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The photoreceptors and visual pigments of the garter snake (*Thamnophis sirtalis*): a microspectrophotometric, scanning electron microscopic and immunocytochemical study

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Abstract Scanning electron microscopy, immunocytochemistry, and single cell microspectrophotometry were employed to characterize the photoreceptors and visual pigments in the retina of the garter snake, *Thamnophis sirtalis*. The photoreceptor population was found to be comprised entirely of cones, of which four distinct types were identified. About 45.5% of the photoreceptors are double cones consisting of a large principal member joined near the outer segment with a much smaller accessory member. About 40% of the photoreceptors are large single cones, and about 14.5% are small single cones forming two subtypes. The outer segments of the large single cones and both the principal and accessory members of the doubles contain the same visual pigment, one with peak absorbance near 554 nm. The small single cones contain either a visual pigment with peak absorbance near 482 nm or one with peak absorbance near 360 nm. Two classes of small single cones could be distinguished also by immunocytochemistry and scanning electron microscopy. The small single cones with the 360-nm pigment provide the garter snake with selective sensitivity to light in the near ultraviolet region of the spectrum. This ultraviolet sensitivity might be important in localization of pheromone trails.

Key words Reptile · Photopigments · MSP · Immunolabelling · Vision

Introduction

The Ophidia or snakes are a highly successful group with some 2300 known living species distributed amongst at least 11 families (Cadle 1987; McDowell 1987; Ford and Burghardt 1993). Their evolution has been characterized by a truly spectacular adaptive radiation culminating in their presence in virtually every portion of the biosphere, with the exception of the polar regions, some islands and the deeper ocean waters (Lillywhite and Henderson 1993). Although the phylogenetic origin of snakes, in terms of both time and ancestry, is not without controversy, two evolutionary characteristics appear to be generally accepted. First, the snakes are most closely related to the lizards. Second, at some point in their evolutionary history snakes adopted a fossorial life style (Walls 1942; Underwood 1970; Rage 1987; Repérant et al. 1992). Aside from the elongation of the body and the loss of limbs, the most compelling evidence for a subterranean episode in the evolution of snakes comes from studies on the ophidian eye (Walls 1942; Carroll 1969; Underwood 1970). Regardless of the identity of the immediate terrestrial ancestor, life in an environment devoid of light would have led to the deemphasis and subsequent degeneration of the ophidian eye in favor of a more appropriate sensory modality such as chemosensation. When, for whatever reason, the snakes returned to the surface, the eye regained its importance and reevolved. Along with touch and chemoreception, vision is thought to be a dominant sense in extant snakes (Ford and Burghardt 1993), which generally have relatively large, well-developed eyes.

It was the relevance of the ophidian eye to our understanding of the evolutionary history of snakes that led to the wealth of knowledge accumulated with respect to the morphology of snake photoreceptors (Walls 1942;

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Underwood 1970). We know, for example, that snakes that are primarily nocturnal in habit have retinas which contain only rod-like photoreceptors. On the other hand, some snakes active primarily in bright light have only cone photoreceptors. Other snakes have truly duplex retinas with photoreceptor populations comprised of both rods and cones. The cone-rich retinas often have cones of several types – large singles, small singles and doubles (Walls 1942; Underwood 1970; Wong 1989). Ironically, despite the abundance of information regarding the structure and distribution of snake photoreceptors, virtually no work has been done to elucidate the functional characteristics of those light-sensitive cells. There is the study of Jacobs et al. (1992) reporting spectral sensitivity measurements for the garter snake, and there are two published studies reporting direct measurements to determine the light absorbing characteristics of the photosensitive visual pigments in the photoreceptors of snakes. In one, Crescitelli (1956) measured the spectral absorbance of a visual pigment extracted from the rods of a rattlesnake, *Crotalus viridis helleri*. In the other, Govardovskii and Chkheidze (1989) described absorbance maxima obtained microspectrophotometrically from the photoreceptors of two viperids, *Vipera berus* and *V. lebetina*, and two colubrids, *Malpolon monspessulanus* and *Coronella austriaca*. This dearth of information regarding the visual pigments is especially unfortunate, considering that snakes live in such a wide variety of habitats with, presumably, different spectral environments. It was the purpose of this study on *Thamnophis sirtalis* to employ microspectrophotometry, immunocytochemistry and scanning electron microscopy to expand our knowledge of the light-absorbing characteristics of snake visual pigments and the photoreceptors in which they are contained, and to relate those characteristics to the known behavior of the garter snake.

Materials and methods

Two subspecies of a common North American garter snake, *Thamnophis sirtalis*, were used throughout the study. Virtually all of the useful data were obtained from *T. sirtalis sirtalis*, but when they were unavailable *T. sirtalis similis* was employed. The snakes were obtained from a commercial supplier (West Coast Reptile, Bellflower, Calif., USA) who shipped them to either U. C. Davis or Cornell University just prior to use.

Scanning electron microscopy

The methods employed for scanning electron microscopy of the garter snake retina have been described in detail elsewhere (Sillman et al. 1990). Briefly, snakes were stunned and then killed by decapitation followed by destruction of the brain and removal of the eyes. After hemisection, the eyecups were immersed in fixative (3% glutaraldehyde in 0.1 mol·l⁻¹ phosphate buffer, pH 7.4) wherein the retinas were removed and separated from the pigment epithelium. Following at least 24 h fixation, the tissue was washed with buffer, postfixated with 2% osmium tetroxide, dehydrated with ethanol and then critical point dried. The tissue was then cracked with forceps, mounted on a specimen stub, sputter-coated with gold and

examined with an ISI Model DS130 dual stage scanning electron microscope.

Microspectrophotometry

Two microspectrophotometers (MSPs), each a computer-controlled, single-beam device capable of accurately measuring the spectral absorbance of a single cone outer segment, were employed in this study. The two instruments were very similar, the important distinction being that the optics of the Cornell MSP allowed measurement of absorbance down to 330 nm, whereas the U. C. Davis MSP was limited to wavelengths of 375 nm and above. The detailed characteristics of both instruments have been published before (Loew and Lythgoe 1978; Loew 1982; Sillman et al. 1990, 1991; Loew and Sillman 1993).

Preparation of tissue for microspectrophotometric analysis has also been described previously (Sillman et al. 1990, 1991). A snake was dark-adapted for at least 2 h (normally overnight) and then killed as before. Following enucleation, the eyes were hemisected and the retina, along with pigment epithelium, removed and placed in a dish of saline (Sigma Modified Minimum Essential Medium pH 7.6). In contrast to other animals we have studied, complete separation of the snake retina from the pigment epithelium proved extremely difficult and often some pigment epithelium remained in the preparation. After removing as much pigment epithelium as possible, a small piece of retina in a drop of saline was macerated with razor blades and sealed between two glass cover slips with silicone grease. The preparation was then transferred to the stage of the MSP where it was positioned, usually with the aid of infrared light but sometimes with dim, very deep red light. Other than this occasional, momentary illumination with deep red light, all operations were carried out in “darkness” with the aid of infrared lamps and infrared image converters.

To determine the wavelength of peak absorbance (λ_{\max}) for different visual pigments, the following procedure was followed. The best MSP records for each class of pigment (i.e., long wavelength, short wavelength or UV sensitive) were chosen on the basis of smoothness of the absorbance curve and signal-to-noise ratio. Smoothing of the data was done by Gauss filtering with a window width of either 1 nm (e.g., Fig. 1A, D; Fig. 2A) or 2 nm (e.g., Fig. 2B). The data from each record were then plotted as absorbance versus log wavelength (λ). Using the method of least squares, the long wavelength limb of the absorbance curve, between 0.72 and 0.30 of maximum absorbance, was fitted with a straight line and the point corresponding to 0.60 maximum absorbance was found. The λ_{\max} was then determined using the following relationship derived from Partridge and DeGrip's (1991) data for the rhodopsin absorbance spectrum:

$$\log \lambda_{\max} = \log \lambda_{0.6} - 0.07705.$$

The process was repeated for each absorbance curve in a class, after which mean $\lambda_{\max} \pm$ standard deviation was determined. Theoretical template or ‘nomogram’ curves were then constructed according to Partridge and DeGrip (1991). The λ_{\max} values placed near the nomogram curves in Figs. 1 and 2 are best-fitting values for the particular recordings shown, and may be slightly different from averages owing to statistical variability.

Immunocytochemistry

Semithin sections

The techniques used were essentially the same as those employed by Loew et al. (1996) in a study on the gecko. Snakes were killed and their eyes removed and hemisected as described above. The eyecups were placed in 1% glutaraldehyde prepared in 0.1 mol·l⁻¹ phosphate buffer, where they remained for 1.5 h, and were then postfixated in 4% paraformaldehyde for 4 h. The samples were then transferred to 1% paraformaldehyde where they remained (up to 2 weeks) during transport from the US to the laboratory in Budapest.

