

[1]Centro de Investigacão em Biodiversidade e Recursos Genéticos, Universidade do Porto, Rua Padre Armando Quintas 7, 4485-661 Vairão, Vila do Conde, Portugal. [2]Ethology Lab. Institut Cavanilles de Biodiversitat i Biologia Evolutiva. Universitat de València. 22085, 46071 València, Spain.

*Author for correspondence (guillem.perez-lanuza@uv.es)

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List of symbols and abbreviations

C	clear oil droplet
ERG	electroretinography
LY	light yellow oil droplet
MSP	microspectrophotometry
SWS1	short-wavelength sensitive type 1
T	transparent oil droplet
UV	ultraviolet
UVS	ultraviolet sensitive
Y	dark yellow oil droplet
λ_{max}	spectral location of photopigment peak sensitivity
$\lambda T_{0.5}$	wavelength at which the transmittance reaches 50%

et al., 2000), birds (Wilkie et al., 1998; Ödeen and Håstad, 2003; Ödeen and Håstad, 2009; Ödeen and Håstad, 2010; Håstad et al., 2005; Ödeen et al., 2010; Ödeen et al., 2011; Ödeen et al., 2012), mammals (Wang et al., 2004; Carvalho et al., 2012) and in the lizard *Gekko gecko* (Shi and Yokoyama, 2003). Compared with MSP, opsin gene sequencing is a high-throughput technique that enables the comparative analysis of large numbers of species (Wilkie et al., 1998; Carleton et al., 2000; Ödeen and Håstad, 2003; Ödeen and Håstad, 2009; Ödeen and Håstad, 2010; Ödeen and Håstad, 2013; Håstad et al., 2005; Ödeen et al., 2009; Ödeen et al., 2010; Ödeen et al., 2011).

Regardless of whether visual pigment spectral sensitivity is measured by MSP or approximated by molecular analysis, determining the light transmittance of the ocular media is a key to assessing a species' ability to perceive stimuli in the UV range. The spectrum of light reaching the retina is affected by the absorption properties of the cornea, lens and ocular humours (Vorobyev et al., 1998; Vorobyev et al., 2001; Endler and Mielke, 2005; van Doorn, 2012; Lind et al., 2013; Lind et al., 2014; Douglas and Jeffery, 2014). For example, some fish species with UVS photoreceptors are actually UV blind because the ocular media block UV wavelengths completely (Siebeck and Marshall, 2001; Siebeck and Marshall, 2007; Nelson et al., 2001).

The retinas of most diurnal birds, turtles and lizards contain oil droplets that act as spectral filters and enhance colour perception (Vorobyev, 2003; Stavenga and Wilts, 2014). There are several types of oil droplet, some of which are coloured and some colourless. Each oil droplet type is characteristically associated with a specific type of cone photoreceptor, acting as a long-pass spectral filter that restricts the range of wavelengths that reach the photopigment and decreases the overlap among cone responses (Govardovskiĭ, 1983; Vorobyev, 2003). Therefore, characterizing the different oil droplet types may provide an indirect route to identifying the different types of photoreceptors present in the retina (e.g. Goldsmith et al., 1984; Bailes et al., 2006). The evidence supporting this oil droplet–cone type specificity is especially strong in birds, but recent research supports a similar specificity for diurnal lizards (Loew et al., 2002; Bowmaker et al., 2005; Macedonia et al., 2009; Fleishman et al., 2011).

Behavioural experiments that probe the animals' ability to discriminate among visual stimuli are an alternative and time-honoured approach to demonstrate colour perception in non-human animals (e.g. Birgersson et al., 2001; Van-Eyk et al., 2011; reviewed in Kelber et al., 2003). Behavioural experiments are based on a discrimination test, where choices between visual stimuli are based exclusively on colour differences (Goldsmith, 1990). Behavioural data are widely recognized as the ultimate and most convincing demonstration of colour discrimination because, when experiments are properly designed, the response of experimental animals will reflect the overall result of physiological and neural processes related to colour perception and discrimination (Kelber et al., 2003; Kelber and Osorio, 2010).

Although colour vision and visual processes in diurnal lizards are believed to be highly conserved (Olsson et al., 2013), most of the available information has been obtained through work with a phylogenetically restricted sample of species (reviewed in Table 1). Available data suggest that the visual system of diurnal lizards is probably tetrachromatic and includes four types of single cone photoreceptors: UV-, short-, medium- and long-wavelength-sensitive photoreceptors (UVS, SS, MS and LS, respectively), which are associated with photopigments SWS1, SWS2, MWS and LWS, respectively [names according to Endler and Mielke (Endler and Mielke, 2005)]. The retinas of diurnal lizards also contain double cones, but their role in colour vision has not been established (Loew et al., 2002; Fleishman et al., 2011). The peak sensitivity of the UVS photoreceptor ranges from 359 nm in *Crotaphytus dickersonae* to 383 nm in *Chamaleo dilepis* (Fleishman et al., 1993; Fleishman et al., 1997; Loew, 1994; Ellingson et al., 1995; Loew et al., 1996; Loew et al., 2002; Bowmaker et al., 2005; Macedonia et al., 2009; Fleishman et al., 2011). Unfortunately, efforts so far have been focused on a few species belonging to Iguania and the extremely variable Gekkota and Serpentes. Other clades remain unexplored with a single study that confirms UV sensitivity in the cordilid *Platysaurus broadleyi* (Fleishman et al., 2011). In fact, Fleishman et al. (Fleishman et al., 2011) have stressed that more data on non-iguanian species are essential for identifying general principles of lizard visual system design and evolution.

The ability of several species of Lacertidae, an Old World family that encompasses more than 300 species, to discriminate colours in the human visual range has been known from behavioural experiments dating back several decades (Wagner, 1932; Swiezawska, 1949; Svoboda, 1969; Dücker and Rensch, 1973). More recent work has shown that lacertids have prominent UV colorations, often sexually dimorphic, that may function as social signals (e.g. Thorpe and Richard, 2001; Font and Molina-Borja, 2004; Molina-Borja et al., 2006; Pérez i de Lanuza and Font, 2007; Font et al., 2009; Pérez i de Lanuza, 2012; Pérez i de Lanuza et al., 2013b; Pérez i de Lanuza et al., 2014), but nothing is known about the visual perception of any lacertid in this wavelength range. Although the results of some behavioural experiments are consistent with the possibility that lacertids perceive UV wavelengths (Martín and López, 2009; Bajer et al., 2010), a conclusive demonstration of UV vision in this large lizard clade is lacking.

