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Light-dependent pigment migration in blowfly photoreceptors studied by in vivo CLSM

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Abstract

The light-dependent migration of pigment granules in the soma of fly photoreceptors has been studied in vivo with a fast confocal laser scanning microscope. Images as well as photometric measurements were obtained in the reflection and fluorescence modes. Measurements at the single cell level were performed by using water immersion. The illumination of dark adapted photoreceptors causes a rapid increase in reflectance due to the migration of light scattering pigment granules toward the rhabdomeres. In the steady-state, the reflection signal strongly fluctuates, indicating that the pigment granules undergo a very rapid fluctuating movements. A major part of the reflection signal is due to light back-scattered by the pigment granules and channeled through the light guiding rhabdomeres. The optical axes of the rhabdomeres can thus be directly traced and appear to be directed toward the optical centre of the corresponding facet lens. Simultaneous with the reflection increase, the fluorescence of the photoreceptors decreases, because the pigment granules accumulating near the rhabdomeres act as a light-controlling pupil. Broad-band, white light filtered by the predominantly blue absorbing pupil causes an increased fraction of the visual pigment in the rhodopsin state.

Keywords: Fly; Vision; Light control; Pupil mechanism

1. Introduction

Exner [1], now more than a century ago, used his ophthalmoscope to study the physiology of the compound eyes of insects and crustaceans [2], and thus analyzed the wide gamut of optical phenomena coined pseudopupils. In the eyes of dipterans, for example a housefly, he observed 'a rather large, poorly defined glowing spot with a much brighter spot standing out in the middle'. We now know that the central bright spot was due to the incident light reflected by small pigment granules, accumulated in the distal part of the photoreceptor cells. The assembly of photoreceptor pigment granules there controls the light flux in the rhabdomeres, i.e., it functions as a pupil mechanism. The first step to this insight was made by Kirschfeld [3], who found, by using an incident light reflection microscope, that illumination of the dark-adapted eye of a housefly induces an increase in reflection, specifically from the illuminated ommatidia. Together with Franceschini [4], he subsequently demonstrated the light-dependent movement of reflecting particles in the photoreceptors, e.g. by sectioning the retina and observing the cut-end of the photoreceptors. Since then several studies have assembled a substantial amount of data concerning the spectral properties and intensity dependence of the pupil mechanism in connection with physiological as well as thefunctional characteristics of fly photoreceptor cells [5–9]. Interestingly, as was already the case with Exner [1], the advance in this research greatly benefited from analyses of the optics of fly eyes, and vice versa. Especially crucial has been the recognition of the deep pseudopupil by Franceschini [5], which is the superimposed image of the distal part of the photoreceptor cells projected by the individual facet lenses at the level of the eye's centre of curvature.

Yet, since these early studies little knowledge has been gained on how the pigment migration occurs at the single cell level. The recent advent of the confocal laser scanning microscope (CLSM) has opened the possibility to optically section biological tissues, and thus we decided to explore the potential of the CLSM for investigating the fly compound eye and specifically its pupil mechanism. We show here that the CLSM enables us to monitor the dynamics of the photoreceptor pigment granules non-invasively and with considerable spatial and temporal detail. Furthermore, we find that the versatility of the CLSM in imaging, combined with the various modes of photometry, e.g. reflection and fluorescence, provides a valuable, modern tool for research on photoreceptors in vivo.

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2. Materials and methods

2.1. Preparation

Blowflies (*Calliphora vicina*), both wild type and mutant chalky, were reared under a 9:15 h dark:light regime. A live fly was put in a loosely fitting tube and then further immobilized by low temperature melting wax with the head glued to the thorax and the tube. The fly was mounted on a microscope slide, with the ventral part of the retina facing upwards.

