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Visual pigments and oil droplets in diurnal lizards: a comparative study of Caribbean anoles

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Summary

We report microspectrophotometric (MSP) data for the visual pigments and oil droplets of 17 species of Caribbean anoline lizard known to live in differing photic habitats and having distinctly different dewlap colors. The outgroup *Polychrus marmoratus* was also examined to gain insight into the ancestral condition. Except for *Anolis carolinensis*, which is known to use vitamin A_2 as its visual pigment chromophore, all anoline species examined possessed at least four vitamin-A_1-based visual pigments with maximum absorbance (λ_{max}) at 564, 495, 455 and 365 nm. To the previously reported visual pigments for *A. carolinensis* we add an ultraviolet-sensitive one with λ_{max} at 365 nm. Five common classes of oil droplet were measured, named according to apparent color and associated with specific cone classes – yellow and green in long-wavelength-sensitive (LWS) cones, green only in medium-wavelength-sensitive (MWS) cones and colorless in short-wavelength-sensitive (SWS) and ultraviolet-sensitive (UVS) cones. MSP data showed that the colorless droplet in the SWS cone had significant absorption between 350 and 400 nm, while the colorless droplet in the UVS cone did not. The pattern for *Polychrus marmoratus* was identical to that for the anoles except for the presence of a previously undescribed visual cell with a rod-like outer segment, a visual pigment with a λ_{max} of 497 nm and a colorless oil droplet like that in the UVS cones. These findings suggest that anoline visual pigments, as far as they determine visual system spectral sensitivity, are not necessarily adapted to the photic environment or to the color of significant visual targets (e.g. dewlaps).

Key words: vision, microspectrophotometry, anoline, lizard, *Anolis carolinensis*, *Polychrus marmoratus*, visual pigment, oil droplet, photoreceptor.

Introduction

Anoline lizards are diurnally active, visually oriented animals that are known to possess an excellent, high-acuity visual system (Underwood, 1970; Makaretz and Levine, 1980; Fite and Lister, 1981). There are over 300 species of anoles and they occupy a variety of habitats, making them a model system for studies of adaptive evolution. Here, we present a comparative spectral study of the photoreceptors of 18 species of anoline lizard of known evolutionary relationships from a variety of different habitats.

Animal visual systems exhibit tremendous diversity. A tenet of the discipline of visual ecology is that this diversity is due, at least in part, to differences among species in the environment in which their visual systems function and in the visual tasks the systems are designed to carry out (see Lythgoe, 1979). A common approach has been to examine the visual and photic environment in which different animals live and to compare it with the design features of their visual systems, looking for correlations that might help explain the functional significance of different elements in the system. As regards spectral sensitivity (overall and of individual sets of photoreceptors), this approach has been used most successfully in studies of fish and other aquatic organisms in relation to the spectral quality of the waters they inhabit, where it has been shown that these features have apparently evolved in response to the color, water clarity and depth at which the animals are found (Loew and Lythgoe, 1978; Bowmaker et al., 1994; Lythgoe et al., 1994; Levine and MacNichol, 1979; Shand, 1993; McDonald and Hawryshyn, 1995; Cronin et al., 1996; Partridge and Cummings, 1999).

Attempts to apply the same process to terrestrial systems have generally not yielded such clear relationships (e.g. Partridge, 1989). For example, Fleishman et al. (1997)

measured overall spectral sensitivity and habitat light spectra for six closely related species of Puerto Rican anoline lizard. Although the species occupied microhabitats with distinctly different spectral irradiances, there was little difference among the species in spectral sensitivity. Two possible explanations were offered for the lack of apparent correlation between photic environment and spectral sensitivity: (i) since the species evolved relatively recently from a single common ancestor, they may all share the ancestral condition and, for whatever reason (e.g. lack of time or lack of appropriate genetic variation), they have not yet diverged in response to habitat conditions; (ii) the visual systems have evolved in response to some specific photic aspect (e.g. background radiance, which in all cases was dominated by green vegetation) that is much more similar among habitats than is habitat irradiance.

To deduce the influences of both ancestral state and environmental conditions on the evolution of specific traits, one would ideally like to compare species with distinctly different ecological conditions that share a common ancestry and, conversely, species under common ecological conditions with distinct ancestry.

The genus *Anolis* has experienced a massive radiation and contains over 300 species. The best-studied species are those that occupy the islands of the Greater and Lesser Antilles. Many of these islands appear to have been colonized once or twice, and from these original colonists a number of new species evolved on each island as the ancestral population diverged into distinctly different ecological niches (see Roughgarden, 1995). Detailed studies of the evolutionary history and ecology of these species have shown that many are found on different islands and occupy very similar ecological niches (referred to as ecomorphs). However, the species on the different islands occupying similar ecomorphs are generally not closely related. In contrast, species from the same island occupying distinctly different ecomorphs are typically closely related, reflecting the pattern of colonization and radiation described above (Jackman et al., 1999; Losos et al., 1998).

The different ecological niches occupied are largely determined by preferred substratum (e.g. tree trunks, grass, twigs, etc.) and range of preferred temperatures (Losos et al., 1998; Rand, 1964; Hertz et al., 1994). An indirect consequence of these shade and substratum preferences is that each species occupies a habitat with a distinct and characteristic photic environment. Fleishman et al. (1997) identified four distinct photic habitats for anoles on Puerto Rico. These included: (i) full shade, closed forest canopy where habitat irradiance is dominated by the green chlorophyll spectrum and total light intensity is quite low; (ii) partial shade, dry, semi-open forest or forest edge where light intensity is intermediate and the irradiance spectrum includes a large short-wavelength component coming from blue sky; (iii) full sun, open habitats such as grassy fields where light intensity is high and the irradiance spectrum is broadband and essentially that of sunlight; and (iv) canopy, species that live near the forest canopy, where the light environment is similar to that of partial shade, except that the background tends to include a mixture of green (vegetation) and blue (short wavelengths from the sky). The anoline species from other islands can, in most cases, also be assigned to one of these habitat types (Fleishman, 2000) (see also Endler, 1992, 1993). However, as described above, the occupants of similar light environments on different islands are not usually closely related. For example, the Puerto Rican grass anole *Anolis pulchellus* is more closely related to the full-shade Puerto Rican species *A. gundlachi* than it is to the grass anoles of Hispaniola or Cuba (Jackman et al., 1999).

The retina of anoles is considered to be pure cone with three classes identified, double cones and large and small single cones. All cells apart from the accessory member of the double cone contain an oil droplet (Walls, 1934, 1967). Visual pigment and opsin sequence data are available for one species, *Anolis carolinensis*. Provencio et al. (1992) reported the presence of three visual pigments: long-wavelength-sensitive (LWS) with maximum absorbance (λ_{max}) at 625 nm, medium-wavelength-sensitive (MWS) with a λ_{max} of 503 nm and short-wavelength-sensitive (SWS) with a λ_{max} of 462 nm, all using vitamin A_2 as the chromophore. An ultraviolet-sensitive (UVS) opsin was later identified and expressed (see Yokoyama and Yokoyama, 1996; Kawamura and Yokoyama, 1997) together with a second pigment similar in λ_{max} to the MWS pigment but more closely related to rhodopsin. However, nothing is known about the visual pigments of other anoles, nor have there been any reports concerning the spectral characteristics of the oil droplets.