The aim of our study is to determine whether lacertids are capable of UV vision, and to evaluate the prevalence and phylogenetic distribution of UV vision across this family of non-iguanian diurnal lizards. We include in our sample species that inhabit different visual ecosystems and belong to all the main clades of Lacertidae. This is, to our knowledge, the first comprehensive (i.e. family level) comparative study of UV vision in diurnal lizards. For this purpose we adopt an interdisciplinary approach that relies on histological, molecular and behavioural methods to provide convergent evidence of UV vision in lacertids.

RESULTS

Ocular filtering

The transmittance spectra of the ocular media are very similar in all the species examined and reveal high transmission of short wavelengths down to around 300 nm. Fig. 1A shows the transmittance spectra for the whole eye. Fig. 1B and 1C compare spectra for the whole eye, cornea, lens and cornea × lens approximation (see below) in *Podarcis muralis* and *Takydromus*

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Table 1. Data on UV sensitivity in squamate reptiles (lizards and snakes) showing the wavelength of peak sensitivity (λ_{max}) for the SWS1 photopigment

Major clades	Species (common name)	λ_{max} (nm)	Technique	Source
Iguania				
Polychrotidae	*Polychrus marmoratus* (many-coloured bush anole)	–	OD	Loew et al., 2002
	Anolis extremus (Barbados anole)	365	MSP	Loew et al., 2002
	A. equestris (knight anole)	–	OD	Loew et al., 2002
	A. sagrei (brown anole)	365	MSP	Loew et al., 2002
	A. bahorucoensis (Bahoruco long-snouted anole)	365	MSP	Loew et al., 2002
	A. conspersus (Cayman blue-throated anole)	365	MSP	Loew et al., 2002
	A. garmani (Jamaican giant anole)	–	OD	Loew et al., 2002
	A. grahami (Graham's anole)	367	MSP	Loew et al., 2002
	A. lineatopus (stripefoot anole)	366	MSP	Loew et al., 2002
	A. opalinus (opal bellied anole)	–	OD	Loew et al., 2002
	A. valencienni (Jamaican twig anole)	–	OD	Loew et al., 2002
	A. cristatellus (Puerto Rican crested anole)	365	MSP	Loew et al., 2002
		–	ERG	Fleishman et al., 1993; Fleishman et al., 1997
	A. evermanni (Puerto Rican emerald anole)	364	MSP	Loew et al., 2002
		–	ERG	Fleishman et al., 1993; Fleishman et al., 1997
	A. gundlachi (Western Antillean anole)	365	MSP	Loew et al., 2002
		–	ERG	Fleishman et al., 1993; Fleishman et al., 1997
	A. krugi (upland grass anole)	365	MSP	Loew et al., 2002
		–	ERG	Fleishman et al., 1993; Fleishman et al., 1997
	A. pulchellus (sharp-mouthed lizard)	367	MSP	Loew et al., 2002
		–	ERG	Fleishman et al., 1993; Fleishman et al., 1997
	A. stratulus (barred anole)	366	MSP	Loew et al., 2002
		–	ERG	Fleishman et al., 1997
	A. carolinensis (green anole)	365	MSP	Loew et al., 2002
			SWS1	Kawamura and Yokoyama, 1996 (AH007736.1)
		358	SWS1-OR	Shi and Yokoyama, 2003
Crotaphytidae	*Crotaphytus dickersonae* (Tiburon collared lizard)	359	MSP	Macedonia et al., 2009
Phrynosomatidae	*Uta stansburiana* (common side-blotched lizard)	–	SWS1-GB	Su et al., 2006 (DQ100325)
Iguanidae	*Dipsosaurus dorsalis* (common desert iguana)	–	BT	Alberts, 1989
Chamaeleonidae	*Chamaeleo dilepis* (flap-necked chameleon)	383	MSP, OD, T	Bowmaker et al., 2005
	C. calyptratus (veiled chameleon)	370–380	MSP, OD	Bowmaker et al., 2005
	Furcifer pardalis (panther chameleon)	375	MSP, OD	Bowmaker et al., 2005
	F. lateralis (jeweled chameleon)		OD	Bowmaker et al., 2005
Gekkota				
Gekkonidae	*Phelsuma madagascariensis* (Madagascar day gecko)	–	SWS1-GB	T. Yuki, H. Osamu, Y. Masao and T. Fumio, unpublished data (AF074045.1)
	Gekko gecko (Tokay gecko)	364	MSP	Loew, 1994
			SWS1	Yokoyama and Blow, 2001 (AY024356.1)
		364	SWS1-OR	Shi and Yokoyama, 2003
	Hemidactylus turcicus (Turkish gecko)	366	MSP, II	Loew et al., 1996
	H. garnotii (Indo-Pacific gecko)	363	MSP, II	Loew et al., 1996
	Teratoscincus scincus (common wonder gecko)	365	MSP, II	Loew et al., 1996
Sphaerodactylidae	*Gonatodes albogularis* (yellow-headed gecko)	362	MSP, ERG, T	Ellingson et al., 1995
Serpentes				
Pythonidae	*Python regius* (royal python)	360	MSP	Sillman et al., 1999
Boidae	*Boa constrictor imperator* (common northern boa)	357	MSP	Sillman et al., 2001
Colubridae	*Thamnophis sirtalis* (San Francisco garter snake)	360	MSP, II	Sillman et al., 1997
	Masticophis flagellum (San Joaquin coachwhip)	362	MSP	Macedonia et al., 2009
	Hypsiglena torquata (night snake)	365	MSP	Loew, unpublished data (cited in Sillman et al., 1999)
Scincoidea				
Cordylidae	*Platysaurus broadleyi* (Augarabies flat lizard)	364	MSP, ERG, OD	Fleishman et al., 2011

BT, behavioural test; ERG, electroretinography; II, immunocytochemical identification; MSP, microspectrophotometry; OD, presence of transparent oil droplet (presumably associated to UVS photoreceptor); SWS1, sequencing of SWS1 genes; SWS1-OR, SWS1 opsin regeneration; SWS1-GB, SWS1 sequence available in GenBank (GenBank accession numbers in parentheses after citation); T, transmittance measurements.

sexlineatus, respectively. In all of the species examined, the lens is the ocular element that most contributes to restricting transmittance of very short wavelengths, with $\lambda T_{0.5}$ values (wavelength at which transmittance reaches 50%) from 313 to 350 nm. Fig. 1C illustrates the effect of freezing (and the consequent dirty appearance of the vitreous humour) on whole eye transmittance measurements, showing an artifactual drop in transmittance between 370 and 440 nm. Table 2 presents $\lambda T_{0.5}$ for all the species examined.