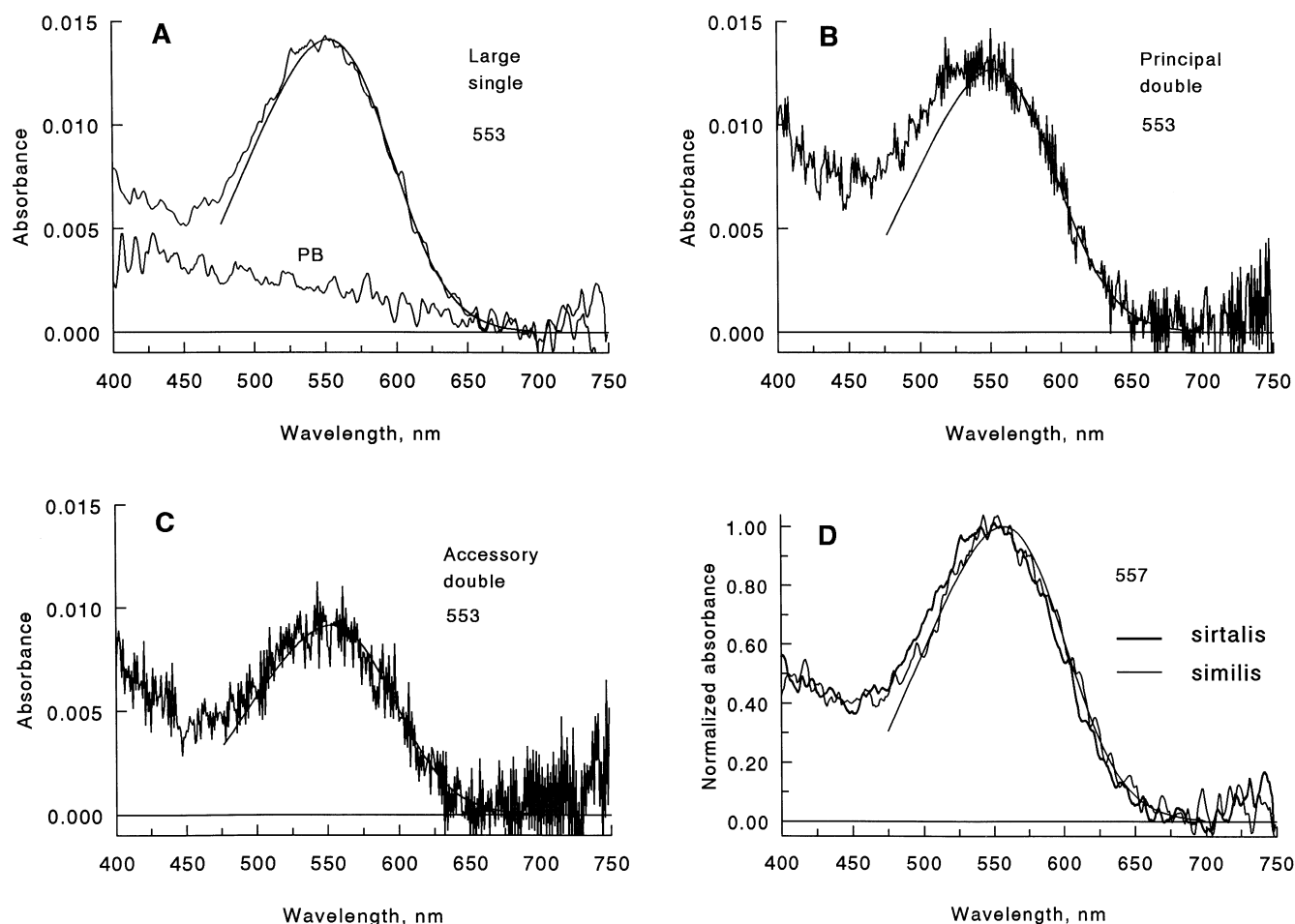


Fig. 1A–D MSP absorbance curves obtained from the large single and double cones of the garter snake. **A** averaged data from 16 large single cones from *T. sirtalis sirtalis*, smoothed as described in the methods and shown with the theoretical 'nomogram' curve for a vitamin A₁-based visual pigment with peak absorbance at 553 nm. **PB** shows absorbance from the same cells after exposure to bleaching light; **B** unsmoothed, averaged data from four principal members of the double cone from *T. sirtalis sirtalis*, shown with the same 553-nm 'nomogram' curve; **C** unsmoothed, averaged data from three accessory members of the double cone from *T. sirtalis sirtalis*, shown with the same 553-nm 'nomogram' curve; **D** smoothed, averaged data from four large single cones from *T. sirtalis similis* (thin line) shown with the theoretical 'nomogram' curve for a vitamin A₁-based visual pigment with peak absorbance at 557 nm. The absorbance curve obtained from the large single cones of *T. sirtalis sirtalis* (from panel A) is provided for comparison (thick line)

The eyecups were then washed thoroughly, first in 0.1 mol·l⁻¹ phosphate buffer and then in 0.1 mol·l⁻¹ TRIS-HCl buffer, after which they were dehydrated with ethanol and embedded in Durcupan ACM epoxy resin. An ultramicrotome (Reichert Ultracut) was used to cut serial semithin (0.3–0.5 μm) radial and tangential sections from the central part of each retina. In general, each series was comprised of about 50 sections, in the case of tangential sectioning, taken from the level of the photoreceptor inner and outer segments. Consecutive sections of a series were then reacted with different antibodies. Micrographs of identical areas of adjacent sections were produced with a Zeiss Axiophot microscope, thus permitting identification of the same outer segments and comparison of their reactivities with the different antibodies.

The characteristics of the antibodies used in this study have been described before. COS-1 and OS-2, both monoclonal antibodies produced against the photoreceptor membranes of the chicken, have been shown to be specific to visual pigments (Szél et al. 1986a; Röhlich and Szél 1993). COS-1, a hybridoma supernatant used in a dilution of 1:40, has been shown to label middle- and long-wavelength-sensitive cones in tetrapods (Szél et al. 1986a, b; Cserháti et al. 1989; Röhlich et al. 1989). OS-2, an ascites fluid diluted 1:10 000, has been shown to bind to almost all photoreceptors in sub-mammalian species (Szél et al. 1986a, b; Cserháti et al. 1989; Röhlich et al. 1989). The binding sites of both monoclonals were found at the C-terminal portion of the appropriate visual pigments (Röhlich and Szél 1993). We also used AO, a polyclonal antibody, mainly N-terminal specific (Röhlich and Szél 1993) produced in rats against bovine rhodopsin and diluted to 1:1500. Two other monoclonal antibodies against bovine rhodopsin were generously supplied by P. Hargrave and G. Adamus of the University of Florida, Gainesville, Fla., USA. They were the N-terminal-specific antibody B6-30 and the 5-6 loop specific antibody K42-41, both used as hybridoma supernatants in a dilution of 1:5 (Adamus et al. 1988). B6 (A. Szél, T. Diamantstein, unpublished observations) was a monoclonal antibody against a mixture of photoreceptor membranes that binds to an unidentified epitope of a rhodopsin-related visual pigment in several submammalian species. The antibody was used as an ascites fluid diluted 1:5000.

