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BERNARD, GD; Stavenga, Doekele; Bernard, Gary D.

Published in:
Journal of Comparative Physiology

DOI:
10.1007/BF00610467

Publication date:
1979

J. Comp. Physiol. 134, 95–107 (1979)

Journal of Comparative Physiology · A

Spectral Sensitivities of Retinular Cells Measured in Intact, Living Flies by an Optical Method

Gary D. Bernard and Doekele G. Stavenga

Department of Ophthalmology and Visual Science, Yale University, New Haven, Connecticut 06510, USA, and
Biophysical Department, Rijksuniversiteit Groningen, Groningen, The Netherlands, and
The Marine Biological Laboratory, Woods Hole, Massachusetts, USA

Accepted August 20, 1979

Summary. The spectral sensitivity of the peripheral retinular cells R1–6 in nine species of intact flies was determined using non-invasive, optical measurements of the increase in reflectance that accompanies the pupillary response. Our technique is to chronically illuminate a localized region of the eye with a long wavelength beam, adjusted to bring pupillary scattering above threshold, then, after stabilization, to stimulate with monochromatic flashes. A criterion increase in scattering is achieved at each wavelength by adjusting flash intensity. Univariance of the pupillary response is demonstrated by Fig. 3.

Action spectra measured with this optical method are essentially the same as the published spectral sensitivity functions measured with intracellular electrophysiological methods (Fig. 4 for *Calliphora*, Fig. 5 for *Drosophila*, Fig. 7 for *Eristalis*, and Fig. 8 for *Musca*). This holds for both the long wavelength peak and the high sensitivity in the UV as was consistently found in all investigated fly species.

Spectral sensitivity functions for R1–6 of hover flies (family Syrphidae) are quite different in different regions of the same eye. There can also be substantial differences between the two sexes of the same species. The ventral pole of the eye of female *Allograpta* (Fig. 10) contains receptors with a major peak at 450 nm, similar to those of *Eristalis*. However, the dorsal pole of the same eye contains receptors with a major peak at 495 nm, similar to those of *Calliphora*. Both dorsal and ventral regions of the male *Toxomerus* eye, and the ventral region of the female eye, contain only the 450 nm type of R1–6 (see Fig. 12). However, the dorsal region of the female eye also contains another spectral type of receptor that is maximally sensitive at long wavelength. Eyes of both sexes of *Allograpta* (Figs. 10 and 11) contain a mixture of spectral types of receptors R1–6.

Introduction

Measuring the spectral sensitivities of the photoreceptors of fly eyes has been approached by a number of different methods, each with its own virtues and shortcomings (Goldsmith and Bernard, 1974). The most popular and most successful in determining the characteristics of single types of retinular cells is the technique of intracellular, electrophysiological recording. Yet, an unavoidable difficulty inherent in the electrophysiological technique is that the experimental animal must be cut, punctured, or partially dissected for the electrode to penetrate the animal's exterior. Furthermore, the micropipette ordinarily penetrates the membranes of many cells in order to reach the cell in which its tip is embedded. Consequently, the length of time one can record from a cell is limited, and the results of measurements can be affected by artifacts of preparation and penetration.

For the last decade, a promising alternative technique for optically probing the photoreceptor cells of completely intact, living invertebrates has remained underdeveloped. We refer to the pioneering work of Kirschfeld and Franceschini (1969) who demonstrated that pigment granules, contained within a photoreceptor cell of a fly, can move upon light-adaptation and congregate next to the rhabdomere, thereby controlling the optical flux in the rhabdomere by absorbing and scattering light. This so-called pupillary response is measurable either as a decrease in transmittance or an increase in reflectance of the eye. Thus, either effect can be used to monitor the sensitivity of photoreceptor cells. The feasibility of the former approach was first demonstrated by Franceschini (1972a, b), who measured the action spectrum for changes in antidromic transmittance caused by orthodromic illumination of the eye of a white-apricot mutant of *Drosophila*.

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Noninvasive, optical techniques for measuring the spectral sensitivity of photoreceptor cells were further developed by Bernard (1976a, b; 1977a, b; 1979) on butterfly species that exhibit tapetal eyeshine. In this case, pupillary responses can be exquisitely measured, since the migrating pigment granules reduce transmittance for both incident and reflected light-paths through the rhabdom. The results of the work on butterflies encouraged our attempt to extend the technique to insect eyes that exhibit optical scattering from pupillary pigment granules.

The phenomenon of pupillary scattering is known not only for Diptera (Franceschini, 1972a, b; Franceschini and Kirschfeld, 1971a, b, 1976; Stavenga, 1975), but also for Hymenoptera, Orthoptera, and a variety of other invertebrate orders (Franceschini, 1975; Franceschini and Kirschfeld, 1976; Stavenga, 1979a, b; Stavenga and Kuiper, 1977; Stavenga et al., 1977).

Species of Diptera and Hymenoptera proved to be suitable subjects, indeed. Our results have been summarized in a published abstract (Bernard and Stavenga, 1977), and our experiments on isolating responses from different spectral types of bumblebee photoreceptor cells have been reported (Bernard and Stavenga, 1978). This paper is devoted to flies.

Methods

The apparatus required for implementation of this method is an incident-light microscope equipped with a photometer head and a specially constructed double-beam illuminator. The "measuring beam" is a steady beam that serves two purposes; it provides the illumination that the photometer head uses to measure the reflectance of the eye, and it sets the resting state of adaptation of the eye. The "stimulating beam" provides monochromatic flashes of adjustable wavelength, intensity, and duration that elicit pupillary responses from the eye.

The remainder of the methods section first describes the apparatus and its alignment, next the preparation and mounting of the fly, and finally the procedure for measuring responses and computing spectral sensitivity.

Microscope-Photometer

We used a Leitz Ortholux microscope equipped with their 0-pak pol-illuminator (including an MPV changing slide), 8×/0.18 P or 5.6×/0.15P objective (or a Zeiss 10×/0.2 Ultrafluar objective), MPV photometer head, and four-axis universal stage. The photometer head contained a centrable measuring aperture, a filter holder, and an EMI 9658RAM red-sensitive photomultiplier.

The optical elements in the measuring beam (see Fig. 1) were a current-stabilized quartz-halogen 45-W lamp (General Electric Q6.6AT2 1/2-CL) operated at 6.5 A in a Schoeffel lamp-housing with quartz condenser, a filter-holder containing a 3 mm Schott KG-3 heat-filter plus neutral-density and colour filters, and a Staeble-Telexon 135 mm f/3.8 telephoto lens.

The stimulating beam contained a Bausch & Lomb high-intensity monochromator (33-86-76) with quartz-halogen illuminator (33-86-39-01) and quartz-fluorite achromatic condenser lens (33-86-53), two neutral-density wheels (6 inches diameter, density range 0–2, metallic coating, made by Kodak) mounted on 1.8° stepper motors, and a Vincent Uniblitz shutter (225). The two beams were combined in a UV-transmitting beam splitter (Oriel A43-564-60-3) before entering the microscope.

The current from the photomultiplier was measured with a Keithly 616 digital electrometer set at one nA/V. the voltage from the 616 was both sampled by a Digital Equipment Corp. LAB/8E computer and monitored by a Tektronix 5103 storage oscilloscope. The shutter of the stimulating beam was controlled by a WPI series 800 pulse generator.