Characterization of oil droplets

Retinas of all the species have the same four types of oil droplets: two apparently colourless types and two yellow types (Fig. 2A–D).

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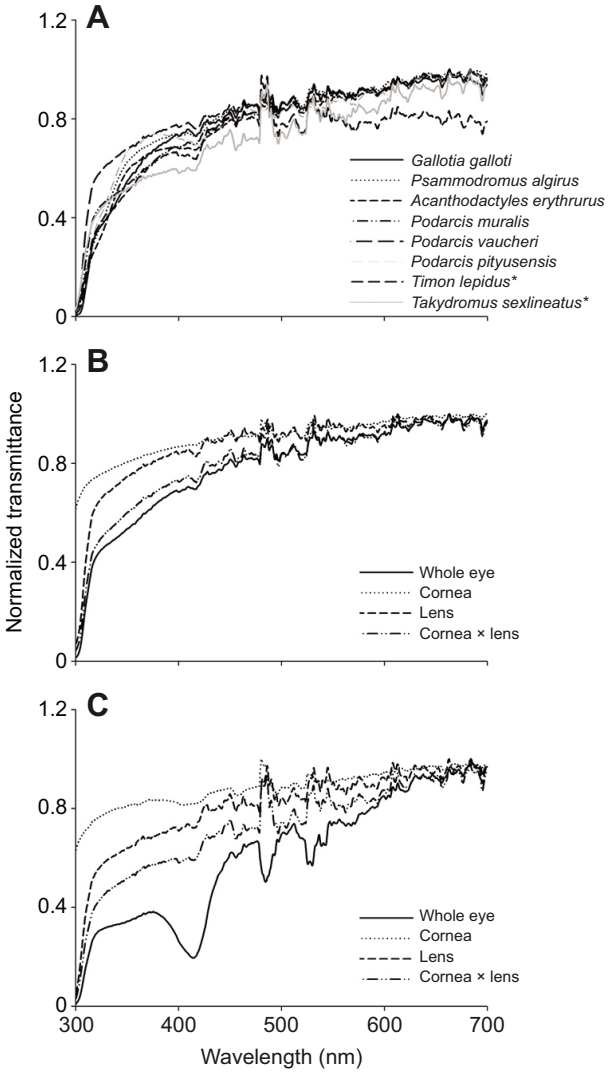

Fig. 1. Transmittance spectra. (A) Whole eyes from eight lacertid species. (B) Whole eye, cornea, lens and cornea × lens approximation from a fresh sample (*Podarcis muralis*, *N*=4). (C) Whole eye, cornea, lens and cornea × lens approximation from a frozen sample (*Takydromus sexlineatus*, *N*=1). The dip between 375 and 450 nm is an artefact caused by fragments of the retina solved into the vitreous humour. *N*, sample size, =number of eyes/2. Asterisks indicate whole eye spectra calculated by cornea and lens spectra integration.

We named the four types as transparent small (T), clear large (C), light yellow (LY) and dark yellow (Y). We also detected a diffuse yellow pigment (YP), which may be associated with the accessory member of double cones as in other lizard species (e.g. Loew et al., 2002). Fluorescence microscopy reveals that T and C droplets respond differently under UV illumination: C oil droplets absorb UV and emit above 400 nm, whereas T droplets do not absorb (i.e. transmit) UV wavelengths (Fig. 2E,F). Based on differences in cut-off wavelengths obtained by the use of microscopy filters (Table 3) and a comparison with previous work with other lizard species, we suggest that T, C, LY and Y oil droplet types are part of the UVS, SS, MS and LS photoreceptors, respectively (Fleishman et al., 1993; Loew et al., 2002; Bowmaker et al., 2005; Macedonia et al., 2009).

Spectral tuning of the SWS1 opsin

Table 4 shows the 13 critical amino acid positions for UV vision in the SWS1 opsin of 30 lacertid species. The 13 sites are, but with two exceptions (*Lacerta agilis* and *Mesalina simoni*), identical to those in the presumed ancestral vertebrate, which possessed UV vision, and similar to those in *Anolis carolinensis*, a species in which UV visual sensitivity has been determined using MSP (λ_{max}=365 nm; Table 1). Interestingly, the only difference between *A. carolinensis* and lacertids corresponds to the replacement V109I, which is identical to that found in *Gekko gecko*, where UV vision has also been confirmed using MSP (λ_{max}=364 nm; Table 1). Moreover, site-directed mutagenesis has suggested that the V109A substitution produces a shift in the λ_{max} of the resulting visual pigment of only 1 nm [from 359 nm in the ancestral pigment to 360 nm in the mutant (Takahashi and Yokoyama, 2005)].

Table 2. Values of wavelength position of transmittance at 50% ($\lambda T_{0.5}$)

	$\lambda T_{0,5}$ (nm)	
Species (common name) (*N*)	Whole eye	Lens
Gallotia galloti (Tenerife lizard) (2)	344.25	327.92
Psammodromus algirus (large psammodromus) (3)	335.91	313.36
Acanthodactylus erythrurus (spiny-footed lizard) (2)	339.54	349.68
Podarcis muralis (common wall lizard) (4)	340.27	312.63
Podarcis vaucheri (Maghreb wall lizard) (5)	314.82	319.92
Podarcis pityusensis (Eivissa wall lizard) (2)	333.01	330.47
Timon lepidus (ocellated lizard) (2)	346.79*	320.28
Takydromus sexlineatus (Asian grass lizard) (1)	339.18*	315.18

*Values obtained from whole eye spectra calculated by cornea and lens spectra integration.
N, sample size, =number of eyes/2.

Visual discrimination experiment

All the experimental males visited the two choice areas, but they spent significantly more time (i.e. scored a higher number of sample points) in the choice area with the UV+ filter than in the choice area with the UV– filter (*N*=10, Wilcoxon matched-pairs test: *Z*=–2.376, *P*=0.018). As experimental lizards were observed only at intervals of 10 min (i.e. time sampling), we did not obtain a complete record of their behaviour. However, during trials, the experimental lizards showed mainly exploratory behaviours (such as tongue-flicking). No agonistic behaviours or escape attempts were observed. Our experimental design does not allow us to determine whether the experimental males' choice was based on the visual appearance of the stimulus males or of the surrounding terrarium. It is also possible that male choice in our experiment was based on brightness differences rather than on the wavelength composition of the two stimulus conditions. However, as the two optical filters (UV+ and UV−) do not differ in transmittance in the 400–700 nm range, the non-random choice of viewing conditions strongly suggests that males of *P. muralis* are capable of UV vision.