The techniques employed for immunolabelling have also been described in detail before (Szél and Röhlich 1985; Szél et al. 1986a, b; Cserháti et al. 1989; Loew et al. 1996) and, therefore, only the essentials are presented here. The epoxy resin was first removed by treatment with sodium methoxide, after which the sections were placed in a 1% solution of bovine serum albumin in phosphate-buffered saline (PBS) to block non-specific binding

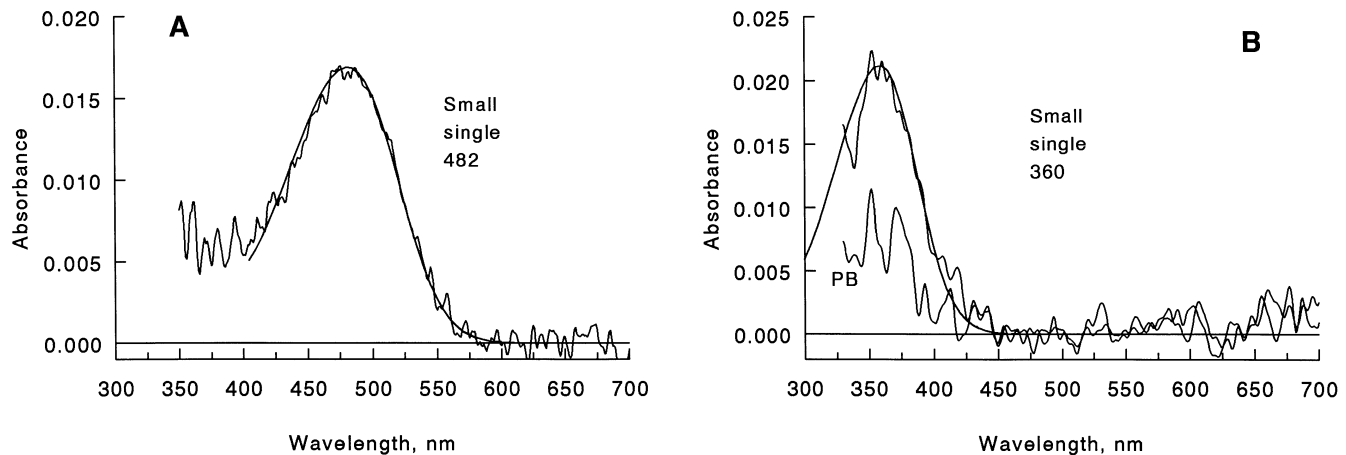


Fig. 2A, B MSP absorbance curves obtained from the small single cones of *T. sirtalis sirtalis*. **A** smoothed, averaged data from nine small single cones containing a short-wavelength-sensitive visual pigment, shown with the theoretical 'nomogram' curve for a vitamin A₁-based visual pigment with peak absorbance at 482 nm; **B** smoothed, averaged data from two small single cones containing an ultraviolet-sensitive visual pigment, shown with the 'nomogram' curve for a vitamin A₁-based visual pigment with peak absorbance at 360 nm. *PB* shows absorbance from the same cells after exposure to bleaching light

sites. The sections were then exposed to the primary antibody for 2 h, with consecutive sections of a series being treated with different antibodies, as mentioned above. To detect the bound antibody, sections were incubated in biotinylated IgG (anti-mouse or anti-rat, diluted 1:300, Vector, Burlingame, Calif., USA) for 1 h and avidin-biotinylated peroxidase complex (Vectastain Elite ABC complex, 1:55, Vector, Burlingame, Calif., USA) for 45 min followed by diaminobenzidine reaction for peroxidase. The sections were mounted in glycerol and photographed in a Zeiss Axiophot microscope using a 500-nm interference filter, differential interference contrast (Nomarski) and a fine-grain B/W film MA8 (ORWO).

Retinal whole-mounts

Eye cups were fixed in 4% paraformaldehyde in 0.1 mol l⁻¹ phosphate buffer for 30 min, after which the retinas were separated from the underlying choroid (with remnants of the pigment epithelium). The retinas were further fixed overnight and then stored in 1% paraformaldehyde in buffer until used for immunocytochemistry. The retinas were then thoroughly washed in PBS and 0.1 mol l⁻¹ TRIS-HCl buffer, and permeabilized 1 h in 0.2% Triton X-100 in PBS. The same concentration of detergent was included in all subsequent reagents and washes. The retinas were treated with 1% bovine serum albumin in PBS and subsequently incubated overnight in a mixture of antibodies COS-1 and OS-2. COS-1 was labeled with rhodamine and OS-2 was conjugated with fluorescein (Röhlich et al. 1994). The retinas were washed in PBS and mounted (photoreceptors upward) in glycerol for observation in the Axiophot microscope. The filter set used for rhodamine was: excitation filter BP546/12, beam splitter FT580, and emission filter LP590. The filter combination for fluorescein was: excitation filter BP450-490, beam splitter FT510, and emission filter LP520. A special filter set for the simultaneous observation of both rhodamine and fluorescein fluorescence was also used, with the following specifications: excitation filter DBP485/20 and 546/12, beam splitter 500/560, and emission filter DBP515/580-630. Retinal areas were photographed with Neofluar 40× and planapochromat 63× objectives on a Kodak 400 ASA color negative film.

Results

Microspectrophotometry

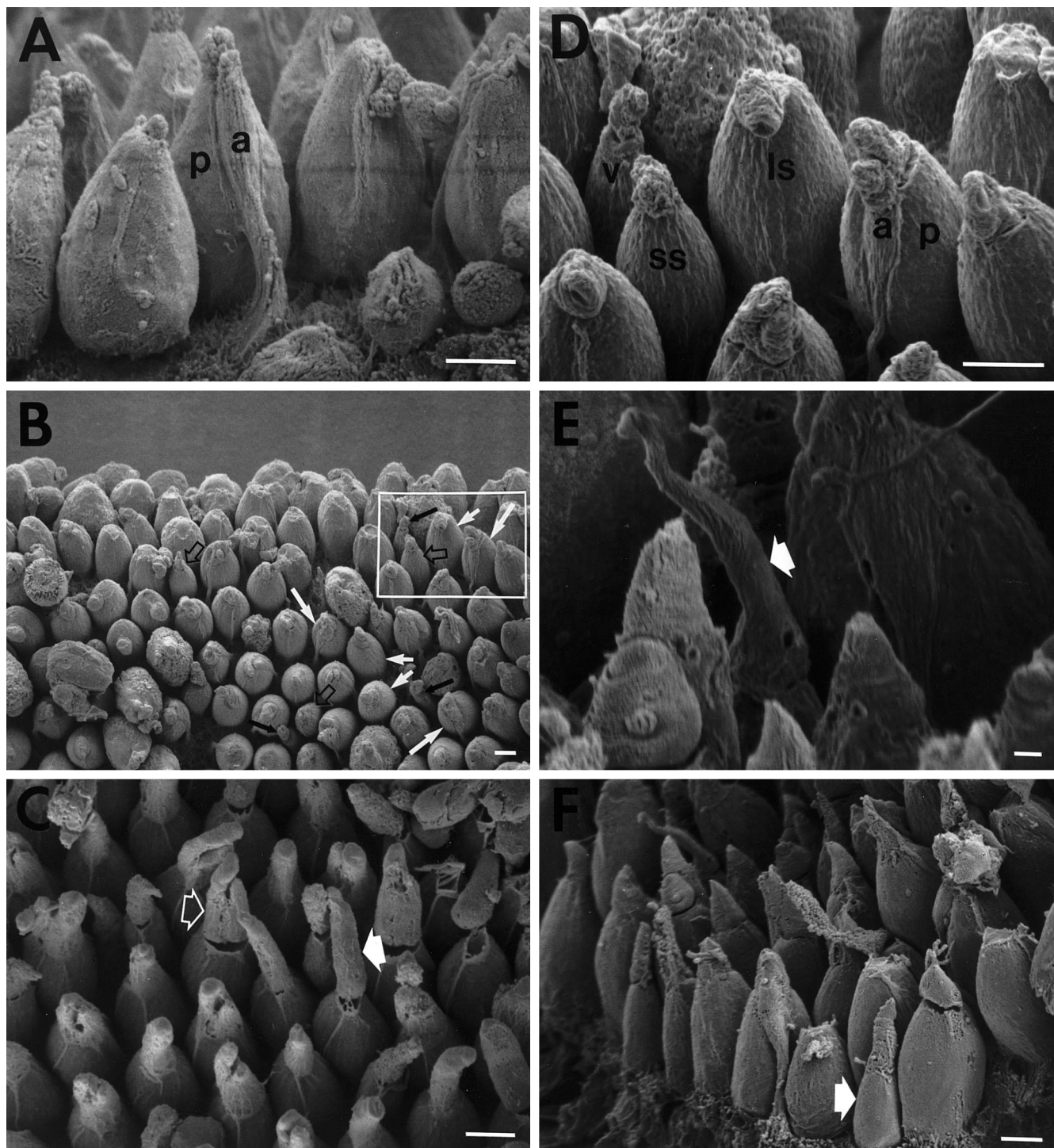
Microspectrophotometric analysis revealed the presence of three different visual pigments in the photoreceptors of *T. sirtalis sirtalis*. The large single cones and both the principal and accessory members of the double cones contained a visual pigment with peak absorbance (λ_{\max}) calculated at 554 ± 3.7 nm. Averaged absorbance curves for this pigment are shown in Fig. 1A, B, C along with the best-fitting theoretical nomogram curve, that for a visual pigment that absorbs maximally at 553 nm and whose chromophore is based on vitamin A₁. Small single cones were found to contain visual pigments with absorbance maxima at shorter wavelengths. The visual pigment within most of these small single cones absorbed maximally at 481.2 ± 1.9 nm (Fig. 2A). Much less common were small single cones containing a visual pigment with absorbance in the near ultraviolet. This UV-absorbing pigment had a λ_{\max} at 358.4 ± 3.2 nm (Fig. 2B). Exposure of the pigment to bleaching light resulted in a large decrease in absorbance (*PB* in Fig. 2B), showing that the pigment was truly a light-sensitive visual pigment and not merely a photoproduct absorbing at short wavelengths. MSP examination of retinas from *T. sirtalis similis* revealed the presence of two vitamin A₁ based visual