In this paper, we present a study of the visual pigments and oil droplets of 17 species of *Anolis* lizard from the Caribbean. In addition, we have included data for *Polychrus marmoratus*, a South American lizard that is widely considered to be one of the closest relatives to *Anolis* (Jackman et al., 1999). The species included are summarized in Fig. 1, which also shows their evolutionary relationships and typical photic habitat. Jackman et al. (1999) identified 17 distinct monophyletic lineages in the genus. Of these, five are represented in our sample. The majority of the species come from two monophyletic clusters of ecologically diverse species, one from Jamaica and one from Puerto Rico, which provide the opportunity to examine the relationship between the light environment and retinal design in closely related species. The other species in our sample provide the opportunity to examine the visual system in the context of the overall evolutionary history of the group. Finally, we have examined the outgroup species *Polychrus marmoratus* in an attempt to identify the ancestral condition (i.e. those features common to all the anoles and *Polychrus marmoratus*).

Materials and methods

The Puerto Rican lizards were collected in 1992–1994 in the vicinity of Luquillo, and El Verde, Puerto Rico, under Puerto Rico DNR permits 92-52, 93-82 and 94-40. The remaining species were either purchased from Glades Herp (Fort Myers, FL, USA) or kindly donated by other biologists including J.

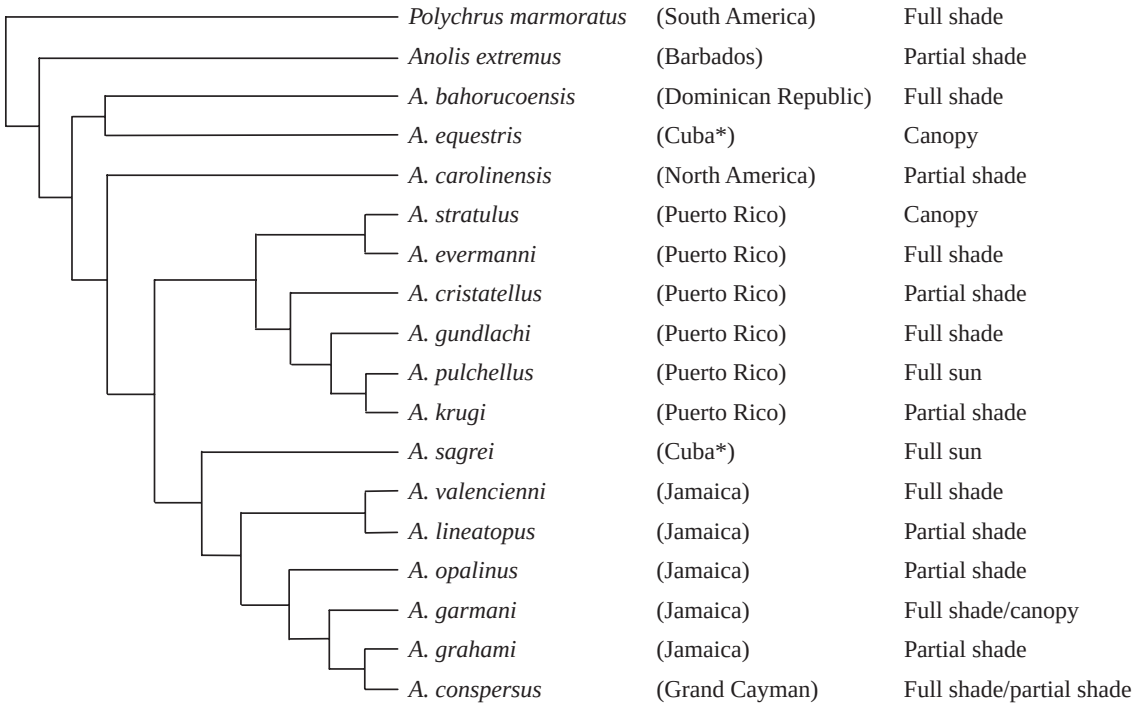

Fig. 1. A cladogram showing the relationships between the species from this study based on Jackman et al. (1999) and T. R. Jackman (personal communication). The lengths of the arms of the cladogram have no quantitative meaning, although we have linked closely related species with short arms. There are other species between those listed in this cladogram: i.e. the closest taxa in this diagram are not necessarily the most closely related species known. Jackman et al. (1999) identified 17 major distinct clades within the genus *Anolis*. Five of these, plus the closely related outgroup genus *Polychrus*, are represented in our sample. **A. sagrei* and *A. equestris* were collected from feral populations in Florida, but are Cuban in origin.

Macedonia, K. Orrell and J. Losos. In each case, we received assurances from the donating individuals that the specimens had been legally collected with proper permits. Lizards were usually used for microspectrophotometric (MSP) measurement and retinoid analyses within a few days of capture after being transported directly to New York (microspectrophotometry) or Virginia (retinoid analysis). In a few cases, lizards were maintained at Union College, New York, in small individual aquaria for periods of up to 2 months in a room illuminated with incandescent and fluorescent lighting on a 12 h:12 h dark:light cycle. All the anoles and *Polychrus marmoratus* were regularly provided with water and fed on crickets. All housing, maintenance and methods of killing the lizards conformed to the recommendations of Pough (1991).

Chromophore analyses

Because vitamin A_2 was found to be the chromophore for the visual pigments in *A. carolinensis* (Provencio et al., 1992), it was deemed important to analyze as many other anoles as possible to determine how widespread this usage is. Procedures identical to those described by Provencio et al. (1992) were used. Briefly, dark-adapted animals were decapitated and enucleated, and the the whole isolated eyes were frozen in liquid nitrogen and stored at –70 °C until analyzed. The 11-*cis* retinoids from whole eyes were converted to their corresponding oxime isomers using hydroxylamine hydrochloride and extracted using the method of Groenendijk et al. (1980). These retinoids were separated from the crude extract by high-performance liquid chromatography (HPLC) through a LichroSorb Si60 analytical column (Chrompack, Raritan, NJ, USA) with a hexane:dioxane mobile phase. Eluent from the first 3 min of the run was collected and evaporated under nitrogen, after which it was redissolved in 200 µl of a 95:5 hexane:dioxane solvent mixture. Injection volumes of 100 µl were run through an identical analytical column using a 95:5 hexane:dioxane mobile phase. A Waters HPLC system including a model 991 photodiode array detector was used for separation and analysis.

Retinoid standards were prepared by dissolving 25 mg of all-*trans* retinaldehyde (A_1; Sigma, no. R-2500) or all-*trans* 3,4-didehydroretinaldehyde (A_2; a gift from Hoffman-LaRoche, Inc.) in 6 ml of ethanol. A 1 ml sample was illuminated with bright light (420–540 nm) for 3 h, after which 5 ml of a 1000-fold molar excess solution of hydroxylamine hydrochloride (1.92 mol l^{-1}, pH 6.5) was added to convert the retinoids to their respective oximes while retaining their isomeric configuration. The oximes were extracted by the method of Groenendijk et al. (1980) and stored at –80 °C.