DISCUSSION

Taken together, the results presented here converge on the notion that UV vision is widespread in lacertid lizards. Ocular media transmittance suggests adaptation to a UV-tuned photoreceptor in the retina of lacertids. In fact, no lacertid examined shows selective filtering of UV wavelengths, and we only found relatively minor differences in the cut-off wavelengths of lens transmittance. In addition, lacertids show a type of cone oil droplet (i.e. transparent small oil droplets) that allows the transmission of UV wavelengths and suggests the existence of a population of UVS photoreceptors. Because violet-sensitive (VS) cones have not been described in the Squamata [lizards (Fleishman et al., 1997; Fleishman et al., 2011;

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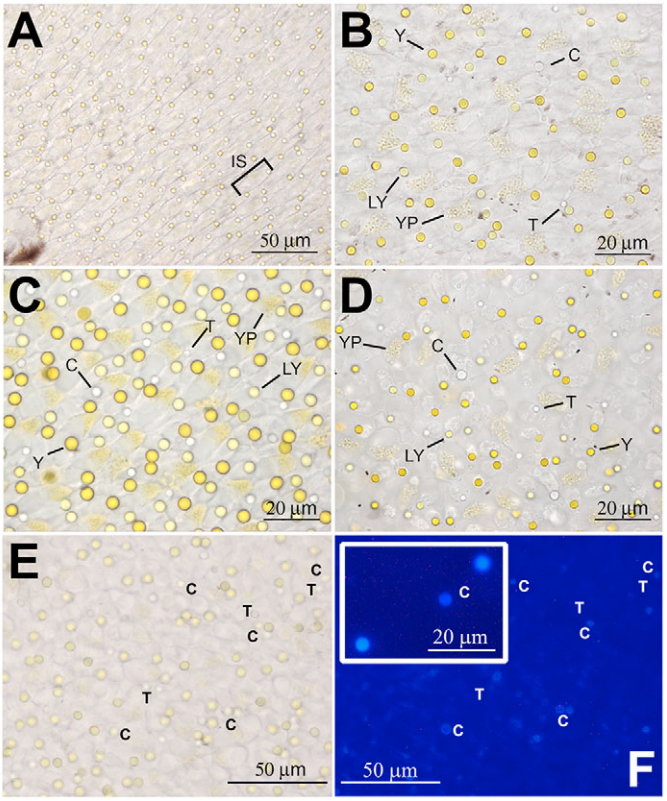

Fig. 2. Microscopic images of lacertid cone oil droplets. Retina viewed under brightfield microscopy: (A,B) *Podarcis vaucheri*, (C) *Gallotia galloti* and (D) *Psammodromus algirus*. (E,F) A portion of a retina of *Podarcis pityusensis* viewed using brightfield (E) and fluorescence (F; shown in detail in the inset) microscopy. Oil droplets: Y, dark yellow; LY, light yellow; C, clear; T, transparent (not visible using fluorescence microscopy); IS, inner segment; YP, putative diffuse yellow pigment of double cones.

Loew et al., 2002) and snakes (Sillman et al., 1997; Sillman et al., 1999; Sillman et al., 2001; Macedonia et al., 2009)], the short-sensitive photoreceptor of lacertids is likely of the UVS type.

Moreover, sequences of the SWS1 opsin gene strongly suggest that this gene expresses a UVS-type opsin instead of the VS type. Molecular analyses reveal that all 30 species that had their SWS1 opsin gene sequenced possess the amino-acid substitutions that are critical for UV perception (see Table 4). For example, substitutions that cause strong changes in spectral tuning [e.g. at F86 and S90 (van Hazel et al., 2013; Ödeen and Håstad, 2013)] are not found. In fact, we found relevant substitutions only at M109 in *Lacerta agilis* and at A118 in *Mesalina simoni*. The I109M substitution is conservative and as such probably has a limited effect, but the S118A substitution is non-conservative, replacing polar with non-polar side chain polarity, and possibly important to spectral tuning, probably increasing λ_{max} (Yokoyama, 2008). However, in general it seems that the SWS1 sequence is conserved throughout lacertids and probably also throughout diurnal lizards. In the absence of more accurate data (i.e. microspectrophotometric analyses), a crude extrapolation of available data from *A. carolinensis* and *G. gecko* (Loew, 1994; Loew et al., 2002) tentatively suggests that the peak of sensitivity of lacertid UVS photoreceptors may be around 360 nm.

Finally, our conclusion is further buttressed by the result of the behavioural discrimination experiment, which demonstrated that *Podarcis muralis* males are capable of discriminating between two

Table 3. Types of oil droplet found in lacertids

Oil droplet	Cut-off wavelength	Putative photoreceptor
T	Cut-off < 340 nm	UVS
C	340–380 nm < cut-off < 435 nm	SS
LY	435 nm < cut-off < 485 nm	MS
Y	485 nm < cut-off < 510–550 nm	LS

T, C, LY and Y are transparent, clear, light yellow and dark yellow oil droplets, respectively; UVS, SS, MS and LS are UV-, short-, medium- and long-wavelength-sensitive photoreceptors, respectively.

visual stimuli differing only in their UV component. This result evidences the ability to detect the near-UV waveband, but it does not provide information about the function of detecting and discriminating the male UV-reflective patches. As agonistic behaviours or other social displays were not observed during trials, we suggest that the experimental males probably chose the side of the experimental terrarium that afforded the more natural view of the stimulus lizard. Further work relying on experimental manipulation of UV-reflective patches and/or natural variation in this character must be conducted to explore the adaptive significance of UV-reflective colour patches.

The use of several complementary approaches strongly reinforces our conclusion that lacertids are capable of seeing in the near UV. Previous studies based on a single technique have sometimes yielded contradictory results. For example, in the ornate dragon lizard, *Ctenophorus ornatus* (Agamidae), results of a behavioural test suggested UV vision (LeBas and Marshall, 2000), but MSP measurements later indicated that this species lacks UVS photoreceptors and is therefore blind to UV wavelengths (Barbour et al., 2002).

Our results lend support to the hypothesis that lacertids have a complex colour vision system encompassing a range of wavelengths from 320 to 700 nm. The presence of four oil droplet types in the lacertid retina is presumably associated with four different types of cone photoreceptor (Loew et al., 2002; Bowmaker et al., 2005; Macedonia et al., 2009; Fleishman et al., 2011). Although research on the lacertid visual system is still in its infancy, possessing four types of cone may indicate that lacertids have four independent channels for processing colour information and therefore a tetrachromatic system of colour vision. However, as the number of different cones does not explain per se the number of chromatic dimensions in a colour visual system (Osorio et al., 1999), future efforts should focus on determining the number of neural channels related to chromatic discrimination in lacertids.