Fig. 3A-F Scanning electron micrographs of the photoreceptors in the retina of *T. sirtalis sirtalis*. **A** *p* denotes the principal member of the double cone; *a* the accessory member; **B** long-shafted white arrows indicate double cones; short-shafted white arrows, large single cones; open arrows, small single cones; black arrows, very small single cones. Boxed area is shown at higher magnification in panel D; **C** open arrow indicates relatively intact outer segments of a double cone; filled arrow, relatively intact outer segment of a large single cone; **D** higher magnification micrograph of the boxed area in panel B, showing the four different types of cone photoreceptors. *ls* denotes large single cone; *a* accessory member of double cone; *p* principal member of double cone; *ss* small single cone; *v* very small single cone; **E** filled arrow indicates an intact outer segment of a single cone; **F** arrow indicates a small single cone with outer segment positioned much more proximally than other cones. Calibration bars: 1 μ m for panel E; 5 μ m for all other panels

pigments, one with λ_{\max} near 557 nm in the large single cones and both members of the double cones, and one with λ_{\max} near 487 nm in small single cones. These pigments were similar to those found in the comparable cells of *T. sirtalis sirtalis*, but may be slightly “red shifted” (Fig. 1D). No cells containing a UV-sensitive visual pigment were encountered in *T. sirtalis similis*, probably due to the small number of cells studied in this subspecies.

Scanning electron microscopy

The photoreceptors in the retina of *T. sirtalis sirtalis* are shown in the micrographs of Figs. 3 and 4. Extensive sampling of the tissue revealed the photoreceptor population to be comprised entirely of cones. Large single cones (ls in Fig. 3D) were very common. These cones had a relatively large inner segment ($8.4 \pm 1.7 \mu\text{m}$ in



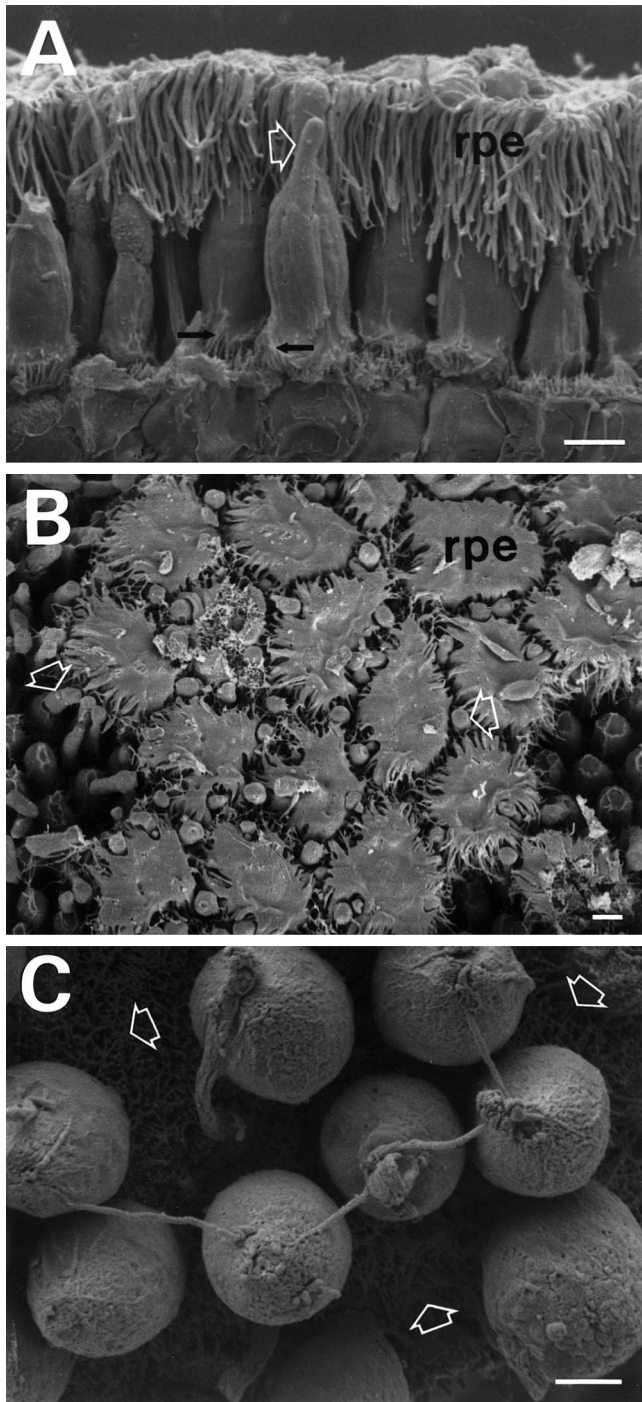


Fig. 4A–C Scanning electron micrographs of the retina of *T. sirtalis sirtalis*. **A** arrow indicates a relatively intact double cone; *rpe* processes of the cells of the retinal pigment epithelium; **B** arrows indicate the tips of cone photoreceptors; *rpe* retinal pigment epithelial cell; **C** arrows indicate the ‘matrix’ of Müller cell fibers at the proximal ends of the cone inner segments. Calibration bars: 5 μm

diameter) from which projected a single long, tapering outer segment (filled arrow in Fig. 3C). Double cones were also quite common. Each double cone was comprised of a principal member (p in Fig. 3D) similar in

size and shape to the large single, and a much smaller accessory member (a in Fig. 3D). Although the two members appeared to be an integral unit at their distal regions, the association between the two inner segments was tenuous closer to the base of the double cone where the inner segment of the accessory member often separated from that of the principal member (Fig. 3A, D, 4C). Less common than either the large single cone or the double cone was a small single cone (ss in Fig. 3D; open arrows in Fig. 3B) with mean inner segment diameter of $5.9 \pm 1.2 \mu\text{m}$. An even smaller single cone was also evident. Since these very small singles (v in Fig. 3D; black arrows in Fig. 3B) were not numerous and were located deeper in the photoreceptor layer, where they were somewhat obscured, we could not obtain an accurate value for inner segment diameter. However, the very small singles generally occurred close to the small singles and, therefore, it was apparent that they were a distinct cone type.

The distal portions of the photoreceptors were enveloped by stringlike processes extending from the cells of the retinal pigment epithelium (*rpe* in Fig. 4A, B). The strong attachment of the pigment epithelium to the photoreceptors often made separation of the two tissue layers very difficult and frequently resulted in damaged cone outer segments. The proximal portions of the photoreceptors were embedded in a matrix of Müller fiber processes (Fig. 4C).

On occasion, but with some degree of consistency, we observed a ropelike structure which connected a group of cones (Fig. 4C). The structure adhered to the photoreceptors near the point where the outer and inner segments join. We could not tell whether or not all the cones so connected were of the same type.

Cell counts from the scanning electron micrographs yielded a value of $18\,280 \pm 1206 \text{ cells mm}^{-2}$ for the packing density of all cones in the retina of *T. sirtalis sirtalis*. The packing density of *T. sirtalis similis* cones was substantially less at $12\,276 \pm 1101 \text{ cells mm}^{-2}$. Otherwise, the retina of *T. sirtalis similis* was very similar to that of *T. sirtalis sirtalis*. It is possible that the difference in packing density actually reflects regional differences within a given retina rather than a difference between the two subspecies (see below). In neither retina were we able to discern from the micrographs a regular pattern of cone distribution.