2.2. CLSM

The CLSM is a normal Nikon Optiphot-2 microscope attached to NORAN-Odyssey laser scanning equipment. Epi-illumination is applied with an Argon-Krypton laser having three lines, at 488, 568 and 644 nm, respectively, allowing measurements of reflection and/or fluorescence (as well as transmission) of the object. X-scanning of the object occurs by an acoustic optical deflector and Y-scanning by a galvanometric mirror. The machine's maximal scan rate is 240 frames s^{-1}. With this frame rate both reflection and autofluorescence images of the blowfly compound eye were rather noisy, even with full laser power, and therefore the images were integrated over time, usually at 30 frames s^{-1}, which appeared to be more than adequate for monitoring the highly fluctuating pupillary reflectance.

2.3. Fly compound eye optics

The blowfly compound eye is a quasi-crystalline array of structurally similar units, the ommatidia, arranged spherically. We investigated the ventral part of the eye. There the back focal distance of the individual facet lenses is about 70 μm [10], whilst the eye radius is about 700 μm. The image seen at the level of the centre of curvature of the eye therefore represents a plane close to the distal ends of the photoreceptors because these coincide with the back focal plane of the facet lens [11]. Superposition of the images of several ommatidia yields the deep pseudopupil (DPP; [5,6]); the number of the participating ommatidia depends on the interommatidial angle and the numerical aperture of the objective. The aperture of the Spindler and Hoyer objective 4, 0.10, used in Fig. 1, embraces *ca* 25 ommatidia. Water immersion largely abolishes the optical power of the fly facet lenses (optical neutralization: [5]). This allows the observation of the separate sets of rhabdomeres in individual ommatidia with antidromic (reverse) illumination [5,12] or alternatively by applying epi-fluorescence [13]. However, with orthodromic (normally incident) illumination the low reflection of the pigment granules is generally swamped by the reflections on the lens surfaces (see however [7]). This difficulty is most conveniently removed in the CLSM. The water immersion objective used was a Leitz SW25, 0.6.

3. Results

3.1. The blowfly pupil mechanism

Illumination of a dark-adapted, wild type blowfly eye with bright green light (568 nm) causes rapid changes in the observed images. Focusing the CLSM at the level of the cornea yields images similar to those observed with a normal epi-illumination microscope (Fig. 1(a) and (b)). In the dark-adapted state (Fig. 1(a)) only the reflection at the front surface of the facet lenses is seen, but upon light adaptation the reflection increases in a limited number of ommatidia (Fig. 1(b)). The corneal reflections obscure the light-induced changes and hence it is preferable to focus at the centre of curvature of the eye, i.e. at the DPP. There the corneal reflections are already greatly reduced in a normal epi-illumination microscope, but in the CLSM they are effectively removed (Fig. 1(c) and (d)). What remains in the DPP are the superimposed reflections from the distal planes of the retinal photoreceptors. The summed reflection is very weak in the dark adapted state (Fig. 1(c)), but it increases strongly upon light adaptation (Fig. 1(d)), due to pigment migration specifically in the photoreceptors R1-6 (e.g. [6]).