Optical Alignment of the MSP

The microscope objective was centered with respect to the optical axis of the microscope. The optics were aligned by selecting the glass plate of the 0-pak illuminator and focussing on a front-surface mirror, adjusting the condenser of the 0-pak to bring its field aperture into focus, then centering the field apertures of both the measuring beam and the stimulating beam. The field aperture of the stimulating beam, as measured in the front focal plane of the microscope objective, was set slightly larger than that of the measuring beam (e.g., 190 μm and 160 μm, respectively). Next, the back focal plane of the microscope objective was examined, and the aperture stop A_a was centered and reduced to about 80% of its full diameter, then the exit-slit of the monochromator was centered with respect to the aperture stop. Finally, both light-sources were adjusted for maximal intensity and uniformity as viewed in the front focal plane. The entrance slit of the monochromator was set to 2.8 mm, corresponding to 10 nm bandwidth. However, because less than 0.5 mm of the exit slit was required to fill the back focal plane of the objective, the illumination at the fly's eye had a bandwidth of 8 nm or less.

Preparing the Fly for Measurements

Flies were collected from Woods Hole and the surrounding countryside, and placed in glass vials prior to preparation. A fly was prepared for measurements by first placing its vial in crushed ice until it ceased to move. Then its wings were fixed together with molten wax of low melting temperature. Next, the fly was fixed to a slotted plastic tube with wax that connected its thorax to the tube, then wax was applied between thorax and head. Finally, the six feet were waxed together, keeping the legs away from the head. Care was taken to avoid waxing the abdomen, the thoracic spiracles, or the mouth parts. The product of the mounting procedure was a completely intact fly with head that was rigidly fixed to the plastic tube, but with movable abdomen and mouth parts. The mounted fly was then offered a drink of honey water. If the fly fed avidly after mounting, it usually survived for several days of measurements, if fed at least twice a day.

Adjustment of the MSP with the Fly in Place

The fly, mounted in its plastic tube, was inserted into the universal stage and placed beneath a Wild M5 stereo-microscope that had been equipped with a vertical-illumination prism (215-972). When the angle of illumination was adjusted to be as close as possible to the angle of view through the right eyepiece, the pupillary scatter from the pseudopupil (as in Fig. 16b of Franceschini, 1975) was easy to observe, even at intermediate settings of the lamp voltage (some species exhibit more scattering than others. See the Results section). After orienting the universal stage to illuminate the desired

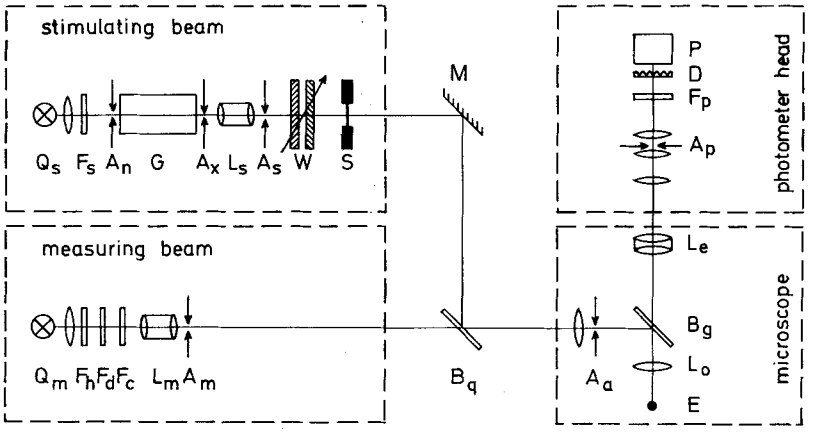


Fig. 1. Diagram of apparatus. The measuring beam contains: Q_m, 45 W quartz-iodine illuminator; F_h, heat filter; F_d, neutral filter; F_c, cut-off filter; L_m, telephoto lens; A_m, field aperture. The stimulating beam contains: Q_s, 45 W quartz-iodine illuminator; F_s, stray-light filter; computer-controlled monochromator G with entrance slit A_n and exit slit A_x; L_s, quartz condenser; A_s, field aperture; W, computer-controlled neutral-density wedge; S, fast electric shutter; M, mirror. The two collimated beams are combined in quartz beam splitter B_q and inserted into the epi-illuminator of the microscope. The microscope contains A_a, aperture diaphragm; B_g, glass plate; L_o, microscope objective; E, eye of intact fly; L_e, 10× eye piece. The photometer head contains A_p, adjustable and centrable photometer diaphragm; F_p, photometer filter; P, photomultiplier; D, micro-prism diffuser

region of the eye, and examining the fly for obvious head movements, the stage was transferred to the MSP. A cut-off filter known to elicit a pupillary response (e.g., OG590) was placed in the measuring beam. Then the beam was switched on and the microscope was focussed on the deep pseudopupil. Subsequently, the trapezoidal region of pupillary scatter was centered with respect to the field stop. The diameter of the field stop was then reduced to be somewhat larger than the trapezoidal region, so that movements of the trapezoid (Franceschini and Kirschfeld, 1971b) stay within the illuminated region. The measuring diaphragm (A_p) of the photometer was centered on the pupillary trapezoid and opened to contain the field stop.

Then the eye was checked for mechanical and optical stability with the following procedure: the measuring beam was turned off and its filter changed to one that elicits a large pupillary response (e.g., OG550). After a few minutes of darkness, the step-response to this bright light was monitored with the photomultiplier. If the fly was able to move its head, it would do so in response to such bright illumination, causing massive fluctuations of the photomultiplier signal. However, if the response increased smoothly and remained high in the presence of the bright light, the experimental preparation was likely to be a good one. If the fly did move its head under these circumstances it was removed and waxed again. If the fly still moved it was discarded and a new fly was prepared. One can work with a healthy, stabile fly for days, so wasting time with a movable retina is ill advised.

Selection of the Measuring Beam

In all experiments the steady measuring beam contained a heat-absorbing filter and a cut-off filter. The particular choice of cut-off filter was governed by the desired state of the unstimulated pupil. Unless otherwise specified, the filter was chosen so that the pupillary response of the dark-adapted eye to onset of the measuring beam was somewhat above threshold (by 0.3 log-unit or so). Having selected a filter for the measuring beam (e.g., RG610), a filter of shorter cut-wavelength (e.g., OG590) was placed in front of the photomultiplier (F_p of Fig. 1), thereby eliminating stimulus artifacts at shorter wavelengths. Because fly metarhodopsins have bathochromically shifted absorbance spectra (Ostroy et al., 1974; Hamdorf, 1979; Stavenga, 1976, 1979a, b) the measuring beam also serves to maintain the titer of rhodopsin at a high level by photoconverting the metarhodopsin to rhodopsin.

After the reflectance to the steady measuring beam stabilized, the photo-multiplier voltage was set to yield about 0.8 nA of current (0.8 V into the oscilloscope) and the vertical gain of the oscilloscope was set to 50 mV/cm, or even 20 mV/cm, so that small changes in reflectance were easily resolved.

Procedure for Measuring Action Spectra

The principle underlying our measurements of spectral sensitivity is the method of response invariance (Rodieck, 1973). The criterion response was achieved at each stimulus wavelength by adjusting the angular position of the circular neutral-density wedge until a setting was found which yielded a criterion response. Some details follow.