Immunocytochemistry

Adjacent semithin sections of a section series, representing both longitudinal and tangential sections of the cones of *T. sirtalis sirtalis*, were treated with different antibodies known to react with the visual pigments within the photoreceptors' outer segments. The staining patterns, shown in Figs. 5–9, revealed the presence of four distinct cone populations. For example, COS-1 reacted well with the visual pigments within the large single cones and both the principal and accessory

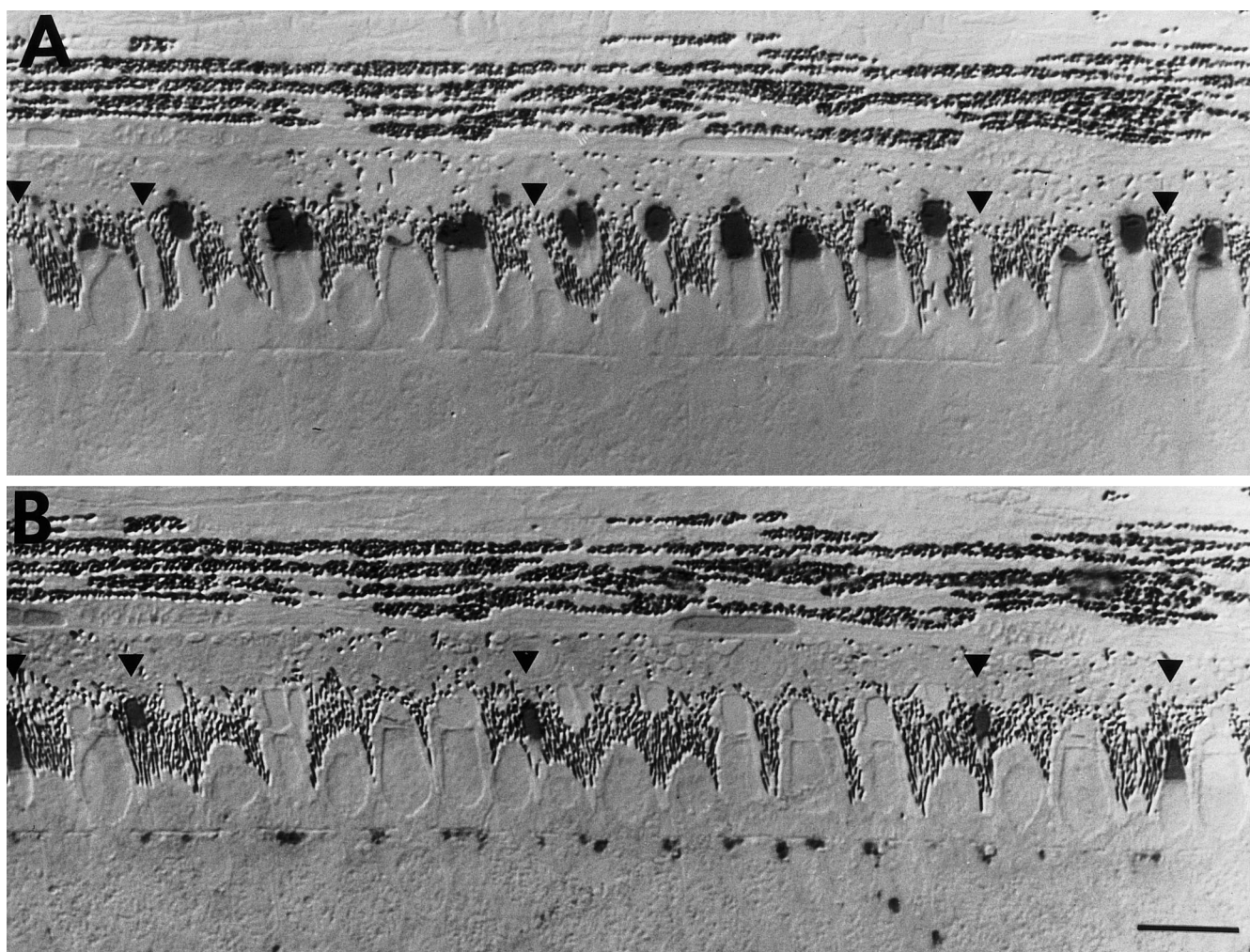


Fig. 5A, B Longitudinal section of photoreceptors showing differential antibody reactivity of cone outer segments from *T. sirtalis sirtalis*. One semithin section was exposed to antibody COS-1 (A), while the adjacent section was stained with antibody AO (B): **A** section stained with COS-1. Outer segments of the large single cones and both principal and accessory members of the double cones are highly reactive (heavily stained) to COS-1. Small single cones (arrowheads) are not reactive to COS-1 and are unstained; **B** adjacent section stained with AO. Outer segments of small single cones (arrowheads) are highly reactive (heavily stained) to AO. Large single cones and double cones are not reactive to AO and are unstained. Calibration bar: 10 μ m

members of the double cones, but did not react at all with the small single cones (Fig. 5A). In contrast, a reverse staining pattern was observed with antibody AO that reacted well with small single cones, but left the large singles and doubles unstained (Fig. 5B). However, not all small single cones exhibited the same reactivity. Thus, some of the small singles left unstained by COS-1 (circled cells in Fig. 6A) were also left unstained by AO (circled cells and arrowheads in Fig. 6B), whereas other small singles were heavily stained by AO (arrows in Fig. 6B). In addition, those small singles which were heavily stained by AO were also well stained by OS-2

(arrows in Fig. 6C) and K42-41 (arrows in Fig. 6D), but the small singles which did not react with AO also did not react heavily with OS-2 (circled cells and arrowheads in Fig. 6C) or with K42-41 (circled cells and arrowheads in Fig. 6D). The difference in immunoreactivity between two classes of small singles is also illustrated, at higher magnification, in Fig. 7A–D. Here the cells that are heavily reactive with AO, OS-2 and B6 (Fig. 7A, C, D) are enclosed in hexagons, while those that exhibit moderate reactivity are encircled. Again, both types of small singles are negative with COS-1 that only stains doubles and large single cones (Fig. 7B).

A double immunofluorescence on retinal whole-mounts (Fig. 8) confirmed the presence of four cone types and also provided information regarding relative numbers. In this experiment, retinas were treated with a mixture of fluorochrome-conjugated antibodies COS-1 and OS-2. COS-1 was coupled with rhodamine and appeared red with the suitable filter set. This antibody heavily stained the large single cone and both members of the double cone. OS-2 was conjugated with fluorescein, giving a yellowish-green fluorescence with the filter set for fluorescein. OS-2 revealed the small single cones with differing staining intensities. Thus, a smaller pro-

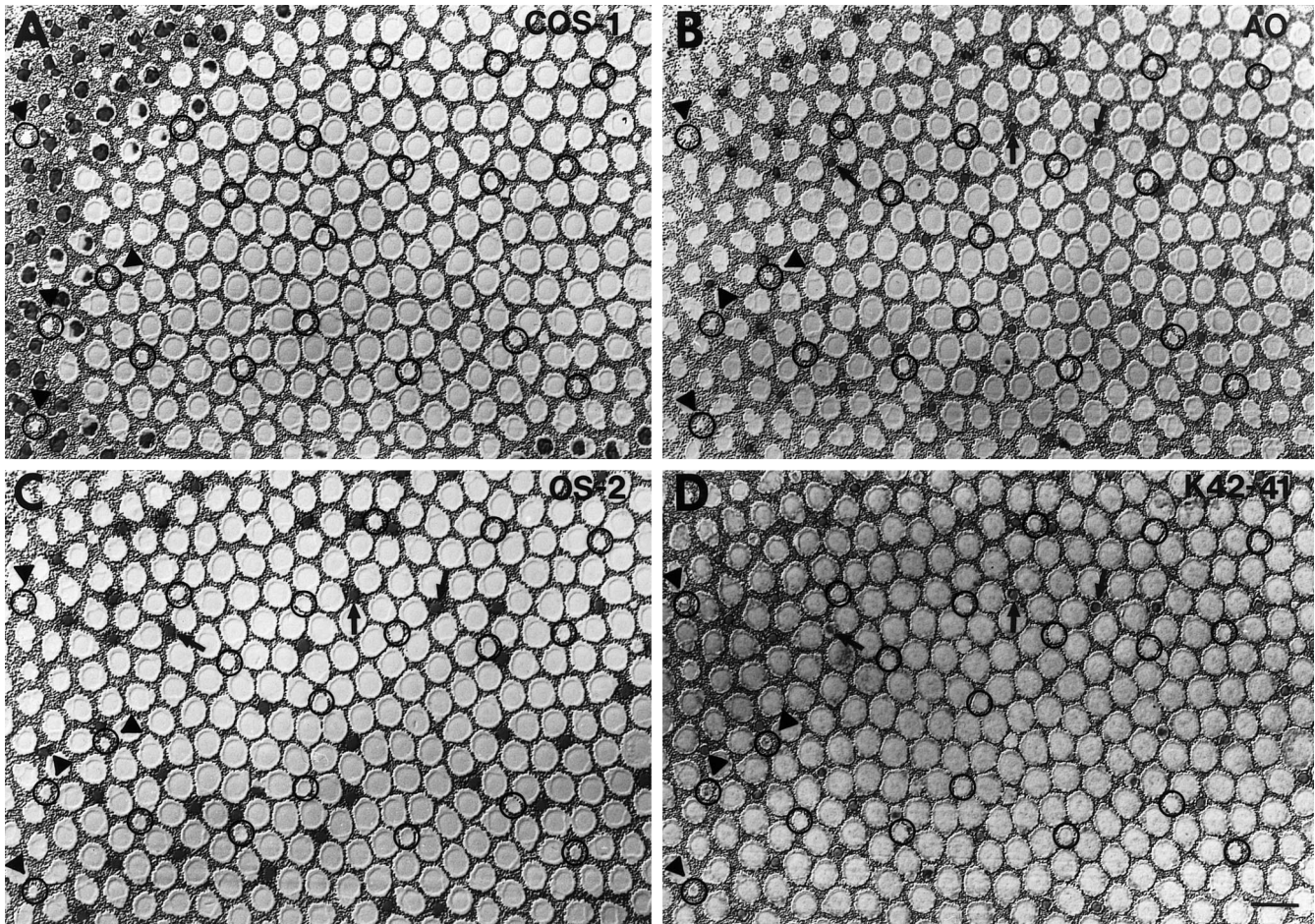


Fig. 6 Transverse sections of photoreceptors on four consecutive semithin sections showing differential antibody reactivity of cones from *T. sirtalis sirtalis*. The antibody with which each section was treated is noted in the upper right of each panel. The circles mark the same small single cones in each panel. See text for explanation. Calibration bar: 10 μm