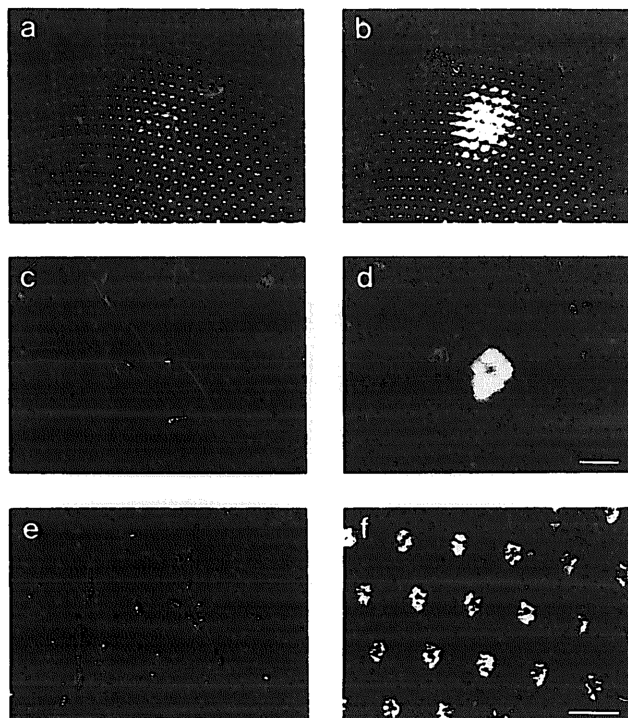

Fig. 1. The blowfly compound eye under green (568 nm) epi-illumination observed with the CLSM in the dark-adapted (a,c,e) and light-adapted state (b,d,f), at the level of the cornea (a,b), deep pseudopupil (DPP; c,d), and photoreceptor tips (e,f), respectively. a) The reflections seen in the dark-adapted state are virtually exclusively from the corneal facet lenses. b) Light adaptation causes an increased reflection from those facet lenses whose photoreceptor cells have visual fields within the aperture of the microscope objective. Background reflections are negligible in the DPP. Scalebars: (a)–(d) 100 μm, (e), (f) 20 μm.

3.2. Reflection by the pupillary granules observed at the single cell level

Because the DPP is a superposition of images, created by several facet lenses from their underlying retina, the events occurring in single photoreceptor cells become integrated there. The CLSM offers a quite convenient possibility to study events in individual cells. As shown in Fig. 1(e) and (f), observation of the eye of a wild type blowfly with the CLSM in the reflection mode using a water immersion objective reveals a lattice of patterns of spots. When the objective is focused ca. 80 μm beneath the corneal surface, i.e. near the distal ends of the photoreceptors, the spots resemble the classical trapezoidal fly rhabdomere pattern. Initially, upon illumination of a dark adapted eye (Fig. 1(e)), the patterns are virtually invisible but within tenths of a second the spots start to twinkle with rapidly increasing intensity. Quite dramatically, in the light-adapted state (Fig. 1(f)), which is reached after several seconds, the intensity of the individual spots continues to fluctuate greatly. The persistent fluctuations in the light-adapted state are demonstrated by Fig. 2(a)–(c), which is a series of three frames separated by a time interval of 0.3 s (each frame takes 1/30 s). The fluctuations in the reflection are reduced by temporal averaging over several seconds (Fig. 2(d): average of 256 frames, i.e. over 8.5 s); note that the fluctuations are also more or less cancelled by spatial averaging over several ommatidia, as is realized in the deep pseudopupil (Fig. 1(c) and (d)).

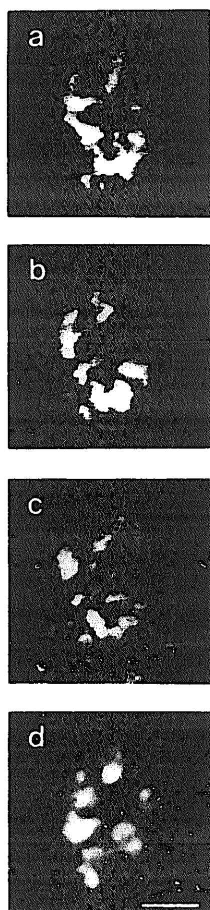

Fig. 2. Reflection from a single ommatidium. The images (a)–(c) were sampled each in 33 ms with interval 300 ms. The image (d) is an average of 256 of those images and hence is sampled during 8.5 s, thus making the rhabdomere pattern recognizable. The images (a)–(c) demonstrate the strong fluctuations in the reflection from the individual photoreceptor cells. Scalebar: 5 μm.