Microspectrophotometry

Microspectrophotometric measurements were performed using methods identical to those described by Loew (1994) and

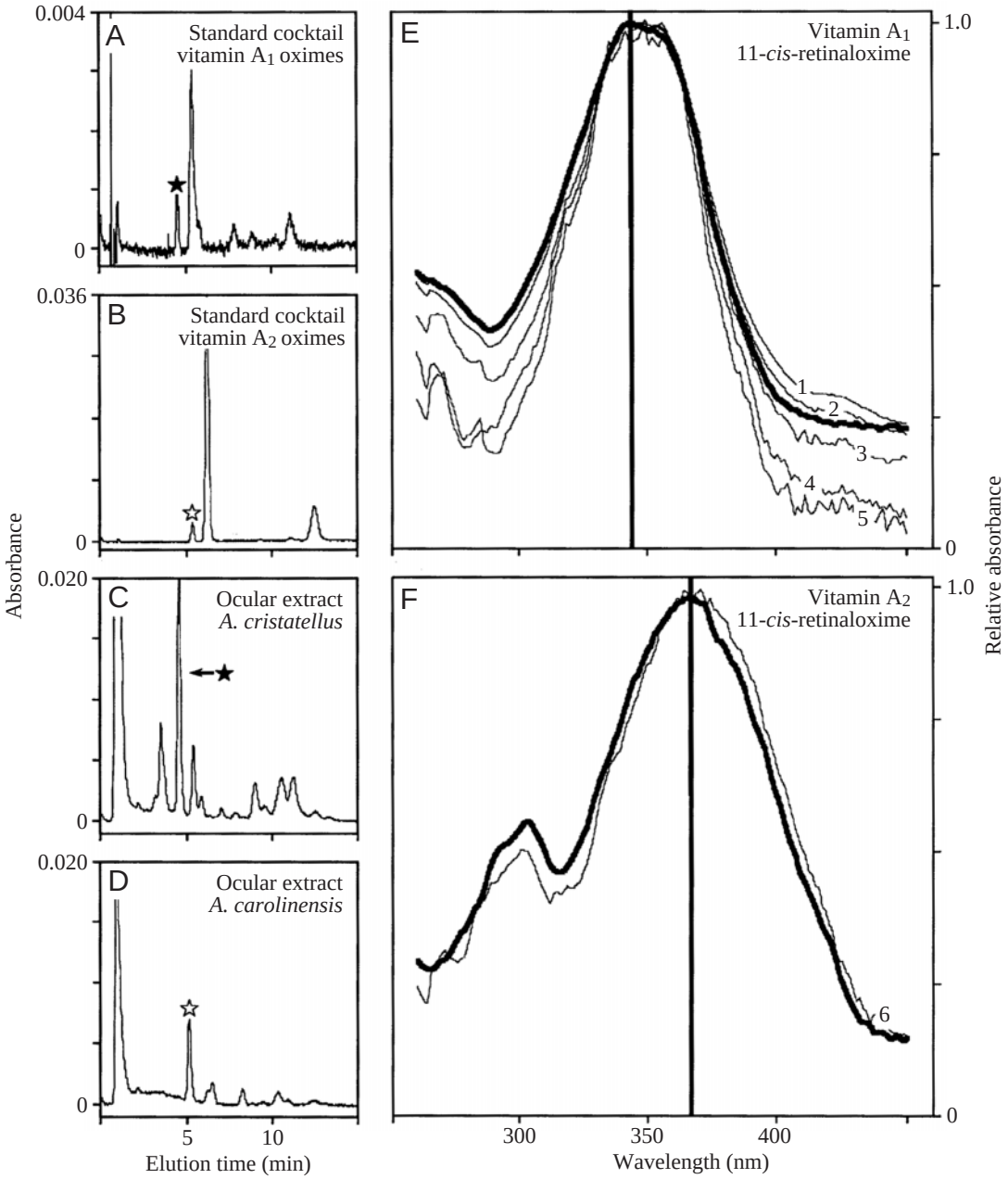

Fig. 2. HPLC chromatograms (A–D) and absorption spectra (E,F) indicating chromophore type for six anoles. Vitamin A_1 (A) and vitamin A_2 (B) standard chromatograms. HPLC chromatograms of whole eye extracts from *Anolis cristatellus* (C) and *A. carolinensis* (D). The peaks corresponding to the 11-*cis* isomers are indicated by stars (filled star, vitamin A_1 11-*cis*-retinaloxime; open star, vitamin A_2 11-*cis*-retinaloxime). The elution time of the vitamin A_1 11-*cis*-retinaloxime is 4.58 min while that of the vitamin A_2 11-*cis*-retinaloxime is 5.16 min. (E) Absorption spectra of the 11-*cis* isomers from whole-eye extracts of *A. cristatellus* and four other Puerto Rican anoles (trace 1, *A. gundlachi*; trace 2, *A. cristatellus*; trace 3, *A. evermanni*; trace 4, *A. pulchellus*; trace 5, *A. krugi*). The bold trace is the spectrum of the vitamin A_1 11-*cis*-retinaloxime standard. (F) Absorption spectrum of the 11-*cis* isomer from whole-eye extracts of *A. carolinensis* (trace 6) and the vitamin A_2 11-*cis*-retinaloxime standard (bold trace). Vertical lines are drawn through the λ_{max} of the spectra in E and F.

Provencio et al. (1992). All procedures were carried out under infrared illumination using appropriate image converters and video cameras. Animals were dark-adapted for a minimum of 2 h, after which they were cooled and decapitated. Enucleated eyes were hemisected, and the posterior segments were incubated for 2 h in Ca^{2+}/Mg^{2+}-free Puck's medium (Gibco) at 5 °C. The retinas were carefully teased from the retinal pigment epithelium and macerated using razor blade fragments and tungsten needles. In some cases, the posterior segment was immersed in a simple Sorensen's phosphate buffer (pH 7.2) with 6 % sucrose or dextran added, and the retina was isolated and prepared as above. No consistent differences in results were noted using these two techniques. As regards cell dispersion, reptile retinas tend to be very 'dirty', with lots of

free and adherent melanin granules and a paucity of free, intact photoreceptors. As a general rule, the more gentle the cutting and teasing, the better the preparation. In some cases, the best results were obtained by folding a small piece of retina over on itself, receptor side out, and working along the exposed edge. A drop of the dispersed retina or the folded piece was sandwiched between two coverslips and transferred to the stage of the microspectrophotometer, which has been described in detail elsewhere (Loew, 1994).

The criteria used for selecting data for inclusion into the analysis pool were the same as those used by Loew (1994). Dichroism was used for differentiating the UVS pigment from the photoproduct. Each acceptable spectrum was normalized by estimating a spectral maximum by eye and fitting a Gaussian function to the data points 20 nm either side of this wavelength. The peak of the Gaussian function was used to normalize the spectrum. Because the number of usable recordings was small, it was decided to sum the individual, normalized spectra at 1 nm intervals, calculate the mean at each nanometer and use this generated spectrum for template-fitting. Smoothing was performed using a digital filter routine ('smooft') (Press et al., 1987). The smoothed spectrum was overlaid on the unsmoothed one and checked by eye to make sure that over-filtering or spurious data points had not shifted the apparent maximum. In cases where there was obvious distortion due to outlier effects, the deviant point(s) was replaced with the mean of the 10 data points surrounding the outlier. λ_{max} was obtained using the method of Mansfield as presented by MacNichol (1986). The templates used were those from Lipitz and Cronin (1988). Wavelength error of the MSP measurements is ±1.0 nm, so whole integer values of λ_{max} are reported here.

Wherever possible, oil droplet or inner segment pigment absorption was measured together with visual pigment absorption in the same cell. Because of the high concentration of pigment in the droplets and, sometimes, the ellipsoids and the resulting high refractive index, it is not possible to obtain true optical density values for most oil droplets. The result is saturation of the MSP signal (the MSP method has been shown to give valid density measurements up to an optical density of 2.0 based on calibrated filters). In cases where this happens, the spectrum is normalized to the highest absorbance, and the wavelength at 50 % of the maximum is reported (see Lipitz, 1984). For non-saturating spectra, the normalization step was omitted (e.g. accessory member ellipsoid).