After choosing the criterion reflectance-increase large enough to reliably distinguish a response from noise, a reference combination of wavelength and wedge setting was chosen (e.g., 490 nm and 108°) that gave criterion responses for five or ten flashes in a row, one every 30 s or 40 s. Having thus achieved stabilization, responses to a new, randomly selected wavelength were measured, adjusting the wedge between flashes until criterion was achieved. After every six or eight flashes a response to the reference combination of wavelength and wedge setting was checked. In this way the entire spectrum was sampled in random order, collecting combinations of wavelength and wedge setting that yield criterion. Near the end of an experiment a few widely spaced wavelengths were checked to guard against systematic errors caused by photochemical conversion.

Another source of systematic errors can be wide-band stray light, and overlapping orders, from the monochromator. These problems are controlled by inserting appropriate filters at F_s of Fig. 1, a Schott UG1 in the UV, and Schott cut-off filters when working at long wavelengths.

Radiometry and the Calculation of Spectral Sensitivity

Having collected raw data for an action spectrum of the pupillary response as combinations of wavelength and wedge setting, the quantum flux at each combination was measured within a day of the experiment. A calibrated[1] U.D.T. PIN10/UV diode was placed beneath the microscope objective, and readings were taken at each combination,

Readings in the ultraviolet were corrected for wide-band stray light by subtracting the reading obtained after inserting a low-fluorescence Schott KV418 cut-off filter at position F_s of Fig. 1. This was necessary because the photodiode is relatively insensitive in the UV compared to the band 400 nm–1000 nm, and because the tungsten light-source is relatively deficient in UV content.

The values of minimal quantum flux (Q_{min}) given in the Table 1 are for individually different numerical apertures of the incident illumination, corresponding to the values for the individual experiments.

The log-sensitivity functions shown in our figures were computed as $^{10}\log[Q_{min}/Q(\lambda)]$.

Results

Properties of the Pupillary Response

When an increase of illumination is delivered to the eye of a fly, the reflectance of the eye increases due to scattering by the pigment granules as they move into the light path. This pupillary response is demonstrated in Fig. 2. In this experiment the reflectance of the eye of a hover fly *Syrphus* sp. was measured with a sub-threshold, red beam (cut-off filter RG630) and recorded with a pen-writer. The responses shown in Fig. 2 were evoked by monochromatic 500 nm flashes of 11 s duration and variable intensity, separated by an interval of 49 s between flashes. This intensity series proves that the maximal reflectance-increase is a monotonic function of stimulus intensity; the dynamic range is 2–3 log-units (see also Franceschini, 1972a, b; Franceschini and Kirschfeld, 1976).

The univariance (Rodieck, 1973) of the pupillary response was investigated by comparing an intensity series of 540 nm flashes to another series of 435 nm flashes, according to the following procedure: first, the neutral-density wedge (W of Fig. 1) was adjusted to yield a 1.6% response to a 5 s, 540 nm flash. Then we searched in the blue for a wavelength (435 nm) which elicited the same response without changing the wedge setting. Next, a series of six, 540 nm flashes were delivered to the eye, decreasing the wedge density by about 0.2 log-units between each flash. Finally, the wedge was returned to its initial setting and a series of six, 435 nm flashes were delivered, making the same changes in wedge setting between each flash as for the former series. The responses to the twelve flashes are shown in Fig. 3. In both series, the maxi-

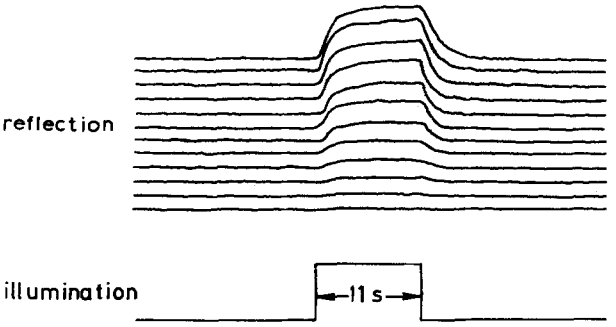


Fig. 2. A series of pupillary responses from a *Syrphus* hover fly. The reflectance of the eye was measured with a broad-band red beam (cut-off filter RG630). Stimulating flashes of duration 11 s and wavelength $\lambda = 500$ nm were delivered every 60 s. The neutral density wedge in the stimulating beam was adjusted between each flash. The density settings were 2.49, 2.34, 2.15, 1.98, 1.78, 1.59, 1.41, 1.18, 0.91, 0.62, 0.28, and 0.00. Zero density at 500 nm corresponds to 2×10^{14} quanta cm^2/s. There is no stimulus artifact because a RG630 cut-off filter was inserted in front of the photomultiplier. The baselines of the traces in this figure are separated from one another by a constant vertical shift for sake of clarity. The baseline of the bottom trace (corresponding to density = 2.49) is coincident with the baseline of the illumination trace. This series proves that the maximal reflectance increase is a monotonically increasing function of stimulus intensity, ranging from 0% at density = 2.49, to 47% at density = 0

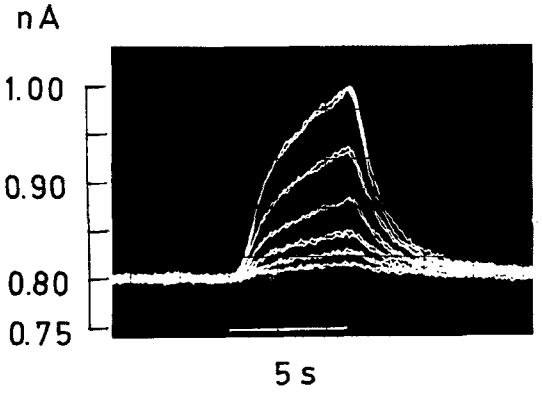


Fig. 3. A 540 nm intensity series matched to a 435 nm intensity series. Oscillograph showing pupillary responses to twelve 5 s flashes comprising one series of six 540 nm flashes and another series of six 435 nm flashes. The resting reflectance corresponds to a photomultiplier current of 0.8 nA. The flash intensities, in units of 10^{12} quanta/cm^2/s are 2.8, 4.3, 6.6, 11, 19, and 36 at 540 nm; and at 435 nm are 0.90, 1.5, 2.4, 3.8, 6.7, and 12.5. The stimuli are matched within 0.05 log-units throughout the entire range, and the responses are matched within 0.6%. This series demonstrates univariance of the pupillary response

[1] The diode was calibrated in 1974 by Optronic Laboratories, Silver Spring, Maryland, USA, and subsequently checked in 1978 against a factory-calibrated UDT-101A radiometer in the Laboratory of Prof. R. Wehner at Zürich, Switzerland. We believe the spectral calibration deviates from the truth by no more than 0.1 log-unit in the band 350–800 nm