3.3. Axial direction of photoreceptors

It is extremely cumbersome to obtain images like those of Fig. 1(e) and (f) with a normal, epi-illumination microscope from the blowfly. Reflections from layers outside the focal plane then contribute to the measured optical signal, seriously degrading the visibility. As we already emphasized above, a distinct, intrinsic property of the CLSM is that the reflection from the out of the focus layers is effectively removed. An obvious interpretation of the spots and their intensity fluctuations is then that the spots are due to the light scattering (reflecting) pigment granules of individual photoreceptors and that the fluctuations result from the granules rapidly moving in and out of the image plane. Because an image represents a thin optical slice of the object, it should thus in principle be straightforward to exploit the CLSM for estimating the distribution of the reflecting pigment granules throughout the photoreceptor and thus for assessing the extent of their contribution to the total reflection that is measured with a conventional light microscope.

The series of reflection images of Fig. 3(a)–(c) shows images at three levels. Fig. 3(b) is at the back focal plane (same level as in Figs 1(e) and (f)) whilst Fig. 3(a) is at 20 μm above and Fig. 3(c) 20 μm below this level. Averaging in the light-adapted state was done during 3 s. Going from distal to proximal, the patterns appear to spread out and fade. Useful images could be obtained only over a limited depth range. A very similar phenomenon is observed when applying antidromic illumination with a normal light microscope with an immersion objective. Light that has propagated through the rhabdomeres is radiated in wave patterns that seem to coalesce near the back nodal plane; the distance between the individual beams increases when focusing proximally (see [14]), in agreement with the anatomical observations that the distance between photoreceptor rhabdomeres increases from the distal tips toward proximally (e.g. [15,16]). Furthermore, the diameter of the beams appears to be minimal at or near the plane of the rhabdomere tips, and the beam widths increase when focusing up or down.

The clear correspondence between the convential light-microscopic observations with those in the CLSM suggested that the light patterns seen in Fig. 3(a)–(c) are actually radiation patterns of the rhabdomeres, rather than direct reflections on (clusters of) pigment granules. To test this hypothesis we used the identical optical situation of Fig. 3(a)–(c) and investigated the blue-induced red fluorescence in the eye of the pigment-less mutant chalky. The fluorescence is emitted by metarhodopsin molecules existing in the

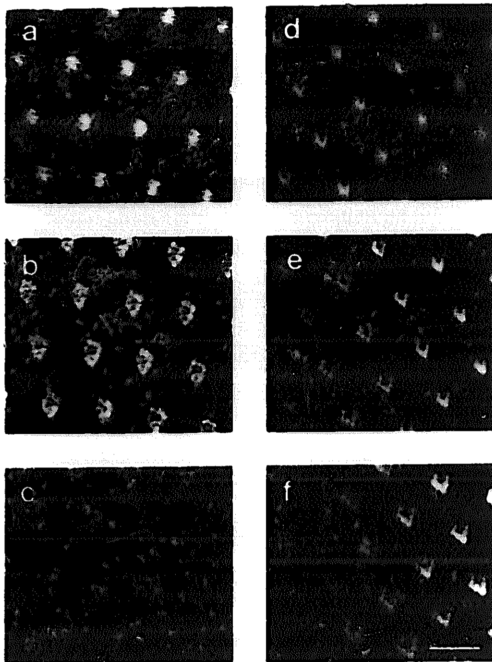

Fig. 3. (a)–(c) Reflection images (568 nm) observed in the eye of a wild type blowfly 20 μm distal of the rhabdomere tips (a), at the level of the tips (b) and 20 μm proximal (c) with water immersion (objective Leitz 25SW, 0.6). The images demonstrate that light beams radiated from the rhabdomeres fuse together at a distal level, near the back nodal plane of the facet lens. (d)–(f) Fluorescence images (excitation 568 nm, emission >660 nm) observed in a white-eyed mutant (chalky) at the same levels as in (a)–(c). Scalebar: 20 μm.

rhabdomeres. The emission beams behave exactly like the reflection beams in the wild type: the intensity of the emission beams is substantial at distal levels well above the rhabdomere tips, and furthermore the beams come together near the facet lens and diverge when focusing proximally.