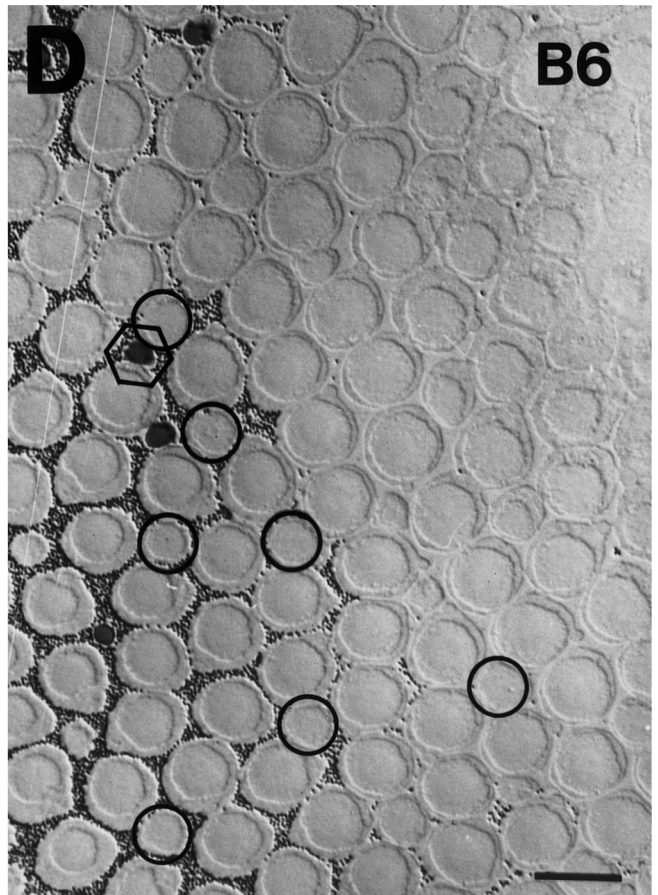
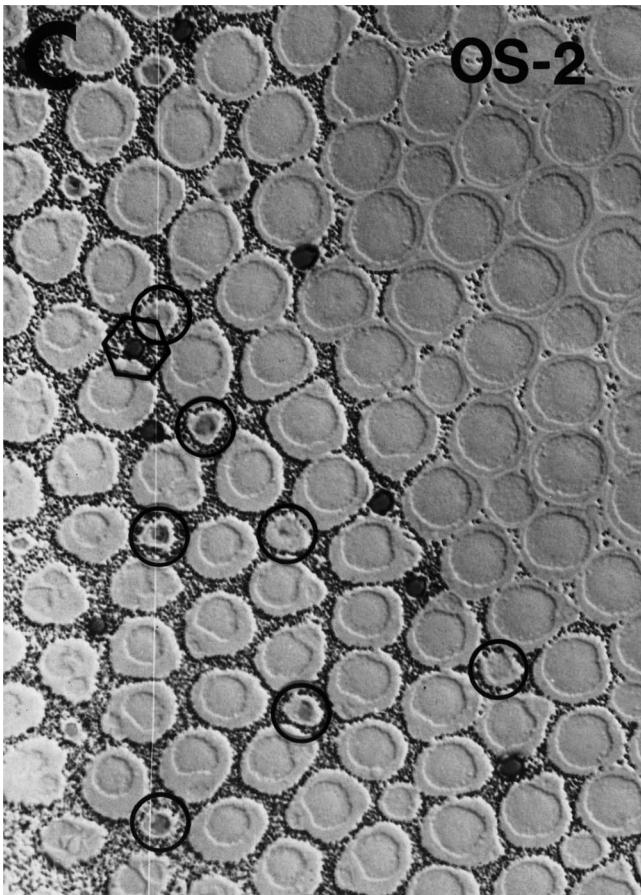
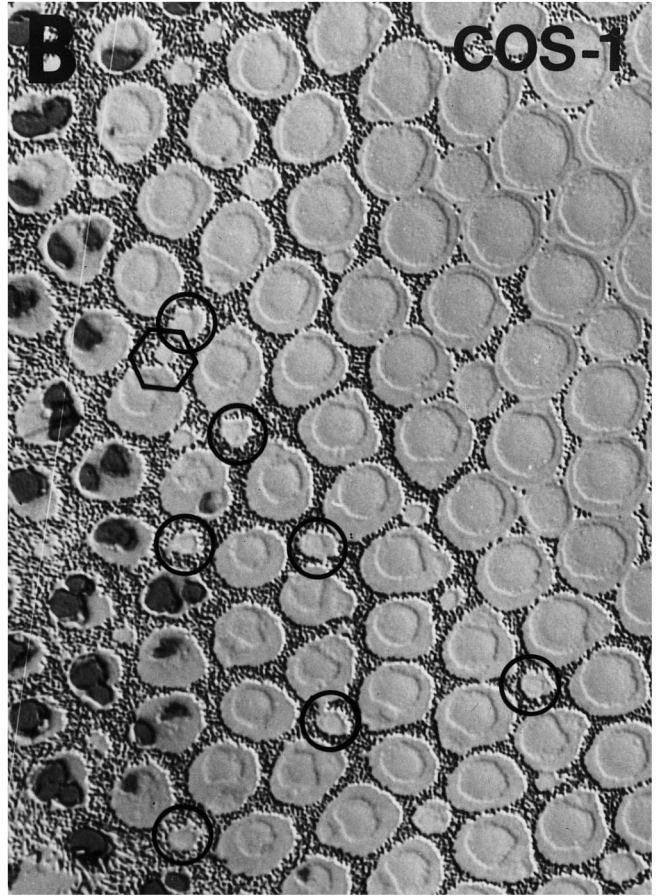
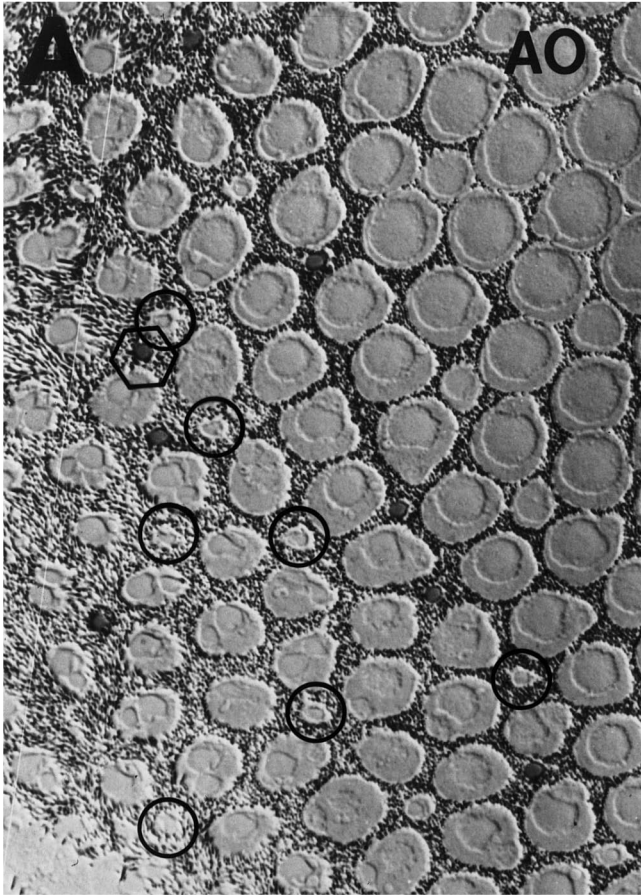
portion of the small singles was very weakly stained (arrowheads in Fig. 8), while a larger proportion of the small singles gave a bright fluorescence with this antibody. The weakly labeled small single cones were usually smaller in diameter and constituted about 5.5% of all cones, while the strongly reacting small single cones comprised about 9%. The relative number for the double cones was 45.5% and that for the large single cones was 40%. The retinal wholemounts also provided the opportunity to estimate the packing density of all photoreceptors in non-dehydrated specimens. The average value in the near central area of the retina was 14 350 cells mm^{-2} . However, there are certainly regional differences in packing density since, in radial sections, we counted more than twice the number of photoreceptors in the central region of the retina than in the periphery.