Relevance of UV vision in lacertids

Several key aspects of lacertid biology may crucially depend on their ability to perceive UV stimuli. For example, visual prey detection and recognition of aposematic colour patterns may be mediated by UV vision. Indeed, insects and arachnids, which constitute the largest part of the lacertid diet (Carretero, 2004), often include conspicuous (or cryptic) colour patterns that reflect or absorb selectively in the UV spectrum (e.g. Oxford and Gillespie, 1998; Kemp et al., 2005; Théry and Gómez, 2010).

UV vision in lacertids is bound to be particularly important in relation to visual communication. As in other lizards (e.g. Lappin et al., 2006; Whiting et al., 2006), the colour patterns shown by many lacertids often encompass UV patches [with a single UV-reflective peak or a secondary UV peak accompanying a main peak in the human visible spectrum (Pérez i de Lanuza et al., 2013b)] that are likely designed for signalling. These colour patches are displayed

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Table 4. The 13 amino acids of the SWS1 opsin deemed critical for UV vision

Species (common name)	Amino acid position													GenBank accession number
	46	49	52	86	90	91	93	97	109	113	114	116	118	
Gallotia galloti (Tenerife lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917087
Psammodromus algirus (large psammodromus)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917083
Dalmatolacerta oxycephala (sharp-snouted rock lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917102
Zootoca vivipara (common lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917094
Iberolacerta cyreni (Cyren's rock lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917100
Takydromus sexlineatus (Asian grass lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917082
Darevskia armeniaca (Armenian lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917099
Timon lepidus (ocellated lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917086
Timon tangitanus (Atlas ocellated lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917101
Lacerta schreiberi (Iberian green lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917104
Lacerta bilineata (western green lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917103
Lacerta agilis (sand lizard)	F	F	T	F	S	V	T	A	M	E	A	L	S	JQ917095
Scelarsis perspicillata (Moroccan rock lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917089
Teira dugesii (Madeira lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917096
Podarcis liolepis (Catalonian wall lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917085
Podarcis vaucheri (Maghreb wall lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917088
Podarcis guadarramae	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917105
Podarcis bocagei (Bocage's wall lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917097
Podarcis pityusensis (Eivissa wall lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917098
Podarcis sicula (Italian wall lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917081
Podarcis muralis (common wall lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ818254
Podarcis melisellensis (Dalmatian wall lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917080
Meroles ctenodactylus (giant desert lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917077
Meroles knoxii (Knox`s desert lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917078
Meroles suborbitalis (spotted desert Lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917079
Ophisops occidentalis (western snake-eyed lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917090
Atlantolacerta andreanszkyi (Atlas dwarf lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917092
Acanthodactylus erythrurus (spiny-footed lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917084
Acanthodactylus boskianus (Bosk's fringe-toed lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	JQ917091
Mesalina simoni (Simon's desert racer)	F	F	T	F	S	V	T	A	I	E	A	L	A	JQ917093
Anolis carolinensis (green anole)	F	F	T	F	S	V	T	A	V	E	A	L	S	AH007736.1
Uta stansburiana (common side-blotched lizard)	F	F	T	F	S	V	T	A	I	E	A	L	S	DQ100325
Phelsuma madagascariensis (Madagascar day gecko)	F	F	T	F	S	V	T	S	I	E	A	L	S	AF074045.1
Gekko gecko (Tokay gecko)	F	F	T	F	A	V	T	S	I	E	A	L	S	AY024356.1

All the lacertid data come from the present study. Other sequences are from various sources: AH007736.1 (Kawamura and Yokoyama, 1996), DQ100325 (Su et al., 2006), AF074045.1 (T. Yuki, H. Osamu, Y. Masao and T. Fumio, unpublished data) and AY024356.1 (Yokoyama and Blow, 2001).

during social interactions, in which males maximize the visibility of these patches by compressing their body laterally and/or raising over the four limbs, offering a lateral view of their body to other lizards. The UV patches are often male-biased, sexually dichromatic characters (Font and Molina-Borja, 2004; Molina-Borja et al., 2006; Arnold et al., 2007; Font et al., 2009; Pérez i de Lanuza and Font, 2011; Pérez i de Lanuza, 2012) that probably evolved under sexual selection pressures relating to male–male competition (Pérez i de Lanuza et al., 2013b). Moreover, visual modelling reveals that the UV-reflective ventrolateral patches of *Podarcis muralis* are responsible for the most contrasted patterns in the visual world of this lacertid, maximizing the detectability of this colour trait (Pérez i de Lanuza, 2012).

Many of the UV patches of lacertids have their peak reflectance between 360 and 370 nm [mean ± s.e.m. peak location=368.5±2.0 nm, range=337–407 nm, calculated from 34 spots with a single UV-reflective peak and 15 spots with secondary UV-reflective peaks, corresponding to 33 species of lacertids; 33 spots from males and 16 from females (Pérez i de Lanuza et al., 2013b)]. These patches often show subtle spectral differences between species, sexes or individuals (Font and Molina-Borja, 2004; Pérez i de Lanuza and Font, 2007; Molina-Borja et al., 2006; Font et al., 2009; Bajer et al., 2010; Bajer et al., 2011; Pérez i de Lanuza, 2012; Pérez i de Lanuza et al., 2013b). Our data suggest that the SWS1 opsin of lacertids shows its maximum sensitivity around 360 nm, probably allowing lacertids to accurately discriminate the sex of conspecifics in species with UV sexual dichromatic patterns (e.g. Molina-Borja et al., 2006; Font et al., 2009), or to assess fighting ability and dominance of competitors (e.g. Pérez i de Lanuza et al., 2014). The proximity between the putative spectral location of maximum sensitivity of the SWS1 opsin and the maximum reflectance of most lacertid UV patches suggests that lacertid signal properties and sensory processes are selectively matched (Bradbury and Vehrencamp, 2011). However, as this coincidence is not necessarily explained by a process of signal matching, more information is needed to test whether selection acts on the chromatic properties of colour signals, on the visual sensitivity of receivers, or drives both traits convergently.

The available evidence thus suggests that the UV component of lacertid coloration may be an essential element in lacertid visual communication. Ignoring the differences between the visual systems of humans and lacertids may thus lead to inadequate interpretations of the evolution and functions of lacertid coloration. For example, in the sand lizard, *Lacerta agilis*, probably the lacertid in which coloration has been best studied (e.g. Anderholm et al., 2004; Olsson et al., 2005), the UV reflectance of the male breeding coloration was ignored until recently (Pérez i de Lanuza and Font, 2007). The inclusion of the UV range of the spectrum in studies of male *L. agilis*' coloration has demonstrated that UV reflectance plays a

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crucial role in intrasexual and intersexual communication (Olsson et al., 2011). Similarly, the presence of UV reflectance may force a revision of conclusions regarding the function and evolution of colour patches in other widely studied lacertid species.