Table 1. Experimental parameters

Fig.	Symbol	Genus	Sex	Eye region	Objective	nA of illumination	Filter	Neutral density	Meas. ap. (μm)	Stim. ap. (μm)	Duration (s)	Criterion (%)	Q_{min} $\times 10^{-12}$	Wavelength (nm)	Date
4	o	*Calliphora*	F	V	8×	0.10	RG630	0.0	126	155	6	2	1.8	490	8.9.77
5	o	*Drosophila*	M	V	10×	0.16	RG630	1.0	106	134	10	1.5	2.4	480	1.3.79
6	C	*Chlorops*	F	E	10×	0.16	OG590	0.6	106	134	7	2	3.3	480	13.8.77
	×	*Dimecoenia*	M	D	5.6×	0.09	OG550	2.6	220	275	22	4	1.0	480	11.7.77
7	o	*Eristalis*	F	D	8×	0.10	OG570	0.3	126	155	5	2	7.9	450	10.9.77
8	o	*Musca*	M	E	5.6×	0.08	OG590	3.1	330	400	22	5	0.62	500	8.7.77
	×							1.3					3.4	490	
9	+	*Syrphus*	M	V	5.6×	0.09	OG570	0.0	220	275	8	2.5	1.6	440	7.8.77
	×											1.5	1.6		
	2			V	8×	0.10	OG550	1.3	150	190		3	3.2	470	9.8.77
	3											4.5	3.1		
	5						OG570	0.0				3	2.9	460	
10	M	*Allograpta*	F	E	5.6×	0.09	OG570	1.3	220	275	9	2	0.72	460	27.7.77
	D			D									1.0	480	
	V			V							7		0.17	450	
11	#	*Allograpta*	M	D	10×	0.16	RG610	0.3	160	190	7	3	0.77	480	14.8.77
	×			V			OG590	0.7					0.63	450	
	*			V			RG610	0.0			5		0.51	460	15.8.77
	+			V									0.21	450	
12	×	*Toxomerus*	M	V	5.6×	0.09	OG550	1.1	220	275	13	2.5	3.2	450	19.7.77

mal changes in reflectance were 1.6%, 3%, 11%, 17%, and 26% for the six flashes. The time-course of each response for 540 nm was the same as its companion response for 435 nm.

Spectral Sensitivity Functions

Nine species of flies were investigated, each from a different genus. Six families are represented. The results given below are organized according to genus, proceeding from the well-studied to the unknown.

The stimulating flashes of monochromatic light used in these experiments were between 5 s and 22 s in duration, eliciting criterion increases in reflectance of between 1.5 and 5.0%, with quantum fluxes at maximal sensitivity of between 8×10^{10} and 8×10^{12} quanta/cm^2/s (normalized to a numerical aperture of 0.1). The parameters for individual experiments are given in Table 1, keyed to the symbols with which the spectral sensitivity functions are plotted. Table 1 contains information on genus, sex, microscope objective, numerical aperture of illumination, filters in measuring beam, diameters of measuring and stimulating beams as measured in the front focal plane of the objective, duration of stimulating flashes, criterion increase in reflectance, quantum flux required to elicit criterion at the long-wavelength peak of sensitivity, the value of that wavelength, and the date on which the measurements were performed.

Calliphora

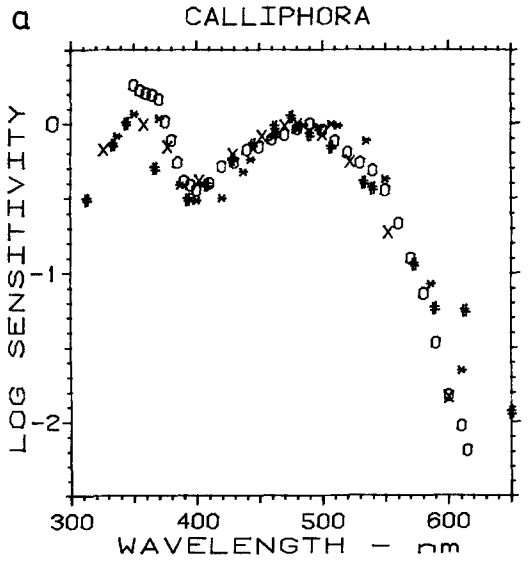
Our results for one female *C. vicinia* Robineau-Desvoidy (this designation is now considered to have precedence over the more familiar name *C. erythrocephala*) are plotted in Fig. 4a with symbol o. The sensitivity at 350 nm is greater than that at 490 nm by a factor of 1.75, while the local minimum at 400 nm is a factor of 0.36 below the latter peak. For wavelengths greater than 550 nm the function is approximately log-linear, decreasing at a rate of 0.35 log-units/10 nm.

Drosophila

Our measurements from one male *D. melanogaster*, Canton-S wild-type are plotted in Fig. 5 with symbol o. The UV peak of sensitivity is at least 1.6 times higher than the peak at 480 nm. The local minimum at 400 nm is quite deep, being a factor of 0.31 below the 480 nm peak. The long-wavelength limb of the function decreases at a rate of 0.3 log-units/10 nm.

Chlorops and Dimecoenia

The flies of Fig. 6 were selected because of a possible visual importance of their irridescent corneas (Ber-



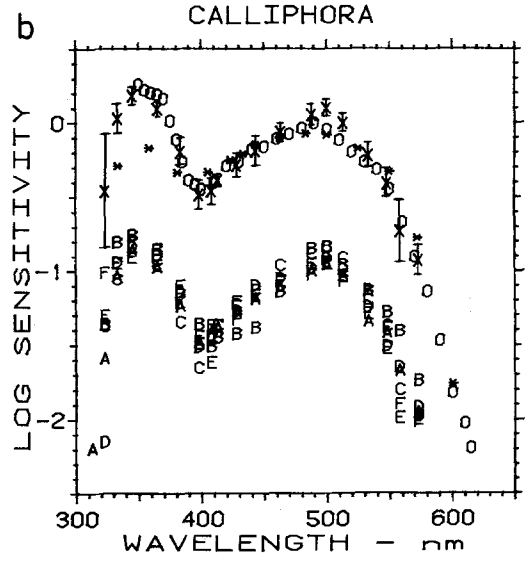


Fig. 4a and b. *Calliphora:* comparison of a pupillary action spectrum to published intracellular electrophysiological spectra. **a** Symbol o, results from our optical method; symbol #, Burkhardt (1962); symbol ×, McCann and Arnett (1972); Symbol *, Dörrscheidt-Käfer (1972). **b** Symbol o, results from our optical method; symbol ×, averaged data from Horridge and Mimura (1975); Symbol *, Hardie (1979); symbols A through F are the data from Fig. 1 of Horridge and Mimura (1975) lowered by one log-unit for clarity

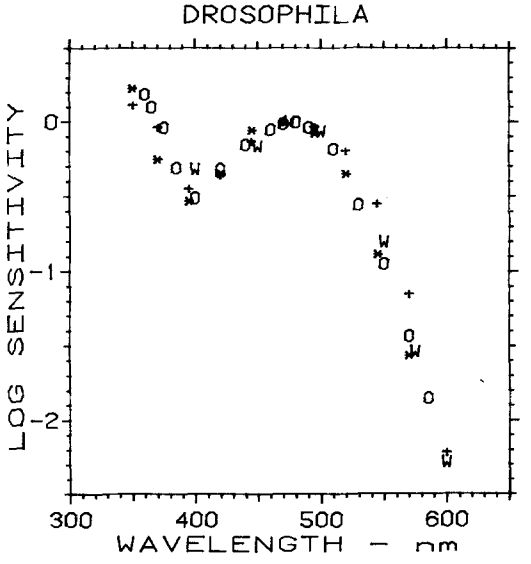


Fig. 5. *Drosophila*: comparison of a pupillary action spectrum to published electrophysiological spectra. Symbol o, results from our optical method; symbol W, Wu and Pak (1975); Symbol *, Harris et al. (1976); symbol +, Stark et al. (1979)