Evidently we have to conclude here that the reflection of the photoreceptor pigment granules is at least in part channeled through the lightguiding rhabdomeres, giving rise to radiation patterns that have considerable intensity at distal levels well above that of the pigment granules in the photoreceptor soma. Clearly, the power of the CLSM to make thin optical slices is much reduced when the radiation (reflection and/or fluorescence) is directional.

3.4. Photometry

Classically, the dynamics of the pupil mechanism is studied by reflection and/or transmission measurements with an epi-illumination microscope from the deep pseudopupil (DPP; see [5–8]). In agreement with the previous studies [5–9], the photometric evaluation of the reflection time course of the DPP with the CLSM shows that the pupil's time constant is in the order of a few seconds (Fig. 4(a)), demonstrating the rapidity in the migration of the pigment granules in the photoreceptor cells.

An attractive, alternative way for studying the pupil mechanism is via fluorescence [17]. Measuring the green-induced

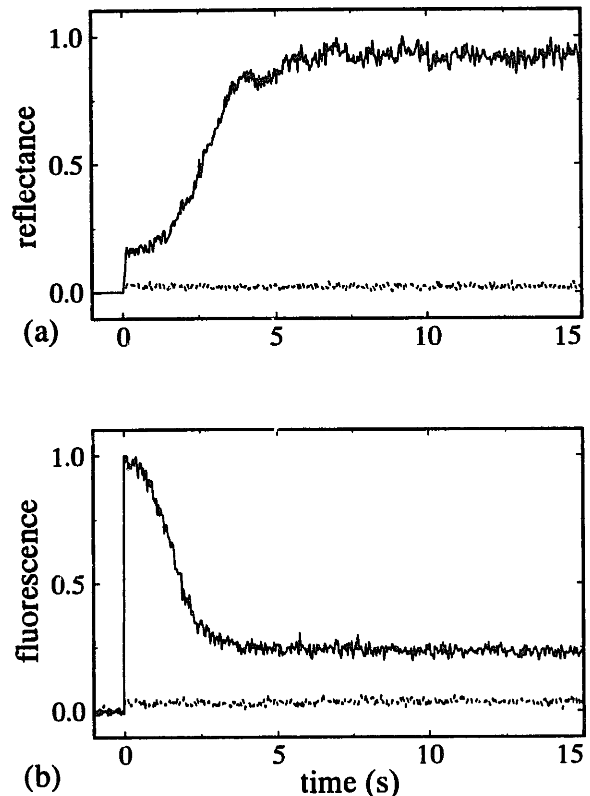

Fig. 4. Photometry of the deep pseudopupil (see Fig. 1(c) and (d)). a) The DPP of a wild type fly was sampled every 33 ms, yielding the reflection time course of light adaptation after 1 min of dark adaptation. b) The illuminating green (568 nm) laser light not only causes reflection but also induces fluorescence. The red emission, predominantly emerging from metarhodopsin in the rhabdomeres, was measured via a long pass barrier filter >660 nm.

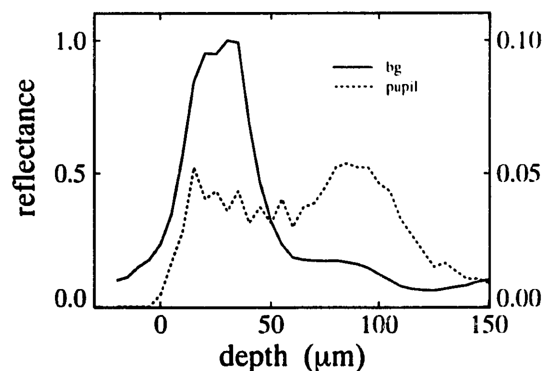

Fig. 5. Integral reflection measured at various levels from about 50 ommatidia in the wild type blowfly eye (water immersion). The continuous curve represents the reflection measured in the dark adapted state, being virtually exclusively background (bg) reflection, due to incomplete neutralization of the corneal reflection, together with reflection from the pigments in the screening pigment cells. The interrupted curve is the reflection increase occurring upon light adaptation, due to accumulation of photoreceptor pigment granules near the rhabdomeres (pupil).

red emission of the DPP in a wild type fly yields a similar rapid time course; however, now a decrease in fluorescence is observed (Fig. 4(b)). The reason is that the intensity of the green excitation light propagating in the rhabdomeres is suppressed by the absorbing pupillary pigment granules. The

red emission, originating from the metarhodopsin molecules in the rhabdomeres, is hence initially high but decreases due to the closure of the absorbing pupil.