Discussion

The photoreceptor population

The results of this study confirm the light and transmission microscopic observations of Wong (1989) that the photoreceptor layer of *T. sirtalis* is devoid of rods and comprised of several different types of cones. In agreement with Wong (1989), we found most of the photoreceptors to be either large single cones or double cones comprised of a large principal member in close association with a smaller accessory member. Also in agreement with Wong (1989), we found small single cones which are much less common than either the large singles or the doubles. Wong's (1989) value for packing density is, in fact, about 30% higher than ours, but the difference is most likely due to different degrees of sample dehydration and to regional differences observed

Fig. 7 Transverse section of photoreceptors on four consecutive semithin sections showing differential antibody reactivity of small single cones from *T. sirtalis sirtalis*. The antibody with which each section was treated is noted in the upper right of each panel. Circles and hexagon mark the same cells in each section. See text for further explanation. Calibration bar: 10 μm



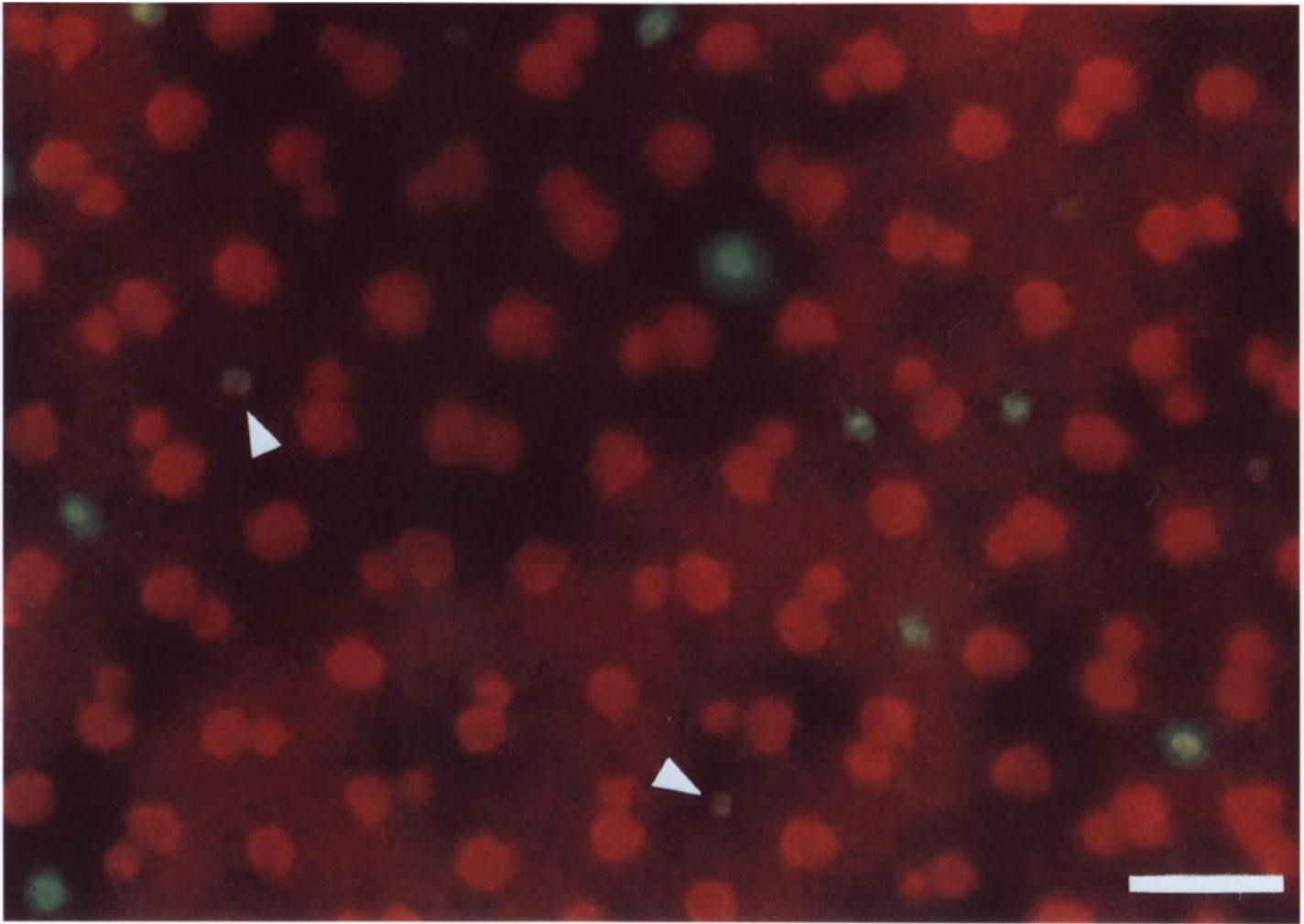


Fig. 8 Fluorescence micrograph of a retinal wholemount of *T. sirtalis*. Four distinct populations of cone photoreceptors can be distinguished. Double cones and large single cones appear red, indicating a strong COS-1 reactivity. Together, they form the overwhelming majority of all cones. Small single cones appear yellowish green (OS-2 reactivity) and can be classified into two distinct populations, one with bright fluorescence and the other with very weak fluorescence (*arrowheads*). See text for further details. Calibration bar: 10 μ m

by Wong (1989) and also revealed in our sections prepared for immunocytochemistry. Unlike Wong (1989), however, we found with the SEM that the small single cones are actually of two types, one type being substantially smaller than the other. The presence of four rather than three distinct populations of cone photoreceptors was revealed also by our immunocytochemical experiments which identified large single cones and double cones which react heavily with the COS-1, an antibody shown to detect medium-to-long wavelength-sensitive visual pigments in most vertebrates (Szél and Röhlich 1985; Szél et al. 1986a, b; Cserháti et al. 1989; Röhlich et al. 1989; Röhlich and Szél 1993; Loew et al. 1996), small single cones which react heavily with AO, OS-2, B6 or K42-41, and small single cones which react

only lightly or not at all with AO, OS-2, B6 or K42-41. The weakly reactive small single cones usually had smaller, and sometimes more vitreally positioned, outer segments than the highly reactive small singles. However, we are reluctant to identify these weakly reactive small singles as the very small singles revealed by the SEM because of possible regional differences in cone morphology, an issue not systematically addressed in the present study.

Since in some snake retinas, Walls (1942) and Underwood (1970) reported the presence of small, rare photoreceptors they suggested might be rods, we considered whether the small single photoreceptors found in the present study might, in fact, be rods rather than cones. We have rejected the possibility for several reasons. First, except for being smaller, the very small single cells resemble all the other cells in the garter snake retina. All the cells have typical cone morphology, that is, a relatively small, tapering outer segment extending from a rather bulbous inner segment. In contrast, rods universally have a long, cylindrical outer segment connected to an inner segment of similar diameter. Second, this cone type morphology is consistent with the fact that every garter snake photoreceptor examined by

Wong (1989) exhibited a lamellar organization of the outer segment. Such a lamellar organization, as opposed to free floating discs, is diagnostic for cone photoreceptors. Third, where the visual pigments of snake rods have been examined (Crescitelli 1956; Govardovski and Chkheidze 1989), they have been shown to have peak absorbance very close to 500 nm. The very small single photoreceptors in the garter snake contain a visual pigment with peak absorbance at either 487 nm or 365 nm. Finally, and perhaps most important, Jacobs et al. (1992) reported no electrical activity in the garter snake retina at scotopic (rod) light levels. Thus, in agreement with both Wong (1989) and Jacobs et al. (1992), we conclude that the garter snake retina is pure cone.