In some cases, enough retina was available to allow the oil droplets from pieces viewed in white or colored light to be categorized. A micrograph was first obtained in white light using a color video camera and frame grabber to show the true color of the droplets. The camera was switched to black and white, and the background illumination was altered with glass cut-off filters until a class of droplet was seen to turn black, indicating that the cut-off for that droplet had been passed. A coincident image was obtained. The background was again adjusted until the next class turned black. The process could not be used to differentiate among the colorless droplet classes as this would have required moving below 400 nm and there was neither enough light nor enough camera sensitivity to work in this wavelength range. Fluorescence has been used to

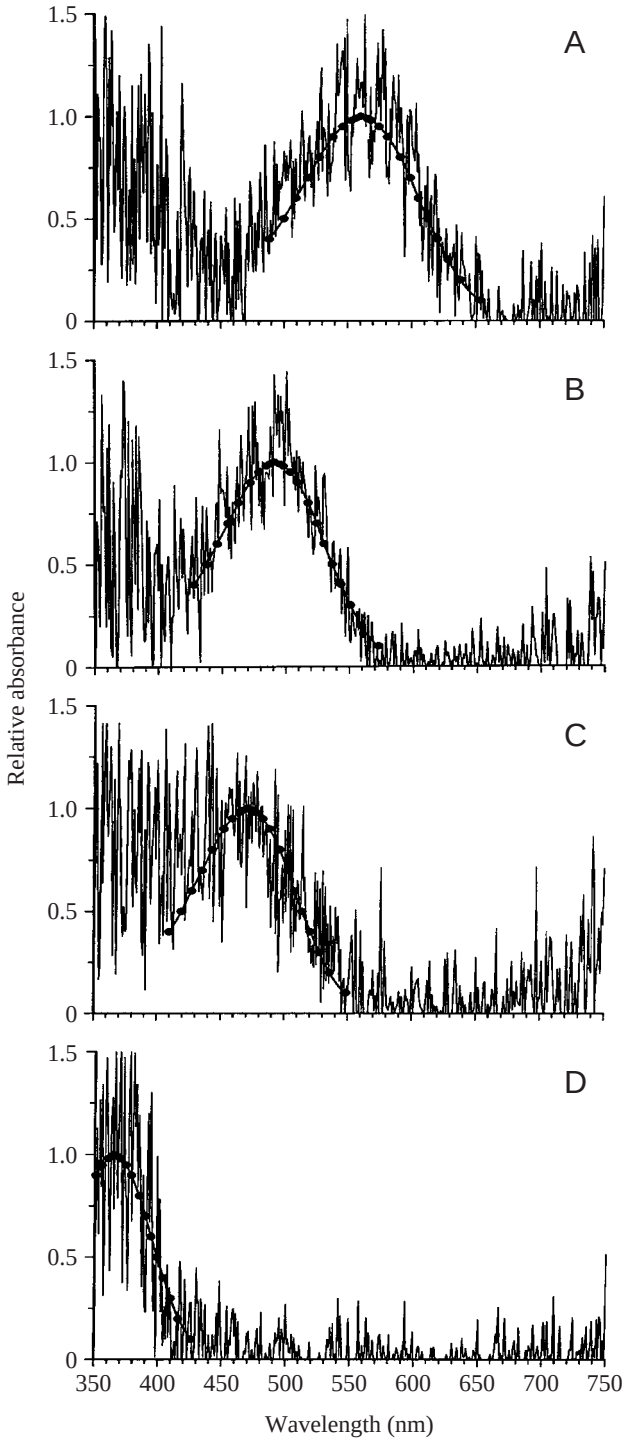

Fig. 3. Typical normalized visual pigment absorbance spectra, in this case from *Anolis cristatellus*. The filled circles and smooth curves are for the best-fit visual pigments calculated from vitamin-A_1-based template data. (A) Long-wavelength-sensitive pigment; (B) medium-wavelength-sensitive pigment; (C) short-wavelength-sensitive pigment; (D) ultraviolet-sensitive pigment.

Table 1. *Visual pigment and oil droplet values obtained by microspectrophotometry*

Species	Nationality	Habitat	Rod?	Visual pigment			
				LWS	MWS	SWS	UVS
Polychrus marmoratus	Central America	Full shade	497±2 (12)	568±4 (9)	490±3 (3)	453±5 (2)	–
Anolis extremus	Barbados	Partial shade		566±5 (8)	487±9 (3)	451±7 (1)	365±7 (1)
Anolis equestris	Cuba	Canopy		565±8 (4)	492±11 (1)	460±9 (1)	–
Anolis sagrei	Cuba	Full sun		567±4 (22)	495±5 (7)	460±6 (8)	365±3 (3)
Anolis bahorucoensis	Dominican Republic	Full shade		569±4 (6)	500±6 (5)	450±6 (2)	365±6 (2)
Anolis conspersus	Grand Cayman	Full sun/partial shade		562±3 (11)	500±7 (4)	460±7 (3)	365±7 (1)
Anolis garmani	Jamaica	Full shade/canopy		565±8 (3)	496±9 (2)	467±10 (2)	–
Anolis grahami	Jamaica	Partial shade		565±6 (4)	495±7 (3)	460±6 (4)	367±8 (1)
Anolis lineatopus	Jamaica	Partial shade		560±2 (7)	498±4 (2)	449±2 (3)	366±5 (1)
Anolis opalinus	Jamaica	Partial shade		566±5 (8)	496±5 (4)	450±5 (2)	–
Anolis valencienni	Jamaica	Full shade		560±9 (5)	500±8 (2)	456±8 (3)	–
Anolis cristatellus	Puerto Rico	Partial shade		562±4 (15)	492±5 (7)	458±4 (10)	365±6 (4)
Anolis evermanni	Puerto Rico	Full shade		565±3 (9)	490±3 (6)	460±5 (5)	364±5 (2)
Anolis gundlachi	Puerto Rico	Full shade		564±5 (7)	490±7 (2)	450±9 (1)	365±7 (2)
Anolis krugi	Puerto Rico	Partial shade		562±4 (11)	490±5 (4)	448±6 (3)	365±5 (1)
Anolis pulchellus	Puerto Rico	Full sun		565±7 (3)	495±8 (2)	446±7 (2)	367±8 (1)
Anolis stratulus	Puerto Rico	Canopy		564±4 (3)	494±6 (1)	454±7 (2)	366±6 (1)
Anolis carolinensis	North America	Partial shade		625±3 (6)	503±8 (3)	462±5 (6)	365±5 (4)
Mean				564±3	494±4	455±6	365±1

