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SYMPOSIUM REVIEW

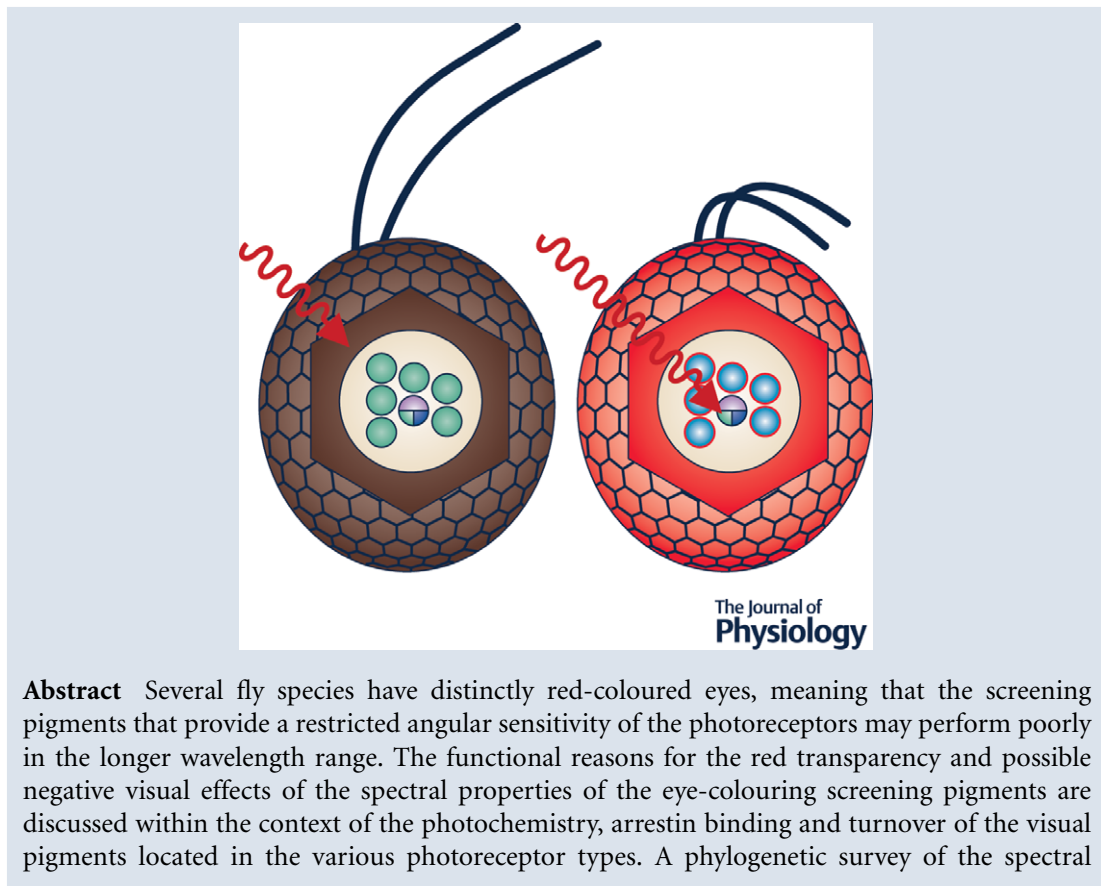
Functional interplay of visual, sensitizing and screening pigments in the eyes of *Drosophila* and other red-eyed dipteran flies

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Abstract Several fly species have distinctly red-coloured eyes, meaning that the screening pigments that provide a restricted angular sensitivity of the photoreceptors may perform poorly in the longer wavelength range. The functional reasons for the red transparency and possible negative visual effects of the spectral properties of the eye-colouring screening pigments are discussed within the context of the photochemistry, arrestin binding and turnover of the visual pigments located in the various photoreceptor types. A phylogenetic survey of the spectral

Doekele Stavenga (left), emeritus professor in Biophysics at the University of Groningen, the Netherlands, studies insect vision using optical methods. Closely related to vision, he also investigates the optics of flowers and animal coloration, of butterfly wing scales, bird feathers, beetle elytra and spider scales, applying microspectrophotometry, a newly developed imaging scatterometer and optical modelling. He enjoys a long-term collaboration with the team of Kentaro Arikawa (Japan). **Ric Wehling** is an amateur entomologist associated with Eglin Air Force Base, Florida. **Gregor Belušič** (right) is an assistant professor of Animal Physiology at the University of Ljubljana, Slovenia. When not teaching, he studies the detection of colour and polarization in the insect retina with electrophysiological recordings and anatomical methods.



properties of the main photoreceptors of the Diptera indicates that the transition of the brown eye colour of the Nematocera and lower Brachycera to a much redder eye colour of the higher Brachycera occurred around the emergence of the Tabanidae family.

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Abstract figure legend Functional interplay of visual and screening pigments in the eyes of long-horned flies (left; Diptera: Nematocera) and short-horned flies (right; Diptera: Brachycera). The seven circles in the central enlarged facet indicate the rhabdomeres, where the six peripheral rhabdomeres contain green-sensitive rhodopsin (Nematocera) with a blue-shifted metarhodopsin isoform (blue circle around the green dot), or blue-sensitive rhodopsin (Brachycera) with a red-shifted metarhodopsin isoform (red circle around the blue dot). The seventh, central rhabdomere (violet, green and blue spot) contains UV-, blue- and green-sensitive rhodopsins with the corresponding blue- or green-sensitive metarhodopsins. The photoreceptor somas contain yellow screening pigment. The dark brown screening pigment (Nematocera) or red screening pigment (Brachycera) in the pigment cells optically isolate the ommatidia and produce the eye colour. The nematoceran dark brown pigment absorbs all UV and visible wavelengths, while the brachyceran red pigment allows red stray light (red arrow) to re-isomerize the metarhodopsin into rhodopsin, thus restoring the peripheral photoreceptors' sensitivity. The red stray light may cause reduction of the angular and contrast sensitivity of the green-sensitive central photoreceptor.