The SEM data and the immunocytochemical data are entirely consistent with the results of microspectrophotometric measurements of absorbance in the cone outer segments. Thus, in *T. sirtalis sirtalis*, the outer segments of the large single cone and both the principal and accessory members of the double cone contain a visual pigment with peak absorbance (λ_{\max}) near 554 nm. The outer segments of the small single cones, on the other hand, contain either a visual pigment with λ_{\max} near 481 nm or one which absorbs maximally in the near ultraviolet (UV) with λ_{\max} near 358 nm. It was not possible with the microspectrophotometer to identify which of the two types of small single cones housed the UV-sensitive visual pigment. However, the fact that the UV-sensitive cells were encountered least frequently suggests that they are the small single cones that reacted only weakly with AO, OS-2, B6 and K42-41 antibodies. This would be in agreement with the identification of the UV-sensitive photoreceptors of geckos as those reacting weakly with AO, OS-2, and K42-41 antibodies (Loew et al. 1996).

Compared to the 554-nm pigment of *T. sirtalis sirtalis*, the long-wavelength-sensitive pigment in the cones of *T. sirtalis similis* appears to be slightly red shifted with a λ_{\max} at 557 nm. Although the biological significance, if any, of this small shift in spectral absorbance is unclear, the difference is certainly not surprising. Visual pigment polymorphism is now well established (e.g., Jacobs 1996), and such a shift can easily result from a single amino acid substitution in the opsin portion of the visual pigment molecule (Yokoyama et al. 1995). Although we are fairly confident in placing the long wavelength pigment of *T. sirtalis similis* at 557 nm, not enough cones containing the shorter wavelength pigment were measured to establish peak absorbance with great accuracy. We can only say that the pigment absorbs maximally near 487 nm but may not be different from the comparable pigment found in *T. sirtalis sirtalis*. We attribute the failure to encounter any UV-sensitive cones in *T. sirtalis similis* simply to their rarity and to the fact that we studied very few cells in that subspecies.

Our data are at odds with the results of Jacobs et al. (1992) who used an electroretinographic procedure to measure spectral sensitivity in two species of garter

snake, *T. sirtalis* and *T. marcianus*. Jacobs et al. (1992) predicted the presence of a visual pigment with λ_{\max} near 556 nm, but found no evidence for the presence of any visual pigment absorbing maximally at shorter wavelengths. However, the small single cones which contain the pigments sensitive to shorter wavelengths are rather rare compared to the cells which contain the 554-nm pigment, and their electrical responses may have been masked. In fact, after increasing stimulus intensity, Jacobs' laboratory is now finding evidence for the presence of short-wavelength-absorbing visual pigments (G.H. Jacobs, personal communication).

Both the absorbance spectrum and the strong reactivity with the COS-1 antibody show that the visual pigment of the large single cones and the double cones of the garter snake is similar to iodopsin, the long-wavelength (λ_{\max} 550–560 nm)-sensitive pigment so widespread among the terrestrial Tetrapoda. Although it is somewhat premature to generalize on the basis of the extremely limited data we have on snake visual pigments, it is nevertheless interesting to note that similar visual pigments, with absorbance maxima between 550 and 560 nm were also found by Govardovskii and Chkheidze (1989) in the large single cones and principal member of the double cones in two viperid and two diurnal colubrid species. The visual pigments in the outer segment of the accessory member of the double cones and of the small single cones have not been identified. However, E.R. Loew (unpublished observations) recently studied the transmuted, rod-like photoreceptors of a nocturnal colubrid, *Hypsiglena*, and found that the large single cells and the double cells contain an iodopsin-like visual pigment, whereas the small single photoreceptors contain either blue- or UV-absorbing pigments. Thus, it may be that the photoreceptor system of colubrid snakes in general is characterized by the presence of the same long-wavelength-sensitive visual pigment in large single cones and double cones (transmuted in nocturnal species), the presence of UV- and/or blue-absorbing visual pigments in small single cones (again, transmuted in nocturnal species), and the absence of a green-sensitive pigment with λ_{\max} close to 500 nm.

The importance of the visual sense

In view of *T. sirtalis*' relatively complex cone population with its multipigment system, it is certainly reasonable to assume that the snake makes good use of its visual sense. It is true that the packing density of the cones, even in the area centralis identified by Wong (1989), would preclude a visual acuity comparable to a typical foveate mammal or bird. Nevertheless, there is sufficient cone density and optics of high enough quality (Land and Snyder 1985) to allow production of a very useful image. This is consistent with observations that vision is important to garter snake behavior. For example, Teather (1991) found that *T. sirtalis similis* is ten times more

successful in catching fish when the prey were presented against a contrasting background. Macias Garcia and Drummond (1995) concluded that *T. melanogaster* uses its visual sense to discriminate prey objects on the basis of size and to determine trajectory, and the vision of *T. sirtalis sirtalis* is at least acute enough to allow the snake to recognize the eyes of potential predators (Bern and Herzog 1994) and to strike at those eyes preferentially (Herzog and Bern 1992). Finally, the importance of the visual sense is attested to by the fact that the eyes of garter snake species that are specialized as strictly aquatic predators have much greater accommodative ability than those not so specialized, presumably to ensure sharp vision under both aquatic and terrestrial conditions (Schaeffel and de Queiroz 1990).

The presence of more than one cone pigment satisfies the most basic retinal requirement for wavelength discrimination or color vision. Unfortunately, we know of no reports describing any color vision capability, or lack thereof, in any snake species. Color vision is so common among the vertebrates (Jacobs 1981) that it would not be unreasonable to assume that the garter snake has color vision. However, the assumption would be premature without direct experimental data since a multipigment system can serve other functions, such as simply increasing sensitivity across a broad spectral range or enhancing contrast (Lythgoe 1979).

The role of the UV-sensitive photoreceptor

The combined data from microspectrophotometric, electrophysiologic and behavioral experiments have made it apparent that sensitivity to UV light is not unusual but, rather, is widespread among the vertebrates. Thus, evidence for UV sensitivity has been found in birds, mammals, fishes, amphibians and reptiles (Jacobs 1992). Since UV light can be damaging to retinal tissue, it stands to reason that mechanisms for filtering out UV light would have evolved if not for an important adaptive value to UV sensitivity (Bennett and Cuthill 1994). Thus, many suggestions, some more speculative than others, have been made as to the specific functional role of UV sensitivity in various species, but the issue is still quite open to debate. This would seem to be especially true with respect to snakes as there are no reports other than the present one which bear directly on the question of UV sensitivity amongst the Ophidia. There is evidence, however, that UV-sensitive cones do contribute to color vision in another reptile, the turtle (Arnold and Neumeyer 1987). The anoline lizard is another reptile known to be sensitive to UV light (Fleishman et al. 1993). Fleishman et al. (1993) found that certain species of anoline lizards, which live in an environment rich in UV light, possess dewlaps with strong UV reflectance. They suggested that the UV reflectance of the dewlap, an expandable throat fan used prominently in display behavior, is important to visual communication in those lizards. UV light is also important to communication in

iguanaid lizards. Alberts (1989) found that the response of desert iguanas to the presence of femoral gland secretions was greatly enhanced by UV light. The secretion contains a pheromone which is assumed to be of importance for marking territories or for locating other members of the species. The substance is a low-volatile material which absorbs UV light, thus permitting the lizard to see the contrast between the dark secretion and the relatively bright UV reflecting surround. While garter snakes do not have an obvious UV-reflecting display structure such as the anoline dewlap, they do extrude a low-volatile pheromone through the skin. The pheromone is known to elicit reproductive activity in garter snakes, and is also thought to be deposited as a trail which guides males to females during the breeding season (Ford and Low 1984). It has been assumed that the snake follows the trail by means of its exquisite chemosensory ability (Ford and Low 1984), but it would be very interesting to see if the garter snake secretion either absorbs UV light as does the femoral gland secretion of the iguanaid lizard, or reflects UV light as does the dewlap of the anoline lizard. It doesn't really matter much which is the case, as only the contrast between the trail and the surround would be important. The use of UV light here would have an important adaptive value. For one, although the trail might be very easy to follow with the chemical sense it is probably much more easily located with the visual sense. As Marler (1967) has pointed out, the directional nature of light ensures that perception is virtually synonymous with spatial localization, something that is not true with chemosensation. Moreover, the ability to locate a trail with the visual sense allows the use of a low-volatile substance which will be more persistent.

The use of UV sensitivity for spatial localization would not require the formation of a fine image. This is important because the garter snake's UV-sensitive cones are few and far between and, therefore, cannot by themselves provide good visual acuity. However, whereas acuity in the UV is ultimately limited by the distance between UV-sensitive cones, sensitivity in the UV might involve proximal mechanisms for 'gain'. Thus, the fact that there are rather few UV-sensitive cones does not mean that UV-mediated activity in higher neural centers is small (Goldsmith 1994). One may debate whether or not such selectively enhanced gain in a UV channel serves as a component of a system for "true" color vision, but certainly it could enable the snake to perform important visual tasks based on visual cues in the UV.

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