UV vision and colour vision in diurnal lizards

Our results double the number of species of diurnal lizards in which UV vision has been described, providing information on colour vision in a previously unexplored lizard clade. Essential aspects of lacertid eyes, such as eye transmittance and oil droplet retinal composition, are similar to those described in other diurnal lizards (Fleishman et al., 1993; Fleishman et al., 1997; Fleishman et al., 2011; Loew et al., 2002; Bowmaker et al., 2005; Macedonia et al., 2009). These results demonstrate that lacertids have a complex colour vision entirely comparable to that of iguanians, and reinforce the hypothesis that colour vision based on four types of cones, including a specific UV sensitive photoreceptor, is shared and conserved by the main diurnal lizard clades, irrespective of their phylogenetic position (Fleishman et al., 2011).

MATERIALS AND METHODS

In order to generate a visual perception, light first needs to be transmitted through the ocular media (including, if any, oil droplets), and absorbed by light-sensitive visual pigments in the photoreceptor cells of the retina. Retinal photoreceptors transduce photic energy into electrochemical information, which is then conveyed to ganglion cells and processed by the central nervous system, where it may ultimately affect the animal's decision-making processes. Our experimental approach is built around these stages of visual perception. Fig. 3 shows a phylogeny of the currently recognized lacertid genera; genera encompassing the species used in this study are identified with an indication of the types of experiments in which they were involved. Although the phylogeny of this family is not completely resolved (e.g. Arnold et al., 2007; Mayer and Pavlicev, 2007; Pavlicev and Mayer, 2009), all the species included in our analyses belong to accepted monophyletic genera.

Ocular filtering

Ocular transmittance data were obtained from eight species representative of the three main lacertid subclades (Fig. 3): *Gallotia galloti*, *Psammodromus algirus* (subfamily Gallotiinae), *Podarcis muralis*, *P. vaucheri*, *P. pityusensis*, *Timon lepidus*, *Takydromus sexlineatus* (subfamily Lacertinae, tribe Lacertini) and *Acanthodactylus erythrurus* (subfamily Lacertinae, tribe Eremiadini).

Measurements were obtained from fresh samples in most species (*G. galloti*, *P. algirus*, *P. muralis*, *P. vaucheri*, *P. pityusensis* and *A. erythrurus*). Individuals were euthanized with an overdose of anaesthesia (15 µl g^{-1} body mass of 50 mg ml^{-1} Ketolar), and their cervical spinal cord was completely transected with surgical scissors to ensure that the animals were dead before eye enucleation. Additionally, we used eyes from specimens dead from natural causes (*T. lepidus* and *T. sexlineatus*), which had been frozen in air at −18°C immediately after death. We conducted preliminary trials with animals preserved in ethanol or paraformaldehyde, but found that the optical properties of their lenses were altered (data not shown).

We measured transmittance of the ocular media using methods similar to those described by Siebeck and Marshall (Siebeck and Marshall, 2000; Siebeck and Marshall, 2001). After enucleation, a window was cut in the posterior part of the eye, removing the sclera and the retina (that were separated and mounted for microscopic processing; see below). Dissection was done carefully to retain at least a portion of the vitreous humour.

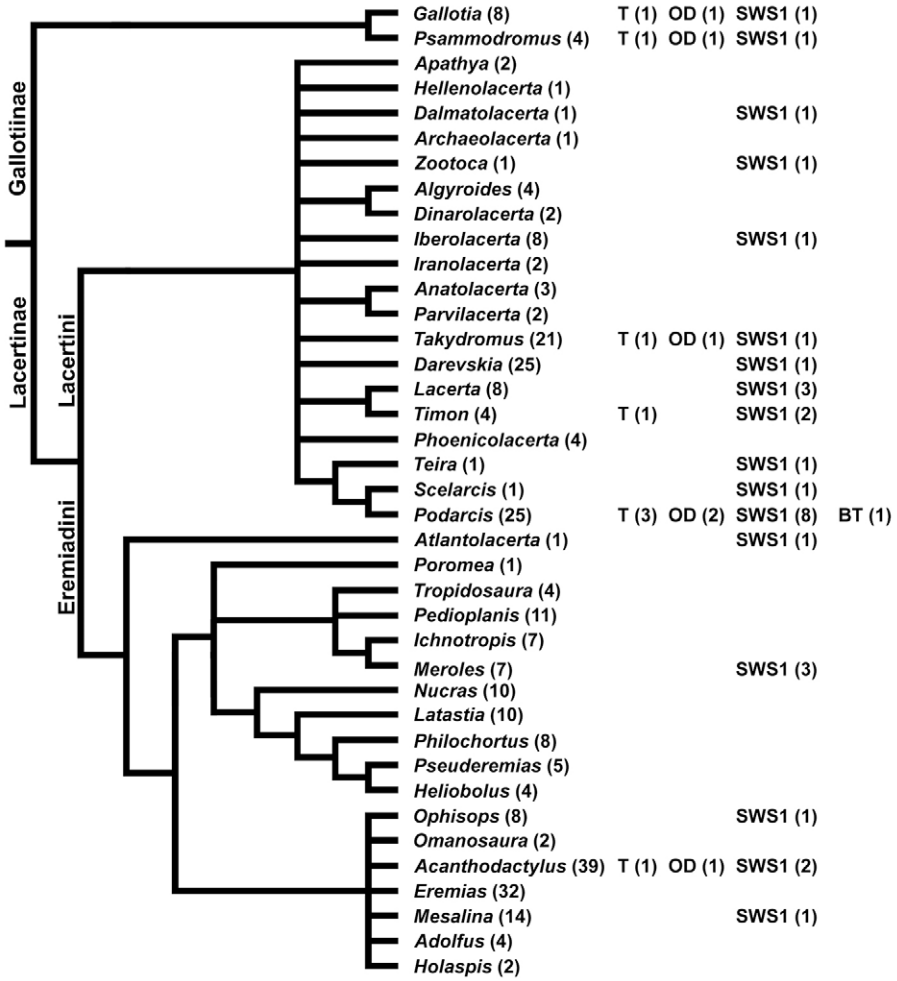

Fig. 3. Phylogeny of Lacertidae showing the genera in which experiments were conducted for the present study. T, transmittance measurements; OD, oil droplet characterisation; SWS1, molecular analysis of the SWS1 opsin gene; BT, behavioural test. Phylogeny adapted from previous studies (Arnold et al., 2007; Mayer and Pavlicev, 2007; Pavlicev and Mayer, 2009). Numbers in parentheses after genus names indicate the accepted number of species in each genus (based on www.lacerta.de database); numbers in parentheses after experimental procedure codes indicate the number of species sampled. Branch lengths are arbitrary.