Species	Nationality	Habitat	Oil droplet				
			G1	G2	Y	C1	C2
Polychrus marmoratus	Central America	Full shade	520±2 (7)	485±5 (4)	462 (1)	368±2 (5)	+ (6)
Anolis extremus	Barbados	Partial shade		488±5 (15)	442±4 (21)	393±3 (3)	+ (3)
Anolis equestris	Cuba	Canopy		506±4 (12)	470±6 (8)	388 (1)	+ (2)
Anolis sagrei	Cuba	Full sun		510±5 (31)	475±3 (22)	376±2 (11)	+ (10)
Anolis bahorucoensis	Dominican Republic	Full shade		500±6 (11)	450±7 (8)	397 (1)	+ (2)
Anolis conspersus	Grand Cayman	Full sun/partial shade		515±5 (21)	475±6 (3)	368±2 (4)	+ (5)
Anolis garmani	Jamaica	Full shade/canopy		492±4 (8)	466±5 (5)	371 (1)	+ (1)
Anolis grahami	Jamaica	Partial shade		505±10 (15)	451±6 (6)	382 (1)	+ (2)
Anolis lineatopus	Jamaica	Partial shade		486±8 (7)	451±4 (3)	367 (2)	+ (2)
Anolis opalinus	Jamaica	Partial shade	521±3 (7)	497±6 (11)	471±5 (9)	375±4 (4)	+ (3)
Anolis valencienni	Jamaica	Full shade	522±2 (5)	505±4 (13)	479±4 (10)	368 (2)	+ (1)
Anolis cristatellus	Puerto Rico	Partial shade		507±5 (17)	463±5 (13)	371 (2)	+ (6)
Anolis evermanni	Puerto Rico	Full shade		515±7 (9)	500±6 (12)	380±4 (5)	+ (3)
Anolis gundlachi	Puerto Rico	Full shade		510±4 (12)	450± 6 (9)	370 (2)	+ (2)
Anolis krugi	Puerto Rico	Partial shade		500±5 (8)	480±5 (5)	370±3 (3)	+ (2)
Anolis pulchellus	Puerto Rico	Full sun		505±6 (6)	475 (2)	390 (1)	+ (1)
Anolis stratulus	Puerto Rico	Canopy		495±5 (3)	467±4 (4)	388 (2)	+ (1)
Anolis carolinensis	North America	Partial shade		507±4 (21)	463±5 (10)	365±2 (7)	+ (8)
Mean			521	502±9	467±15	378±10	

The values under each cone type (LWS, long-wavelength-sensitive; MWS, medium-wavelength-sensitive; SWS, short-wavelength-sensitive; UVS, ultraviolet-wavelength-sensitive) are the mean λ_{max} values obtained by template-fitting ± 1 S.D., with the number in parentheses being the sample size (*N*). In cases where *N*=1, the mean and S.D. are based on the 70 estimates of λ_{max} obtained by template-fitting the single microspectrophotometric recording using the Mansfield/MacNichol method.

The oil droplet values are the mean 50%-of-peak cut-off wavelengths ± 1 S.D., with the number in parentheses being the sample size (*N*). Only droplets that were identifiable as to cone type are included. Where *N*=1, no calculation of S.D. was possible, so none is reported.

Since the C2 droplets have no measurable cut-off over the measurement range 340–750 nm, their presence in identified cells is indicated with a '+'.

The values in the mean rows were calculated using the column values for all entries except for *Polychrus marmoratus* and *Anolis carolinensis*.

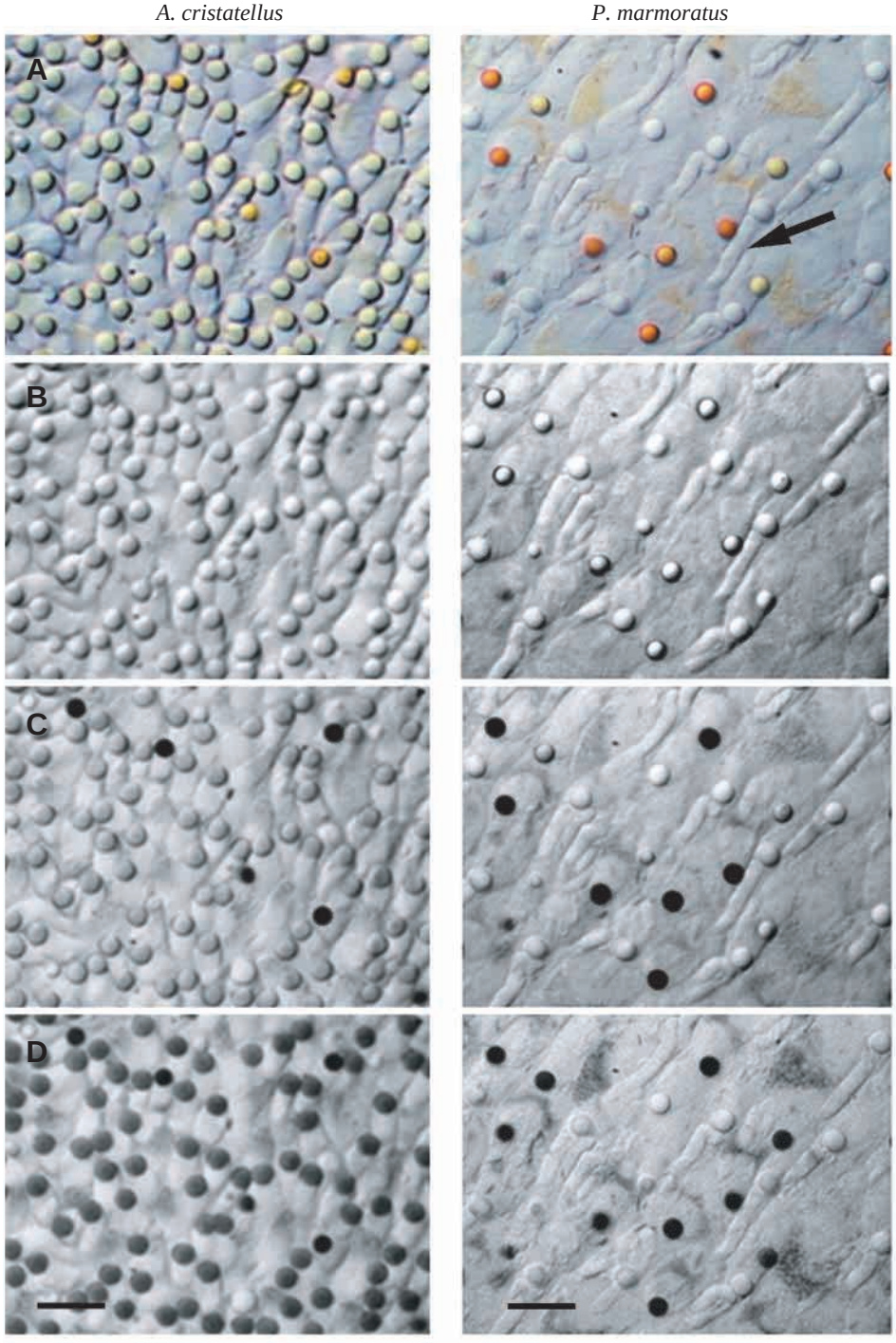

Fig. 4. Multispectral micrographs of pieces of flattened retina from *Anolis cristatellus* and *Polychrus marmoratus*. (A) Color images in white light. The diffuse yellow pigment present in the accessory member of the double cones is easily seen in the *P. marmoratus* images. (B) Black-and-white broad-band images of the same areas as in A. (C) The same areas imaged through a 500 nm low-pass interference filter. This is below the cut-off for the G droplets, which appear dark. (D) The same areas imaged through a 450 nm cut-off interference filter. In this case, both the Y and G classes of oil droplet appear dark. The droplets that remain light are the C class. Note, in particular, the rod-like outer segments seen in the *P. marmoratus* images (arrow) and their colorless oil droplets. Scale bars, 10 μm.

differentiate the colorless droplet classes (see Kolb and Jones, 1987); however, a system suitable for this purpose was not available for this study.

Results

Chromophore analysis

As shown in Fig. 2, the five Puerto Rican anolids sampled (*A. cristatellus*, *A. gundlachi*, *A. pulchellus*, *A. krugi* and *A. evermanni*) all use retinal as the visual pigment chromophore. This is in contrast to *A. carolinensis* (Fig. 2F, trace 6), which remains the only fully terrestrial vertebrate found to date that uses exclusively vitamin-A_2-based chromophores. The chromophore of the visual pigments for the other species reported here was not identified using HPLC. However, since the λ_{max} values of the longest-wavelength pigments were all in the region of 565 nm and were best fitted by a rhodopsin template curve (see below), there is no reason to suspect that any of the other anoles studied use vitamin-A_2-based chromophores unless it is in very small amounts or segregated into a small population of cells missed by microspectrophotometry.