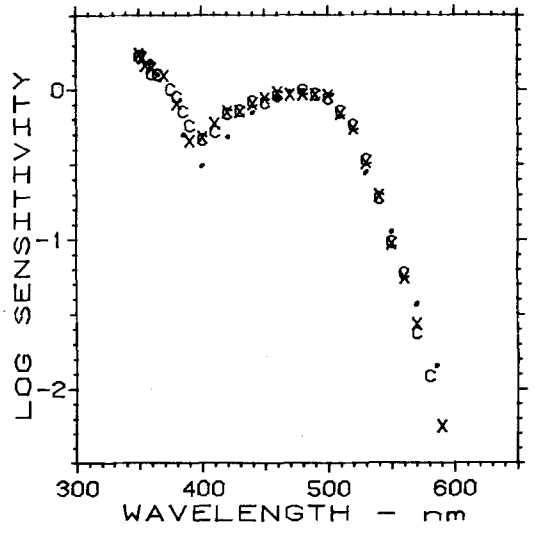


Fig. 6. Pupillary action spectra from a *Chlorops* fly (symbol C) and a *Dimecoenia* shore fly (symbol ×), compared to *Drosophila* (dots)

nard, 1971). The fly *Chlorops* sp., a female about the size of *Drosophila,* is a member of family Chloropidae. The shore fly *D. spinosa* (Loew), a male, is a member of family Ephydridae. We find that both species have eyes with a reflectance spectrum that changes only by a factor of two within the spectral band 390 nm–750 nm, with a broad peak in the far red, a minimum in the green, and a secondary maximum in the violet. Fig. 6 shows that the spectral sensitivity functions for both flies is the same as for *Drosophila* except for a somewhat shallower dip at 400 nm.

Eristalis

The circles in Fig. 7 show our results for a dorsal region of the eye of a female *E. arbustorum* (L.). We were unable to make measurements in the UV because

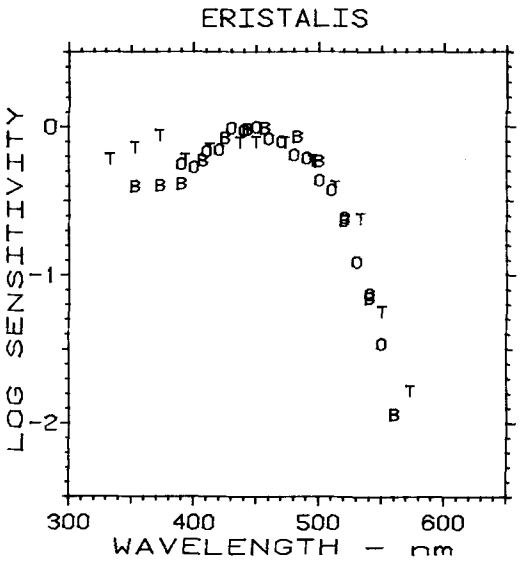


Fig. 7. *Eristalis:* comparison of a pupillary action spectrum to published intracellular electrophysiological spectra. Symbol o, results from our optical method; symbol B, Bishop (1974); symbol T, Tsukahara and Horridge (1977)

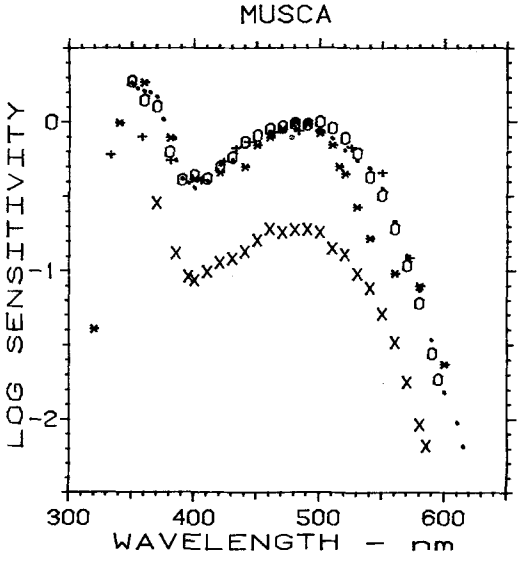


Fig. 8. *Musca:* several comparisons of pupillary action spectra. Symbols o and ×, results from our optical method with the eye in two different states of adaptation; o, measuring beam 0.2 log units above pupillary threshold of thoroughly dark-adapted eye; ×, measuring beam 1.86 log-units more intense than for 0. Symbol +, intracellular electrophysiological results of Hardie (1979); symbol *, optomotor results of Eckert (1971); dots, results of our optical method for *Calliphora*

relatively large intensities were required to elicit a measurable pupillary response; there is a great deal of diffuse, background reflection that makes the deep pseudopupil of *Eristalis* difficult to observe. In the spectral range that we did explore, the peak falls near 450 nm, and the long-wavelength limb decreases at a rate of 0.31 log-units/10 nm.

Musca

Figure 8 shows two spectral sensitivity functions for the equatorial region of the eye of a male *M. domestica*, which differ according to the state of pupillary adaptation. The symbol o is for a measuring light that was 0.2 log-units above pupillary threshold of the thoroughly dark-adapted eye. The symbol X is for a measuring light that was brightened by removing 1.86 log-units of density from the beam. In both cases the eye was allowed to stabilize before measuring sensitivity. The only difference between the results of the two measurements is a vertical shift of the log-sensitivity function by about 0.7 log-units; the shapes of the functions are the same.

Comparisons to the dots in the same Fig. 8 show that the function for *Musca* is essentially the same as for *Calliphora*.

Syrphus

A male *Syrphus* sp. was studied to learn if there were differences in spectral sensitivity among the retinular cells R1–6. Measurements were restricted to selected portions of the deep pseudopupil by masking all but the desired reflections with a movable, adjustable diaphragm (A_p of Fig. 1). Two regions in the medio-ventral part of the eye were studied, at elevations –20° and –30°, where 0° elevation corresponds to the equator. At –20° elevation, functions were measured for R2, R3, and R5, respectively, shown at the top of Fig. 9. The function for R3 is somewhat higher at long wavelengths compared to the others.

At –30° elevation, the sensitivity function for R1+R6 was compared to that for the entire trapezoid, as shown at the bottom of Fig. 9. There is no difference between the two functions.