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Dissected eyes were placed looking down in a purpose-made metallic eye-holder painted with black non-reflective paint. The eye-holder was then situated between the emissive probe (down) and the receptive probe (up) of a portable spectrophotometer (see below), allowing light to pass from the cornea to the posterior (retinal) part of the eye. Emissive and receptive probes included a single 200 µm diameter optical fibre (QP200-2-UV-BX, Ocean Optics, Dunedin, FL, USA). Both probes were placed perpendicularly to the eye surface and aligned to the anteroposterior axis of the eye. After measuring the transmittance of the whole eyes, the lenses and the corneas were carefully removed and washed in saline solution (Cl^-: 154 mequiv. l^{-1}; Na^+: 154 mequiv. l^{-1}). Spectral transmittance data from lenses and corneas were obtained as for the whole eyes. Whole eyes, lenses and corneas were always measured in air (Douglas and McGuigan, 1989).

Spectral measurements were obtained using a portable fibre spectrophotometer USB2000 optimized for human visible/near-UV detection and a PX-2 light source (both from Ocean Optics). Before each measurement, the spectrophotometer was calibrated with both white (i.e. light source) and black (absolute darkness) references. The white reference was taken through a hole of the eye-holder at the same distance that ocular measurements were taken, and without filters or other objects that could alter light transmission. The black reference was obtained by blocking the light path to the receiver probe with a completely opaque black object. Values of transmittance were taken as percentages at 0.37 nm steps between 200 and 850 nm. Each measurement represents the average of 60 spectra taken in a single series of consecutive measurements without re-positioning the eye in the holder. The smoothing level was 10 and integration time was fixed in the range of 30–40 ms. We used OOIBase32 and SpectraSuite (both from Ocean Optics) as spectral acquisition software. For analyses, spectra were restricted to the 300–700 nm range and normalized by dividing all the values by the maximum transmittance. To characterize transmittance spectra, we calculated the wavelength at which transmittance reaches 50% ($\lambda T_{0.5}$).

Eyes from frozen animals occasionally showed injuries to the retina, and at least a portion of this tissue detached and disaggregated into the ocular humours during the dissection, generating artifactual spectra for the whole eye (see an example in Fig. 1C). When this happened, the spectra were rejected for subsequent analyses. However, we calculated an approximation to the transmittance of the whole eye by integrating (i.e. multiplying) the spectra from the cornea and the lens (Siebeck and Marshall, 2000), which were not optically altered by freezing (see Results).

Characterization of oil droplets

We characterized the retinal oil droplets of *Gallotia galloti*, *Psammodromus algirus*, *Podarcis vaucheri*, *P. pityusensis*, *Takydromus sexlineatus* and *Acanthodactylus erythrurus* (Fig. 3). After dissection, retinas were separated from the sclera and the black pigment layer and were rinsed in saline solution. Fresh retinas were mounted on UV-transparent microscope slides, using a droplet of saline solution to prevent folds in the tissue. We used brightfield microscopy techniques to identify the different oil droplet types present in lacertid eyes. Fluorescence microscopy was also used in order to identify oil droplets that selectively transmit or absorb UV wavelengths (Ohtsuka, 1984; Wilkie et al., 1998; Kram et al., 2010; Coyle et al., 2012; Moore et al., 2012). In addition, we used coloured filters for brightfield microscopy to determine the wavelength range encompassing the cut-off wavelength (i.e. filtering threshold) of each oil droplet type.

The retinas were observed and photographed with a standard light microscope (Eclipse E800, Nikon, Tokyo, Japan) equipped with a digital camera (DXM1200F, Nikon). For fluorescence microscopy, a DAPI filter (excitation spectra: 340–380 nm; dichromatic mirror: >400 nm; passband; 435–485 nm) was used. Besides the DAPI filter, three other filters were used to estimate the cut-off wavelengths of the oil droplets: FITC (passband: 510–550 nm), G-2A (passband: over 590 nm) and Texas Red (passband: 600–660 nm).

Spectral tuning of the SWS1 opsin

We extracted total DNA from tail tissue samples using the DNeasyTissue Kit (QIAGEN, Venlo, The Netherlands). Most tissue samples were preserved in ethanol (70 or 90%), but some were frozen. Tissue samples from a total of 30 species were analysed (see Fig. 3).

We amplified exon 1 of the SWS1 opsin gene (292 nucleotides with primers, 256 nucleotides without primers) because this fragment encompasses the 13 amino acid positions relevant for UV vision: F46, F49, T52, F86, S90, V91, T93, A97, I109, E113, A114, L116 and S118 [numbers are standardized by those of the bovine rhodopsin (Shi and Yokoyama, 2003; Takahashi and Yokoyama, 2005; Yokoyama, 2008)]. We designed degenerate PCR primers based on the sequences coding for the SWS1 opsin gene from *Anolis carolinensis* (GenBank accession no. AH007736.1). Primers are SWS1.2f: 5′-CCARTACCACATCGCCCC-3′ (at positions 160 and 177) and SWS1.VSR: 5′-GTGGCAGGTAAAARSCCY-3′ (at positions 434 and 451).

PCR conditions were: a cycle of denaturalization (2 min at 94°C), 35 cycles of hybridization and extension (30 s at 94°C, 30 s at 60°C and 90 s at 72°C), a cycle of final extension (7 min at 72°C) and the final infinite cycle at 4°C. Visualization of amplified products was carried out in 1.4% agarose gel + 5 µl gelred. High Pure PCR Product Purification Kit (Roche, Basel, Switzerland) was used for the purification of the amplified products. PCR products were sequenced with an ABI PRISM Big-Dye Terminator v3.1 system (Applied Biosystems, Foster City, CA, USA) in the automatic sequencer ABI 3730. All PCR products were sequenced in both directions with the amplifier primers and were assembled to obtain the complete region. Sequences were verified, corrected and assembled with the STADEN software package. Sequence alignments were made with ClustalW software implemented in the Mega4 v.4.0.2 package.