Cell types, visual pigments and oil droplets

Table 1 summarizes the MSP results. All anoles possessed an LWS pigment with a λ_{max} between 560 and 570 nm (625 nm for *A. carolinensis*), an MWS pigment with a λ_{max} between 487 and 503 nm and an SWS pigment with a λ_{max} between 446 and 467 nm. All but five of the species had a UVS pigment with a λ_{max} between 364 and 367 nm. For these five species, no cells meeting the inclusion criteria were measured. However, the presence of type C2 droplets in these species strongly suggests that they, too, have a UVS pigment (see below). Except for *A. carolinensis*, all spectra were best fitted by a vitamin-A_1-based pigment template. Fig. 3 shows typical MSP recordings for the four pigment classes; in this case, for *A. cristatellus*. While the number of cells that met the selection

criteria for analysis was small, many spectral scans were made and recordings obtained from both intact cones and isolated outer segments that could at least be identified as to class even if the exact λ_{max} could not be calculated.

The three morphological cone classes known to exist in anoles were easily identifiable in the MSP preparations, although only rarely were cones found free with the outer segment attached. The rarest finds were free intact double cones. As expected from the previous *A. carolinensis* study (Provencio et al., 1992), the long single cone and both members of the double cone contained the LWS pigment. The finding of many free accessory members of the double cones, distinguished by their granular ellipsoid, suggests that many of the isolated long single cones measured may actually have been principal members of separated doubles. The only way a cell could be identified as a true long single cone was when measurements could be made along retinal edges. The short single cones containing the SWS and UVS pigments were morphologically identical. While these same cone morphotypes were observed in the MSP preparations of *Polychrus marmoratus*, a previously unreported photoreceptor class with relatively large, cylindrical, rod-like outer segments was also observed in great numbers. However, unlike true rods, these have a large oil droplet, as can be seen clearly in Fig. 4. The outer segments contain a visual pigment with a λ_{max} at 497 nm, a typical terrestrial vertebrate rhodopsin position. However, bleaching of this pigment with white light did not yield a long-lived photoproduct as usually seen for 'true' rods on the microspectrophotometer.

Three classes of oil droplet were easily distinguishable in retinal pieces from anoles viewed in white and colored light (see Fig. 4). These have been classified according to the spectral position of their cut-off wavelength and visual appearance as yellow (Y), green (G) and colorless (C). On the basis of MSP measurements, the G and C classes can be further divided into two sub-classes each, G1, G2 and C1, C2. Typical spectra for the five spectral types of oil droplet found in the anoles and *Polychrus marmoratus* are shown in Fig. 5AB together with the absorbance spectra recorded from the ellipsoid of the accessory member of the double cone. As seen in Table 1, all species had the G2 (502 nm cut-off), Y (467 nm cut-off), C1 (378 nm cut-off) and C2 (ultraviolet-transmissive) droplets. Only three species examined had the G1 (521 nm cut-off) type, but in these they were quite common. This suggests a real species difference in oil droplet complement, although failure to find the G1 class in other anoles could be due to sampling error and lack of adequate material for whole-retina flat preparation analysis using spectral imaging.

From Fig. 4, it would appear that the most numerous droplet class in anoles is class Y. This is found in the principal member of the double cones and a class of single cone. The G1 and G2 classes are found only in single cones. The C types are the least numerous and are found only in small single cones. In *Polychrus marmoratus*, C droplets are the most numerous because of their association with the rod-like receptors that are present in high density. This is followed by the G and Y classes.

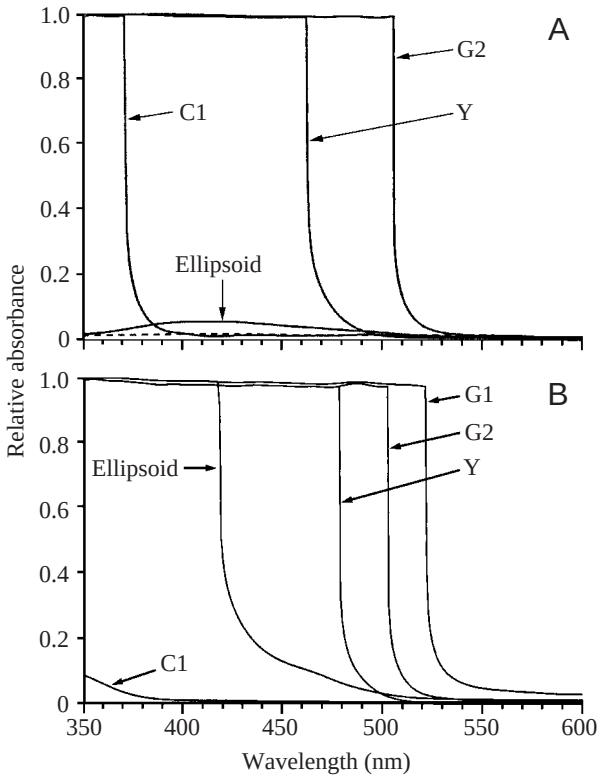

Fig. 5. Typical absorbance spectra of oil droplets from the cones of *Anolis cristatellus* (A) and *A. valencienni* (B). The absorbances of the ellipsoid region of the accessory member of the double cones are also shown. All absorbances that saturated the microspectrophotometer output (optical density >2.5) were normalized to 1.0, while absorbances below saturation are shown relative to the normalized absorbances. Thus, the ellipsoid absorbance in A indicates a very pale ellipsoid and the absorbance of the C1 droplet in B indicates a pale droplet. Although the G and Y droplets always show very steep cut-offs, as seen here, the ellipsoid and C1 absorbances can be quite variable. The absorbance of the C2 droplet associated with the ultraviolet single cone is the dashed line in A and falls on the zero line in B.

Fig. 6 summarizes pictorially the correlation between cell type, visual pigment and oil droplet type in anoles. Both members of the double cone house the LWS pigment, with a Y droplet in the principal cone and dispersed yellow pigment in the accessory cone. Three classes of large single cone were always present. Two contained the LWS pigment with either the Y or G2 droplet and one contained the MWS pigment with the G2 droplet. As mentioned above, three species had an additional large single class with the MWS pigment and the G1 droplet. The MWS pigment was never associated with the Y or C1/C2 droplets. Small single cones contained either the SWS pigment with the C1 droplet or the UVS pigment with the C2 droplet. The strict association between the UVS pigment and the C2 droplet makes us confident that, even in those five species in which we failed to record spectra from any ultraviolet-sensitive cells, the finding of C2 droplets supports their presence.

Discussion

Number and spectral location of the visual pigments

The data show that all anoline species examined possess at least four visual pigments: an LWS pigment centered at 564 nm (except for *A. carolinensis* with its 625 nm vitamin-A_2-based pigments), an MWS pigment centered at 495 nm, an SWS pigment centered at 455 nm and a UVS pigment centered at 365 nm (Table 1; Fig. 3). While there are small differences among the λ_{max} values within a cone class for the different species, the variation within each species was typically as large or larger than the overall variation among all species. Although we cannot rule out the possibility that, given a larger sample size, we might have been able to find differences, there is no evidence from this sample that significant differences among the species exist. To represent the basic pattern, we have taken the mean of each cone class excluding *Polychrus marmoratus* and *A. carolinensis*.