Allograpta

Three experiments were performed on one female *A. obliqua* (Say), to explore regional variations in spectral sensitivity. Figure 10 shows measurements from dorsal (D), equatorial (M), and ventral (V) regions. The measuring beam was the same for all three experiments. Whereas the ventral function is the familiar blue-sensitive function similar to that of *Eristalis*, the dorsal and equatorial functions are much higher at long wavelengths than expected. The shape

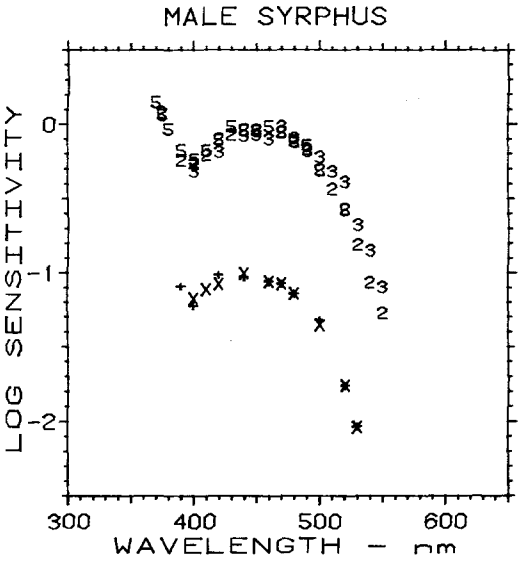


Fig. 9. Male *Syrphus:* pupillary action spectra from single types of photoreceptors. Symbol 2, from R2 only; symbol 3, from R3 only; symbol 5, from R5 only. All measured at elevation of −20°. Symbol ×, whole trapezoid; symbol +, R1 plus R6 only. These two functions were measured at elevation of −30°, and are plotted with a vertical shift of one log-unit to separate them from the other set of three functions

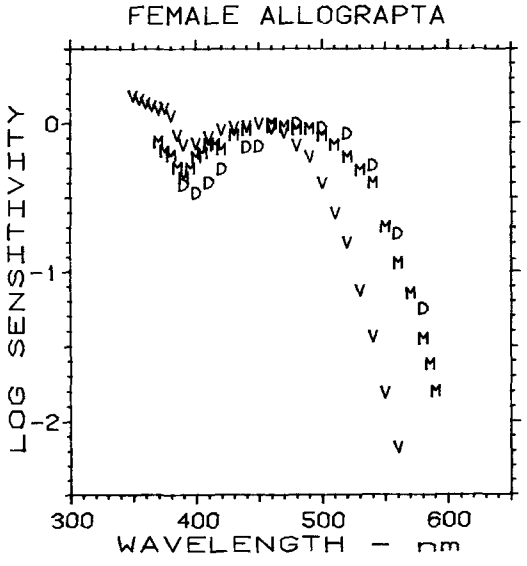


Fig. 10. Female *Allograpta*: pupillary action spectra from different regions of the same eye. Symbol V, ventral pole; symbol M, equatorial region; symbol D, dorsal pole

of the dorsal function is similar to that of *Calliphora*, while the equatorial function is broader, with a depressed UV peak. The long-wavelength, log-linear limb of the dorsal curve is shifted by 50 nm from that of the ventral curve.

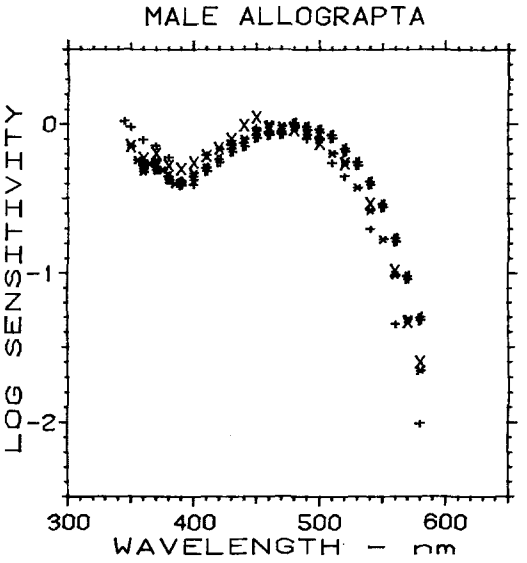


Fig. 11. Male *Allograpta:* pupillary action spectra from different regions of the same eye. Several regions of the eye that view different elevation angles, in a vertical plane of constant 15° azimuthal angle, were measured. Symbol ♯, 5° above the equator; symbol ×, 5° below; symbol *, 10° below; and symbol +, 45° below the equator

Four experiments were performed on one male *A. obliqua*, to explore the possibility that there is a sudden change in spectral sensitivity across the eye's equator. Sensitivity functions were measured at the following elevations in the vertical plane that makes a fifteen degree angle with the body's axis: +15°, +5°, −5°, −10°, and −45°, where 0° elevation corresponds to the equator. The functions shown in Fig. 11 are similar to the equatorial function of the female (M in Fig. 10). The functions for the dorsal region are only slightly shifted from those of the ventral region.

Additional experiments were performed at +5° and +15° to explore the possibility that changes in state of pupillary adaptation are responsible for the differences in spectral sensitivity functions. The measuring beam that was used to determine the function at +5° elevation elicited only a 4% pupillary response at onset. After having measured the function shown as ♯ in Fig. 11, the measuring beam was made brighter by replacing the RG610 filter with OG590+density 1.26, which elicited a 12% pupillary response at onset. After allowing the pupil to stabilize, sensitivity was measured at 400 nm, 420, 460, 500, 520, and 560 nm. The result of this experiment was no detectable change in sensitivity function.

Then the eye was rotated to +15° elevation, and sensitivity was measured with two different measuring beams: a) with filter RG610, flash duration 7 s, and

criterion 2.5%, sensitivity was determined at 470 nm, 490, 510, 520, 530, and 550 nm. The shape was identical to that at +5° elevation; b) with filter RG610 + density 0.29, flash duration 4 s, and criterion 2.5%, sensitivity was determined at 370 nm, 410, 430, 450, 470, 500, 510 and 530 nm. Again, the shape of the function was the same as that at +5° elevation.

Toxomerus

Near the end of the summer, having realized that Syrphidae may have males and females with differences in spectral sensitivity even though members of the same species, we sought to investigate this point further. Unfortunately, the season for *Allograpta* had ended. However, the smaller *Toxomerus marginatus* (Say) were still available. In mid July we had measured sensitivity of the ventral region of a male's eye. That function (Fig. 12) is the same as the ventral function for female *Allograpta*.

In September we collected several of both sexes and assayed the dorsal and ventral regions for differences in spectral sensitivity functions. The procedure was to obtain criterion responses at 430 nm and at 520 nm, and compare relative sensitivity to known spectral types. Results for both ventral and dorsal regions of male eyes, and from ventral regions of female eyes, were within 0.05 log-units of the value

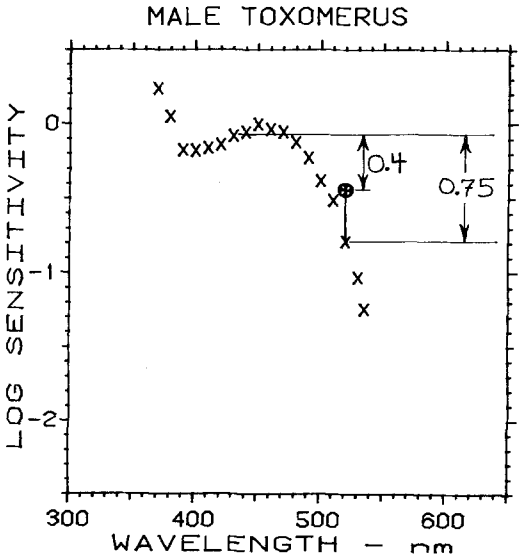


Fig. 12. *Toxomerus*: evidence for a sexual difference in spectral sensitivity. Symbol ×, action spectrum from the ventral region of a male eye. The sensitivity at 430 nm relative to 520 nm is 0.75 log-units for both dorsal and ventral regions of the male eye, and for the ventral region of the female eye. However, as marked by the cross, the ratio for the dorsal region of the female eye is only 0.4 log-units

0.75 log-units that is expected for the 450 nm type of receptor. However, measurements from the dorsal region of female eyes demonstrated much higher sensitivity at 520 nm, being only 0.40 log-units below that at 430 nm instead of 0.75 log-units.