Visual discrimination experiment

To establish the ability of lacertids to detect near-UV wavelengths, we conducted a visual discrimination experiment with the common wall lizard, *Podarcis muralis*. We chose this species because its behaviour and coloration are relatively well studied and because males exhibit conspicuous UV-reflective ventrolateral patches (Pérez i de Lanuza et al., 2013a; Pérez i de Lanuza et al., 2013b; Pérez i de Lanuza et al., 2014). Twenty adult males from a Pyrenean population (42°28′N, 1°57′E) were captured during the breeding season (June 2010) and transported to the laboratory. Lizards were housed in individual glass terraria (20×40×25 cm, width × length × height) and were maintained under conditions of illumination and temperature matching natural spring conditions. Refuges, tiles for thermoregulation and water were always available in the terraria. The males were acclimated to laboratory conditions for 2 weeks prior to the experimental trials. During the acclimation and experimental periods, lizards were fed *Tenebrio molitor* larvae three times weekly and water was provided *ad libitum*.

The experimental terraria (70×40×30 cm, width × length × height) were each divided into a stimulus area (S; 440 cm^2) and an experimental area (1920 cm^2; Fig. 4A). The latter contained a no-choice area (NE; 1120 cm^2) and two choice areas (E1 and E2; 620 cm^2 each). An opaque plastic barrier separated the two choice areas. One side of the stimulus area was separated from the choice area in the same side by a filter transmitting UV light down to 250 nm (UV+; Plexiglas GS 2458, Evonik Industries AG, Essen, Germany). The other side was separated from the choice area by a UV-opaque filter (UV–; Plexiglas GS 233). Fig. 4B shows the transmittance spectra of both filters. The amount of light transmitted in the 300–700 nm range of wavelengths (adding the percent of transmitted light across this range of the light spectrum) is 116,203 for the UV+ filter and 96,466 for the UV– filter, resulting in a difference of 17%. However, this difference is much smaller (<1%) in the human visible spectrum (i.e. 400–700 nm), with amounts of transmitted light of 88,885 and 87,603 for the UV+ and the UV– filters, respectively. As the UV-opaque filter allowed some transmission of UV at longer wavelengths (Fig. 4B), the difference in transmission between the UV– and UV+ stimulus was pronounced at short wavelengths and very small closer to 400 nm. We used two identical experimental terraria to allow simultaneous running of two trials.

In each trial, two males were used: an experimental male and a stimulus male (each lizard participated in only one trial). Eighteen hours before each trial, both males were introduced into the experimental terrarium with their own refuges, thermoregulation tiles and water dish. The experimental male was restrained in the no-choice area by an opaque plastic removable partition. During the 18 h period, four pieces of filter paper were placed in the stimulus area (two under the refuge and two over the thermoregulation

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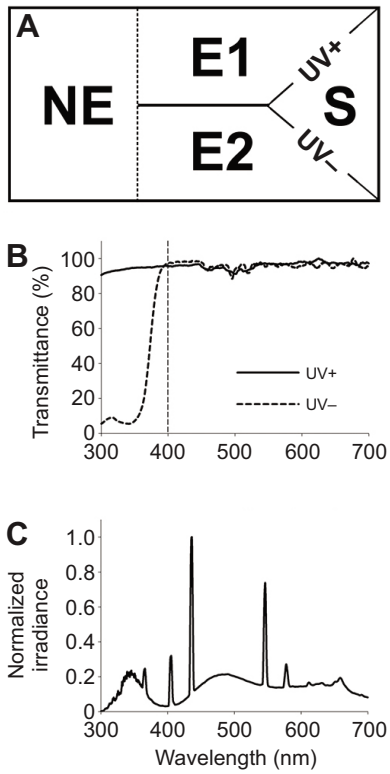

Fig. 4. Experimental setup. (A) Experimental terrarium. NE, no-choice area; E1 and E2, choice areas; S, stimulus area; UV+ and UV–, UV-transmitting and UV-absorbing filters, respectively; continuous horizontal line, opaque wall; vertical broken line, opaque divider used during the 18 h period before trials to avoid visual contact between lizards. (B) Transmittance spectra of UV+ and UV– filters. (C) Normalized irradiance spectrum of experimental light source.

tile) to collect chemical stimuli from the stimulus male. Immediately before each trial, one piece of paper from the tile and another from the refuge were moved into each choice area. Thus, the experimental male had access to chemical and visual stimuli of the stimulus male on both sides of the experimental terrarium (Font et al., 2012). However, chemical stimuli were identical in the two choice areas whereas visual stimuli differed in the presence/absence of UV cues. We predicted that the experimental lizard would spend more time in the UV+ side of the terrarium, which offered a chromatically more realistic view of the stimulus lizard. To begin a trial, the partition separating the choice and no-choice areas was removed. All trials took place during the period of maximum activity of lizards (9–14 h) and lasted 5 h. Experimental terraria were illuminated with full-spectrum tubes (300 to 700 nm, Reptistar, Sylvania, London, UK; Fig. 4C) powered by high-frequency electronic ballasts (Quicktronic® Professional 2×36/230-240 OSRAM, Munich, Germany). The position of the UV+ and UV– filters in one experimental terrarium was opposite that in the other terrarium. The location of the experimental male (scored as NE, E1 or E2) was recorded every 10 min by an observer who was unaware of the position of the filters, resulting in a total of 30 sample points per lizard. A trial was considered valid if the experimental lizard entered the two choice areas. In between trials, the experimental terraria were washed with ethanol to remove any chemical stimuli left by the experimental or stimulus males. A Wilcoxon matched-pairs test was used to compare the number of sample points spent by experimental lizards in each of the two choice areas. After experiments, all lizards were released unharmed at their place of capture.

Ethical standards

Experimentation was carried out according to requirements of the University of Valencia's Ethics Committee. Samples were taken with official permissions of the Conselleria de Medi Ambient, Aigua, Urbanisme i Habitatge (Spain) and the Direction Departamentale des Territoires et de la Mer, Service Environnement, Forêt, Sécurité Routière (France). To minimize our research's impact on natural populations, where possible we used lizards collected for other purposes that could not be released back into the field (*Podarcis vaucheri*), or lizards from introduced populations (*Podarcis pytiusensis* from the Basque coast). The *Takydromus sexlineatus* sample corresponds to a dead individual obtained from captive breeding. For the behavioural experiment, we obtained *Podarcis muralis* males from a dense population (Pérez i de Lanuza et al., 2013a; Pérez i de Lanuza et al., 2014) in which the temporary subtraction of some individuals is unlikely to have serious ecological consequences.

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Competing interests

The authors declare no competing financial interests.

Author contributions

G.P.L. and E.F. conceived and designed the experiments. G.P.L. performed the experiments and the analyses. G.P.L. and E.F. wrote the manuscript and approved the final version.

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