Clearly, if there are no differences in λ_{max} among the species and the species come from a full range of photic habitats, there can be no correlation between visual pigment λ_{max} and habitat. This situation contrasts with that for fish, in which strong correlations between habitat light and visual pigment λ_{max} have been reported (for reviews, see Bowmaker, 1998; Partridge and Cummings, 1999). Endler (1993) demonstrated that there are large differences in the spectral quality of downwelling irradiance in terrestrial habitats with different degrees of shade. Our failure to find differences in visual pigment λ_{max} suggests either that the anoline visual system is, for some reason, highly conservative in an evolutionary sense or that the irradiance differences are not as important to vision in terrestrial species as one might have predicted. However, if one examines background lighting (i.e. horizontal radiance) rather than downwelling irradiance, the differences in spectral quality among these different habitats are greatly reduced since all are dominated by green vegetation (Fleishman et al., 1997). This is not the case in aquatic systems, in which horizontal radiance can show a great deal of variation.

Of course, other factors besides visual pigment absorption, such as optical filtering, waveguiding or scatter, will affect spectral sensitivity (see Loew, 1995). However, even using electrophysiological techniques that would be influenced by these other factors, all anoles studied to date, except the A_2-utilizing *A. carolinensis*, have similar spectral sensitivities (Fleishman et al., 1997) (L. J. Fleishman, unpublished data). HPLC failed to detect vitamin A_2 in any of the Puerto Rican species tested, and the similarity in λ_{max} between this group and the other anoles measured with microspectrophotometry makes the widespread use of a vitamin A_2 chromophore doubtful. However, this does not exclude the possibility of vitamin-A_2-based visual pigments being present in species other than *A. carolinensis*. A small or localized population of vitamin-A_2-based cones could have been missed because of the sampling limitations of microspectrophotometry or could have been below the detection level of the HPLC technique.

As mentioned above, five different opsins have been identified in *A. carolinensis* and have been sequenced and expressed using the COS-1 cell cDNA system (Kawamura and Yokoyama, 1998). They have been designated $SWS1_{AC}$, $SWS2_{AC}$, $RH1_{AC}$, $RH2_{AC}$ and LWS_{AC} (see Kawamura and Yokoyama, 1997) and are orthologous to the chicken violet, blue, rhodopsin, green and red opsins, respectively. Visual pigments reconstituted from these products using 11-*cis* retinal were spectrally characterized and, after calculation of the expected effect of substituting A_2-aldehyde using the formula of Harosi (1994), were found to have expected values of λ_{max} at 377, 444, 511, 517 and 626 nm, respectively. However, the MSP data of Provencio et al. (1992) and those presented here identified only four classes of cone in *A. carolinensis* with values of λ_{max} at 365, 462, 502–503 and 625 nm. Even given the admittedly low signal-to-noise ratio of the microspectrophotometry recordings from the small anole cones (Fig. 3) and the low values of *N* (Table 1), both conspiring to produce large standard deviations, the correlation between the expressed and reconstituted pigments and those measured *in situ*, except for the LWS pigment, is not very good.

The match is even worse if the conversion formula of Whitmore and Bowmaker (1989) is used. Assuming that Kawamura and Yokoyama (1998) are correct in saying that $RH2_{AC}$ corresponds to our reported MWS pigment, none of the mean values for this class seen in Table 1 comes very close to the λ_{max} of 511 nm calculated for $RH2_{AC}$. We agree with Kawamura and Yokoyama (1998), who suggested that the discrepancy could be due to inappropriateness of the A_1-to-A_2 conversion formulae with regard to anoles. However, it is also possible that the discrepancy arises from differences between

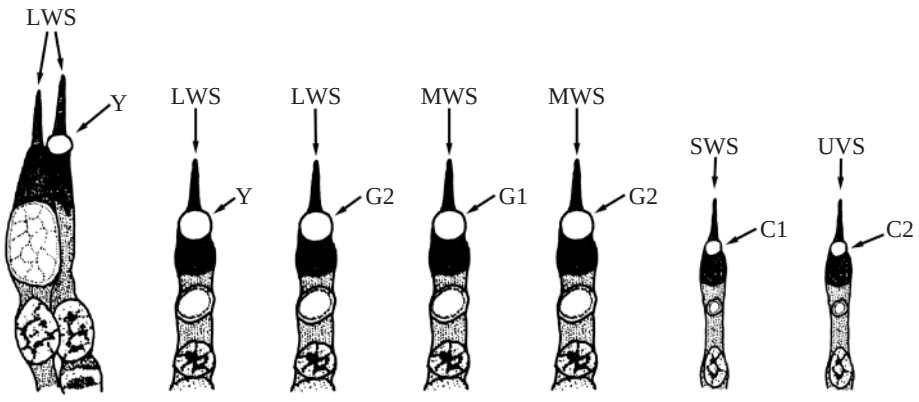

Fig. 6. Drawings of the photoreceptor cells typical of anoline lizards (see Crescitelli, 1972) showing the associations between cell type, visual pigment and oil droplet classes. The *Polychrus marmoratus* pattern is like that shown here for the anolids except for the presence of the rod-like cell containing a medium-wavelength-sensitive (MWS) pigment and a colorless oil droplet. LWS, long-wavelength-sensitive cell; SWS, short-wavelength-sensitive cell; UVS, ultraviolet-sensitive cell; C1, C2, G1, G2 and Y are classes of oil droplet.

the biochemical/biophysical environment of the visual pigment in the expression system and those *in situ*. λ_{max} aside, there is also the problem of locating the photoreceptor class expressing $RH1_{AC}$. This is like classic 'rod rhodopsin' and differs very little from $RH2_{AC}$, which is expressed in cones. Given a difference of only 6 nm in λ_{max} between the two RH pigments and the noisy nature of the microspectrophotometry data, it is possible that both exist in separate, but morphologically indistinguishable, cones both classified as MWS. It is also possible that only one MWS class exists, but that it is expressing both opsins. The data from *Polychrus marmoratus* are useful in this context for here there is evidence for two very similar pigments differing in λ_{max} and clearly present in separate types of cell. We therefore feel that the anoles studied here have two RH-containing photoreceptor types, an MWS photoreceptor containing $RH2_{AC}$ and a morphologically similar cell containing $RH1_{AC}$. However, unlike *Polychrus marmoratus*, the $RH1_{AC}$-containing cell has a colored oil droplet.

The outgroup *Polychrus marmoratus* was added to this study to try to identify the ancestral condition. The fact that its cone pigments and oil droplets are similar to those of the anoles and use vitamin A_1 as the visual pigment chromophore suggests that the A_2 condition is a derived characteristic. However, its retina is unlike that of any of the anoles studied because it contains a class of single photoreceptor with large, rod-like outer segments in addition to the four cone classes found in the anoles (see Table 1 and Fig. 4). Neither Walls (1967) nor Underwood (1968) describe such a cell in any of the numerous diurnal reptiles they studied. The outer segment is attached to an inner segment containing a large, colorless oil droplet similar to the C2 droplet of the UVS cone in that it shows no appreciable absorbance below 400 nm. The cell's appearance is somewhat similar to the cells in nocturnal geckos such as *Aristelliger praesignis* (see Crescitelli, 1972), but in these retinas the outer segments in all photoreceptor classes are large, as befits the nocturnal condition. Several interesting questions arise from these observations. (i) Is this cell functionally a 'rod' or a 'cone'? (ii) Where does this cell fit within the reptilian visual cell sequences created by Walls (1967) and Underwood (1968) and would they call this a 'transmuted' rod or a 'transmuted' cone? (iii) What happened to this class of photoreceptor during the evolutionary transition to the anoles? (iv) How general is this pattern among other species of *Polychrus*? It would be very interesting to apply the techniques of Kawamura and Yokoyama (1998) to *Polychrus*.