To be sure that this difference was not a consequence of differences in state of adaptation, the comparison was repeated with six different measuring beams, containing first OG590 + density 1.26, then OG570 + density 0.29, then OG570, then OG550, then OG550 + density 0.29 and finally, RG610. In all cases the sensitivity ratio was 0.4 log-units.

Discussion

Spectral sensitivity functions for fly retinular cells R1–6 are known to have two peaks separated by a minimum at 400 nm. The peak in the UV is usually near 350 nm, while the other peak ranges from about 440 nm to 500 nm depending on the species. For wavelengths well beyond the latter peak, log-sensitivity decreases linearly with wavelength. Maximal sensitivity is usually in the UV, but this is a variable finding. The most thoroughly studied fly in these respects in the blowfly *Calliphora* and its white-eyed mutant.

Calliphora

Figure 4a compares our results for *C. vicinia* to those of Burkhardt (1962), Dörrscheidt-Käfer (1972), and McCann and Arnett (1972). Figure 4b compares our results for *C. vicinia* to results for its Australian counterpart *C. stygia*, obtained by Horridge and Mimura (1975) and by Hardie (1979).

The spectral sensitivity function for R1–6 of *Calliphora* has peaks near 350 nm and 495 nm. There are substantial differences among the results of these five electrophysiological papers, which we will discuss before comparing the results of the optical method.

The data of Burkhardt (1962, Fig. 6A) are for a single cell of a wild-type fly, illuminated with an extended source. The substantial elevation of values at 616 nm and 650 nm is caused by transparency of shielding pigments at long wavelengths. Subsequent investigations have alleviated this problem by working with either white-eyed mutants or light sources of small angular extent.

McCann and Arnett (1972, Fig. 5) present averaged data ($N=27$) for white-eyed mutants. Their curve is quite similar to Burkhardt's in the band 330–550 nm, with the exception of higher values near 380 nm and 400 nm.

Dörrscheidt-Käfer (1972, Abb. 2) presents an averaged function ($N=11$) for the white-eyed mutant "chalky". It is an unusually shaped curve, shifted to longer wavelengths compared to results of former workers.

The results of our optical method applied to a wild-type female are shown with symbol o in Fig. 4a. The comparison to the electrophysiological results is good except for a somewhat higher UV peak.

The paper of Horridge and Mimura (1975) is a valuable contribution because it presents a series of six spectral sensitivity functions for individual cells. The functions were obtained from wild-type *C. stygia* using a one-degree light source. Furthermore, these were the first experiments that controlled the photochemical state of a fly eye during intracellular measurements of spectral sensitivity. Unfortunately, Horridge and Mimura did not present an averaged function, so we computed one from their Fig. 1 according to the following procedure: the six curves were converted to log-sensitivity versus wavelength, then a normalization constant for each curve was found by fitting it to the function of McCann and Arnett. Our Fig. 4b shows all six functions, plotted with symbols A through F, but lowered by one log-unit for clarity. A mean of the log sensitivity, and its standard deviation (S.D.). was computed at each wavelength. The values for the mean are plotted with symbol $\times$ at the top of Fig. 4b, with error bars corresponding to ± 1 S.D. Two other functions are also shown at the top of Fig. 4b. The symbol $*$ plots the averaged data ($N=6$) from Hardie (1979, Fig. 2) for dark-adapted wild-type. The symbol o is for our measurements of *C. vicinia*, also shown in Fig. 4a.

The averaged function from Horridge and Mimura's data is consistent with earlier electrophysiological results except for a higher UV peak, and aberrantly elevated values near 500 nm. Hardie's function is similar for wavelengths greater than 400 nm, but exhibits a substantially depressed UV peak which is significantly lower than found by other investigators. The difference in height of the UV peak is more than a factor of two when comparing Hardie (1979) to Horridge and Mimura (1975). This is difficult to understand considering that both investigations were conducted in the same laboratory.

We believe that our intracellular optical method is measuring the same spectral sensitivity function as the intracellular electrophysiological method. Examination of Fig. 4a and b shows that the largest differences are in the ultraviolet region, where our function is slightly higher than all others. We attribute this to our procedure for measuring UV quantum flux, in which wide-band stray light is excluded. Without such precautions the UV peak would drop by 0.2 log units, to a level equal to that of early electrophysiological results. It is possible that part of the variability in electrophysiological measurements of the UV peak might be a consequence of this type of systematic error.

Some of this variability must be physiological as well. The long wavelength portion of the spectral sensitivity function is determined by the absorption spectrum of the visual pigment rhodopsin, as follows from the good agreement between physiological and spectrophotometric data (for reviews, see Hamdorf, 1979; and Stavenga, 1979). The UV-peak is probably due to UV-absorption by both the rhodopsin and a photostable, sensitizing pigment that somehow harnesses its absorbed energy to produce isomerization of rhodopsin (Kirschfeld et al., 1977). Possibly a component of the variability in relative height of the UV-peak is a variability in the relative concentrations of sensitizing pigment and rhodopsin.

Drosophila

The technically difficult task of intracellularly measuring spectral sensitivity of *Drosophila* retinular cells was accomplished by Wu and Pak (1975; Fig. 8). Their averaged data from white-eyed mutants is shown in Fig. 5, plotted with symbol W.

Genetic dissection and electroretinography have been used to determine spectral sensitivity functions for R1–6 of *Drosophila*. The function for R1–6 of white-eyed, sevenless mutants, taken from Harris et al. (1975; Fig. 4), is shown in Fig. 5 with symbol $*$. A later estimate of the function for R1–6, from Stark et al. (1979; Fig. 2), is shown in Fig. 5 with symbol $+$.

Our results for a wild-type *Drosophila* of the Canton-S strain, which are plotted in Fig. 5 with symbol o, are quite similar to the electrophysiological results. Both methods show the UV peak to be substantially higher than the long wavelength peak.

Chlorops and Dimecoenia

The spectral sensitivity functions for R1–6 of these flies are shown in Fig. 6. Both are quite similar to our function for *Drosophila* which is plotted with dots in the same figure. Therefore, it is likely that the corneal irridescence of *Chlorops* and *Dimecoenia* does not significantly affect the shape of the spectral sensitivity functions of the underlying photoreceptors, but only affects the external appearance of the eyes.

For further discussions on this theme, see Bernard (1971), Bernard and Miller (1968), Goldsmith and Bernard (1974), Miller (1979), and Stavenga (1979a).

Eristalis

The drone fly *E. tenax* is exceptional among flies that have been studied with intracellular electrophysiology because R1–6 have peak sensitivity in the blue, near 450 nm, and are relatively insensitive at long wavelengths. Furthermore, the variability in the relative height of the UV peak is greater than for other flies.