In order to substantiate the observations in Fig. 3 with quantitative photometry we performed reflection measurements with water immersion at different depths. The eye was first dark adapted during 1 min and then the integral reflection of *ca* 50 ommatidia was measured during the subsequent 30 s of light adaptation. The reflection in the initial, dark-adapted state as well as the increase due to light-adaptation was evaluated (Fig. 5). The reflection by the screening pigments in the pigment cells is maximal around 30 μm proximally to the facet lens, whereas the light-induced reflection increase, due to the accumulating photoreceptor pigment granules, peaks near the facet lens' focal plane; yet the reflection increase is substantial up to near the facet lens.

4. Discussion

Classical light microspectrophoto(-fluoro)metry has proven that the DPP is a convenient and sensitive tool in the study of insect pupil mechanisms (e.g. [7,8]). Because the background reflections, mostly due to the facet lenses, are virtually completely removed in the CLSM, this sensitivity is substantially improved (Fig. 4). Similarly, fluorescence measurements of, for example, the visual pigments in the photoreceptors are facilitated by the CLSM because of reduced background fluorescence [18].

The reflection by the photoreceptor pigment granules is very low in the dark adapted state, but it increases strongly upon light adaptation. Clearly, initially these pigment granules are withdrawn toward the cell periphery [4], behind the diaphragm that the primary pigment cells form in front of the photoreceptors [7]. Upon light adaptation the granules are driven toward the rhabdomere. This occurs with considerable agitation, reflected by strongly flickering spots. The pigment granules are evidently in a highly dynamic equilibrium, continuously subjected to pushing and pulling forces, i.e. driving them toward and away from the rhabdomeres, respectively. Whilst in the dark the latter forces hold the granules back, in the light adapted state the former forces are prevailing [19,20].

How are these forces differentially modulated? The pigment migration in the photoreceptor cells is believed to be dependent on the influx of calcium [21], and therefore we can hypothesize that the local intracellular calcium concentration is the agent that modulates the strength of the two opposing forces. The scintillating reflections perhaps indicate that the local calcium concentration rapidly fluctuates. Alternatively, the forces acting at the pigment granules are stochastic due to a limited number of contractile protein molecules. We note that Wilcox and Franceschini [22] found that colchicine causes a complete standstill of the pigment granules, so ending the fluctuating reflections. Presumably, therefore, microtubules are involved in the pigment migration machinery. We note that a fluctuation analysis of the reflection time course will yield valuable information about the movements of the pigment granules.

With a normal light microscopy study of the dynamics of the pigment migration at the single cell level and hence of the forces involved is difficult, but with the CLSM this is greatly facilitated and thus the CLSM opens up new challenges. The highly fluctuating position of the granules may cause a distinct fluctuation in the transmitted light flux that is transduced into a visual signal and hence may increase the receptor noise. However, intracellular recordings [23] demonstrated that the pupil improves the photoreceptor signal:noise ratio at least at very high intensities.

The present results (Fig. 5) demonstrate that even in the CLSM the pupillary effect is well measurable at levels distinctly above the photoreceptor somas. Obviously, this behaviour is due to the complex optics in the combination of the rhabdomere and the aperture formed by the pigment cells when investigated by CLSM. It follows also from Fig. 5 that little reflection is contributed from levels more than some tens of μm below the rhabdomere tips. This will be partly due to the decrease in the illumination efficiency, due to vignetting by the primary pigment cells. But the pigment migration clearly occurs predominantly in the very distal part of the photoreceptor cell as is strongly suggested by the close correspondence of the effective optical density of the pupil and the reduction of the receptor sensitivity measured electrophysiologically [24].