Oil droplets

Except for the G1 and C2 classes, there was considerable variability among the species within an oil droplet class (Table 1). We believe this variability is real and not due to the problems of measuring the high-optical-density, highly refractive droplets. The differences were obvious in those species in which there was enough material for small pieces of retina to be examined microscopically in white light. No retinal whole-mounts were examined, so it is not possible to say whether droplet color or density was uniform over the entire retina. For two of the Jamaican anoles and *Polychrus marmoratus*, in which it occurs, the G class of droplet clustered into two non-overlapping groups, hence the creation of the G1 and G2 classes. However, as pointed out above concerning the presence of vitamin-A_2-based pigments, the failure to identify G1 droplets in other species may be due to selection error. The C1 droplet was identifiable by having a cut-off below 400 nm, so it appears colorless to the human eye. The C2 class showed no appreciable absorbance down to 340 nm, where MSP measurements stopped (Fig. 5A,B).

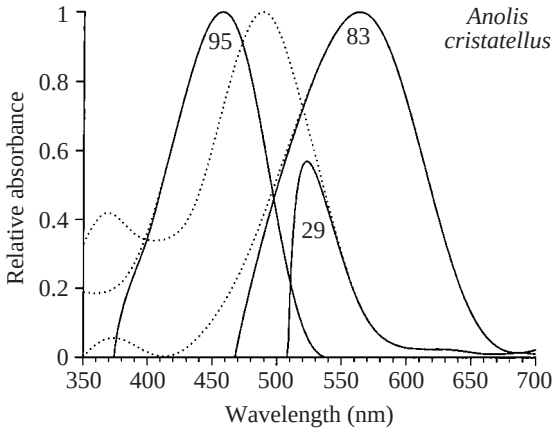

Fig. 7. Effects of oil droplet filtering on the theoretical capture area of *Anolis cristatellus* visual pigments. The solid curves were obtained by multiplying smoothed, normalized visual pigment spectra by the associated smoothed, normalized oil droplet transmission spectra. The dotted lines are the smoothed visual pigment spectra. The numbers are the calculated capture areas under the filtered visual pigment spectra obtained by integration and expressed as a percentage of the unfiltered pigment capture area. The effect of the oil droplets on the long-wavelength-sensitive and medium-wavelength-sensitive (MWS) cones is to reduce short-wavelength absorbance while reducing overall absorbance by 17 % and 5 %, respectively. In these cases, there is no change in the absorbance maximum. However, the position of the oil droplet cut-off of the MWS cone reduces the capture area to 29 % of that of the unfiltered pigment and moves the absorbance peak from 443 to 525 nm. This also produces a much steeper short-wavelength cut-off, which could improve color discrimination in an opponent processing system (see text).

As mentioned above, the accessory members of double cones do not contain an oil droplet, but instead a dispersed, yellow pigment (Fig. 5A,B). However, even here, there was considerable variation in the density ('yellowness') among the species. The functional significance of this dispersed pigment is unknown, but Underwood (1968) made the intriguing suggestion that it could play a role in polarized light detection as a result of scatter.

The presence of oil droplets changes the spectral sensitivity of the cells that contain them and is believed to improve color discrimination (Govardovskii, 1983) (see also Vorobyev et al., 1998). By multiplying the normalized visual pigment

absorbance by the normalized oil droplet transmission, an approximate spectral sensitivity can be calculated, as seen for *A. cristatellus* in Fig. 7. It is easy to rationalize the pairing of Y and G2 droplets with the LWS pigment since the overall effect is to remove the blue and ultraviolet sensitivity. The reduction in ultraviolet sensitivity is obviously the reason for the SWS/C1 pairing. The association between MWS and the G1 and G2 droplets greatly decreases the capture area for the MWS cone while moving the sensitivity peak to longer wavelengths (Fig. 7). We can only assume that the improvement in color discrimination provided by this pairing outweighs the reduction in capture area.

Ecological considerations

The spectral location of the visual pigments reported here could be rationalized using the same model and reasoning recently applied to birds by Vorobyev et al. (1998) and to bees by Vorobyev and Menzel (1999). Even spacing of pigments across the spectrum would allow for reasonable color discrimination with no particular spectral region being singled out for increased discrimination. Thus, the spacing fits well with a 'gray world' model. However, models including green leaves among the targets and backgrounds to be discriminated favour an LWS pigment further into the red than 570 nm (Zhang, 1997, 1999), the apparent long-wavelength cut-off for vitamin-A_1-based visual pigments (see Loew, 1995). Sensitivity to longer wavelengths would take advantage of the steep increase in reflectance for chlorophyll-containing targets that exists between 700 and 800 nm (see Gates, 1980), which could increase contrast for non-infrared-reflecting or -absorbing objects viewed against the bright leaf background. In fact, the LWS pigments predicted by the Zhang (1997, 1999) model for viewing non-chlorophyll-containing targets against a leafy background would have a λ_{max} extending above 650 nm. Only *A. carolinensis* comes close to satisfying this prediction.

It was hoped that the finding of a broader use of vitamin-A_2-based visual pigments among the anoles might shed some light on the possible utility of the dramatic red-shift in spectral sensitivity noted in *A. carolinensis*. In particular, Provencio et al. (1992) speculated that the use of vitamin A_2 might be related to the red color of the *A. carolinensis* dewlap. However, *A. sagrei* and *A. pulchellus* also have red dewlaps (Fleishman, 2000), yet do not use A_2 as their chromophore. Thus, having vitamin-A_2-based pigments is not a prerequisite for 'seeing' red dewlaps. The use of vitamin A_2 also does not correlate with such obvious variables as body color or habitat selection. Thus, we remain ignorant as to what benefit, if any, *A. carolinensis* derives by having extended red sensitivity.

It is clear from the *Polychrus marmoratus* results and the cladogram (Fig. 1) that vitamin A_2 is not the ancestral condition among the anoles. Jackman et al. (1999) identified 17 distinct clades among the anoles. In our sample, *A. carolinensis* is the only representative of its clade. It would be of obvious interest to study other members of this (mostly Cuban) radiation to determine whether A_2 utilization is shared by close relatives and to try to pinpoint where in the anole radiation the use of vitamin A_2 evolved.

The possibility that oil droplet color is the adaptational variable in anoles cannot be discounted. While there was no apparent correlation between cut-off position and photic habitat or dewlap color, there could be other visual tasks we have not considered driving oil droplet cut-off position.

These results, together with recent studies of birds (see Bowmaker et al., 1997; Vorobyev et al., 1998), draw into question the fundamental assumption made by visual ecologists that visual pigment λ_{max} should correlate with the photic environment, at least for terrestrial vertebrates. Rather than concentrating on irradiant and visual pigments, one can look for relationships between visual signals and tasks and overall spectral sensitivity. This seems to work for rationalizing the presence of UVS cones in birds and anoles with ultraviolet-reflecting/absorbing targets such as feathers and dewlaps (Bennett et al., 1997; Fleishman et al., 1993). As anoles have radiated over evolutionary time, there have been only relatively modest changes in the design of their visual system. In contrast, what is known to be a highly significant visual signal, the colored dewlap, shows a great deal of variation among the species. We find no support for the idea that signal color variation evolved as a result of spectral sensitivity. This further supports that idea that the anoles are 'color generalists' with regard to the positioning of their spectral sensitivities.

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