Bishop (1974) found fifteen cells with a peak in the UV, and low sensitivity at longer wavelengths. He also found three photoreceptor cells with a peak in the neighbourhood of 450 nm, and low UV sensitivity. The sensitivity function for one of the latter units, taken from Bishop's Fig. 2c, is plotted in our Fig. 7 with symbol B.

Horridge et al. (1975) also found Bishop's 450 nm type of cell, but infrequently. They most often recorded from photoreceptor cells exhibiting a peak at 350 nm as well as 450 nm, but found considerable variation in relative height of the two peaks. In a subsequent, related publication Tsukahara and Horridge (1977) reported on a large number of intracellular measurements, finding double-peaked units in a continuous series of S(360)/S(450) ratios ranging from 0.35 to 2.0. One of their sensitivity functions, for dark-adapted "cell 2", is plotted in our Fig. 7 with symbol T. Tsukahara was able to hold this cell continuously for nine hours. Compared to Bishop's function, the long wavelength peak of Tsukahara and Horridge's function has a peculiar flattened, broadened appearance.

Our results from the dorsal region of the eye of a female *E. arbustorum* are plotted in Fig. 7 with symbol o. This function is essentially the same as Bishop's at wavelengths greater than 400 nm. Unfortunately, technical difficulties prevented our measuring UV sensitivity, so we can say nothing about the UV peak in this case.

Musca

The spectral sensitivity of the house fly has recently been measured with intracellular methods by Hardie (1979). His averaged data ($N=3$) from dark-adapted, white-eyed *M. domestica* are shown in Fig. 8 with symbol +. Earlier data from optomotor experiments of Eckert (1971) are shown in the same figure with symbol *. Our data from a dark-adapted, wild-type fly are plotted with symbol o. Our function is similar to Hardie's function for wavelengths greater than 400 nm, and similar to Eckert's function for shorter wavelengths.

Note that our curve for *Calliphora*, plotted with dots in Fig. 8, is the same as our curve for *Musca*, plotted with o. Hardie's data shown in his Fig. 2, lead to the same conclusion; his dark-adapted functions for the two species differ by no more than 0.1 log-unit. Yet, our functions and Hardie's do not agree. The UV peak is different by a factor of two, and the shape of the long wavelength peak has the same peculiar, flattened appearance as Tsukahara and Horridge's cell 2, discussed just above for *Eristalis*. Considering the foregoing comparisons made for *Calliphora*, we suppose that Hardie's data for both species are systematically low in the ultraviolet.

Syrphus

The purpose of experiments with the male *Syrphus* was to investigate the degree of homogeneity among cell types R1–6 (see Fig. 9). At $-30°$ elevation the function for R1–6 is identical to that for the entire trapezoid, and both are similar to the 450 nm function of *Eristalis*. Therefore, it appears that ommatidia in this region of the male eye contain only a single spectral type of R1–6.

At $-20°$ elevation the functions for R2, for R3, and for R5 are quite similar. However, the shape of the sensitivity function at this elevation is not well-fit by the 450 nm function, as if some of the eight or ten ommatidia contributing to the pupillary response contain a long-wavelength type of receptor.

Allograpta

The sensitivity function for the female *A. obliqua* is strikingly non-uniform over the eye, as shown in Fig. 10. Whereas the function from the ventral region is similar to the 450 nm function of *Eristalis*, that of the dorsal region is similar to the 495 nm function of *Calliphora*.

The function measured from the equatorial region (Fig. 10, symbol M) has a unique shape, as if it were the composite spectrum of a mixture of spectral types. Similarly, all of the sensitivity functions measured from the male *A. obliqua* appear to be composite spectra, as shown in Fig. 11. The male eye probably contains a spectral variety of receptors too.

Toxomerus

All of our results from male *T. marginatus* can be explained by the existence of a single spectral type of R1–6 over the entire eye having the typical, syrphid 450 nm function. This is not true for the female; the substantial elevation of S(520)/S(430) by 0.35 log-units

above the ratio expected for a 450 nm function argues for the presence of a long wavelength type of receptor in the female eye.

Alternative Hypotheses

We find that syrphid flies exhibit a diversity in the shape of the action spectrum of pupillary responses, and hypothesize that its basis is a diversity in sensitivity functions of individual receptors, as caused by a diversity of visual pigments. Several alternative hypotheses have been considered and dismissed.

The central cells, R7 and R8 might be contributing to the measured response, but are not. The brightest levels of illumination available in our apparatus are below threshold for contributions to optical scattering from the central cells.

Another reasonable hypothesis is that the action spectrum depends on the state of pupillary adaptation. Since the pupillary pigment granules preferentially absorb in the blue, a closed pupil should suppress sensitivity more at short wavelength than at long wavelength (Stavenga et al., 1973). Could our results be attributed to a single spectral type of receptor acting in concert with a pupillary filter? We think not, for the following reasons.

Our results for *Musca* show no measurable distortion when the adapting light is two log-units above pupillary threshold. The dual intensity-series for *Syrphus* (Fig. 3) exhibits univariance for responses as large as 26%. The sensitivity ratio S(520)/S(430) of the "composite function" from the dorsal region of female *Toxomerus* was essentially unaffected when measured with a variety of adapting lights. The intracellular recordings of Hardie (1979) did demonstrate a modest shift toward shorter wavelength upon light-adaptation which he attributed to the pupillary mechanism of the retinular cell. We specifically attempted to measure a spectral shift owing to pupillary adaptation, in several species. Within the range of intensities possible in our apparatus, the higher states of adaptation reduced the response to immeasurable values before a spectral shift was noticeable. We conclude, therefore, that pupillary distortions of spectral sensitivity functions were negligibly small for the regime of intensities and responses employed in our experiments. The question of whether or not the pupillary action spectrum is distorted at extreme states of light-adaptation remains unanswered.

The diversity of visual pigments in Syrphidae was recently confirmed directly with in vivo microspectrophotometry (Stavenga, 1979b). All regions of the eye of *Syrphus balteatus* females contain a visual pigment absorbing maximally around 450–460 nm, similar to the pigment encountered in *Eristalis* (Stavenga, 1976). However, this pigment is found only in the ventral part of the male eyes, whereas the dorsal part contains visual pigment absorbing maximally at 490 nm.

Conclusions

The most important conclusion from our experiments with *Calliphora, Drosophila, Eristalis,* and *Musca* is that the spectral sensitivity function of retinular cells R1-6, measured with the new optical method, are essentially the same as functions measured with intracellular electrophysiological methods.

The most important conclusions from experiments with syrphid flies is that R1-6 can have quite different spectral sensitivity functions in different regions of the same eye, and that there can be substantial differences between the two sexes of a given species. The variations range from the 450 nm function already known from *Eristalis* to one that is like the 495 nm function of *Calliphora.*

This study proves the suggestion of earlier reports (Bernard and Stavenga, 1977, 1978) that optical measurements of pupillary scattering are a useful tool for investigating the properties of visual receptors. The most important advantage of this optical method is that the completely intact animal can be studied non-invasively. It therefore offers the opportunity to study photoreceptors of a healthy animal for days, weeks, or even months.

We thank Dr. Chris Maier of the Connecticut Agricultural Experiment Station, for determination of the Syrphidae. This work was supported by grants EY01140 and EY00785 from the National Eye Institute, U.S.P.H.S., (to GDB), by the Connecticut Lions Eye Research Foundation (to GDB), and by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.), (to DGS).

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