The distal location in the photoreceptor of the pupillary granules has important consequences both for the efficiency of controlling the light flux and for the photochemistry of the visual pigment. Since the pupil absorbance spectrum peaks in the blue [25], a white light beam, inducing closure of the pupil, is thus effectively changed by the pupil into a yellow light stimulus. This has a direct effect on the population of the visual pigment molecules, i.e. whether they exist in the native rhodopsin state, which absorbs mainly in the blue-green, or in the metarhodopsin state, absorbing mainly in the orange wavelength range [26,27]. As demonstrated experimentally [28], the rhodopsin-metarhodopsin ratio resulting with bright white light and with an open pupil is distinctly modified upon the pupil closure. The effect of the closing pupil on the visual pigment composition, i.e. the decrease in the metarhodopsin fraction occurring when the pupil closes, can now be easily calculated by using the template curves for visual pigments [29], taking into account the contribution of the ultraviolet-absorbing sensitizing pigment (Fig. 6(a); [30,31]), and the pupil absorbance spectrum [32]. Fig. 6(b) shows that a pupil with peak absorbance of three log units reduces the metarhodopsin fraction from ca. 33% to less than 10%. The red-leaky screening pigment cells further help to reduce the metarhodopsin fraction in essentially the same fashion [9,24]. The two long-pass pigment filters clearly work in concert, but which is most dominant is presently unclear. At any rate, the resulting increase of rhodopsin implies an enhanced sensitivity. However, enhanced sensitiv-

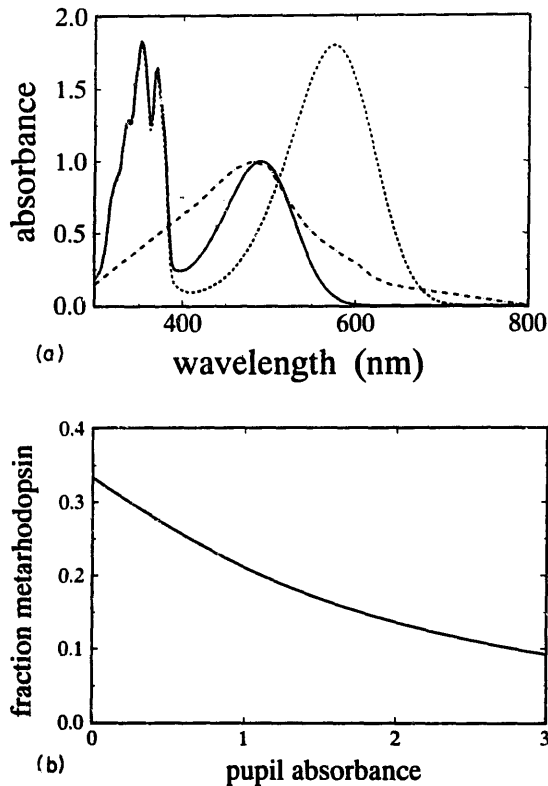

Fig. 6. a) Absorbance spectra of fly rhodopsin (continuous curve) and metarhodopsin (dotted curve) sensitized by the ultraviolet absorbing sensitizing pigment (based on data from [30–32]) together with the pupil absorbance spectrum (from [1]). b) Metarhodopsin fraction as a function of peak absorbance of the blowfly pupil when blowfly visual pigment is illuminated by white (day)light.

ity in the order of a few percent does not make much sense when in comparison, the light flux is diminished by a few log units. Apparently it is beneficial to reduce metarhodopsin as much as possible. In most eyes this is done by enzymatic degradation of the metarhodopsins. Flies clearly utilize their photostable, screening pigments, and specifically the pupil in the photoreceptor cells, for a more economic removal of metarhodopsin molecules.

Acknowledgements

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