Abbreviations A, arrestin; ERG, electroretinography; k_b , arrestin binding constant; k_f , rate constant of the enzymatic degradation; k_g , rate constant of rebuilding active rhodopsin; k_M , rate constant for photoconversion of M_a and M_i ; k_R , rate constant for photoconversion of R_a and R_i ; I , relative photon flux; M , metarhodopsin; M_a , active metarhodopsin; M_i , concentration of active metarhodopsin; M_i , inactive metarhodopsin; PDA, prolonged depolarizing afterpotential; R , rhodopsin; R_a , active rhodopsin; R_a , concentration of active rhodopsin; R_i , inactive rhodopsin; R_0 , number of visual pigment molecules in a microvillus; R_h , rhodopsin; R1–6, peripheral or outer photoreceptors; R7,8, central or inner photoreceptors; R7p, R8p, central photoreceptors in pale rhabdomere; R7y, R8y, central photoreceptors in yellow rhabdomere; UV, ultraviolet.

Introduction

Insect compound eyes analyse the light distribution in their environment with their photoreceptors, commonly packed in sets of eight to nine per ommatidium. In the apposition eyes of diurnal insects, the facet lens of each ommatidium focuses incident light onto the underlying photoreceptor cells, which transduce the absorbed light into an electrical signal, thus starting the visual process. To ensure that only approximately axially entering light reaches the photoreceptors, strongly light-absorbing screening pigment cells envelop the individual ommatidia, effectively blocking off-axis stray light. With their numerous ommatidia arranged in a more or less spherical shell, insect compound eyes thus sample almost their complete surroundings with good spatial resolution (Land & Nilsson, 2002). The spatial resolution principally depends on the size of the eye (and in turn of the animal), but can furthermore distinctly vary across the eye, especially in predatory insects. For instance, whereas the range of the interommatidial angles of the small fruitfly *Drosophila* is ~ 2.5 – 7 deg (Hardie, 1985), in the also rather small robber fly, *Holcocephala fusca*, this range is ~ 0.3 – 5 deg (Wardill *et al.* 2017).

In most insects, the screening pigments have a high density, yielding a blackish eye colour, thus forming perfect optical screens around the ommatidia; remarkably,

the screening pigments of red-eyed flies belonging to the dipteran suborder Brachycera are quite transparent in the long-wavelength range. Consequently, they do not perform very well in preventing red stray light from entering the photoreceptors, thereby potentially degrading spatial resolution. The screening pigments' poor absorption in the longer wavelength range is of historical interest, because in the early days, when spectral sensitivities were measured in fly eyes by electroretinography (ERG), a distinct red peak in the ERG of *Calliphora* blowflies was interpreted as representing a class of red-sensitive receptors, although its participation in colour discrimination could not be demonstrated (Autrum & Stumpf, 1953). Goldsmith later demonstrated, by measuring ERGs in wild-type and white-eyed mutant *Musca* houseflies, that the red receptor was an artefact due to long-wavelength stray light, leaking through the red-transparent screening pigment, thus stimulating large numbers of photoreceptors (Goldsmith, 1965). Subsequent intracellular recordings of blowfly photoreceptors confirmed this view (Streck, 1972). In the main visible wavelength range, wild-type photoreceptors were found to have angular sensitivities with an acceptance angle (i.e. the half-width of the Gaussian approximation to the angular sensitivity characteristic) of 2.8 deg. In the long-wavelength range, in which sensitivity is

comparatively low, the acceptance angle widened to 4 deg (at 625 nm), and the angular sensitivity function had a distinct tail, which extended to large off-axial values (Streck, 1972).

Consequently, red-eyed flies exposed to direct sunlight experience a considerable challenge. The acceptance angle of the photoreceptors is generally much larger than the sun's angular diameter so that photoreceptors facing the sun are saturated within milliseconds. In the rest of the eye, the light entering photoreceptors from the visual surroundings that they face is several log units less intense. The screening pigments must hence be very dense, to prevent light from the sun from overwhelming the signals coding other parts of image.

A most notable example of a distinctly red-eyed fly is the fruitfly *Drosophila*. Its red eyes must mean poor vision in the long-wavelength part of the visual spectrum, which raises the question why the red screening pigments have evolved. To understand the flies' screening pigment transparency and thus poor visual performance in the red we have to recall the organization of fly ommatidia (Hardie, 1985; Hardie & Postma, 2008). Each ommatidium contains a set of six so-called peripheral or outer photoreceptors, R1–6, and two central or inner photoreceptors, R7 and R8 (Fig. 1A and B). A photoreceptor's principal organelle is the rhabdomere, harbouring the visual pigment as well as the phototransduction apparatus. The rhabdomeres of the R1–6 cells extend the full length from the distal to the proximal retina, while the rhabdomeres of R7 and R8 cells form a tandem with the R7 rhabdomere sitting distal to the R8 rhabdomere. Two primary pigment cells surround the transparent pseudocone, located proximally of the facet lens, as well as the distal part of the photoreceptors. Secondary pigment cells surround the primary pigment cells and the photoreceptors, along the whole length of the ommatidia (Fig. 1A and B).

Screening pigment and pupil mechanism in fly eyes

The various cells contain different screening pigments, as can be demonstrated by epi-illumination light microscopy of a fly eye (Fig. 1C–E). A light-adapted eye observed at the level of the cornea displays reflections from the lattice of convex facet lenses together with a main brown-red eye colour and a central yellowish area (Fig. 1C). Focusing at the level of the eye's centre of curvature reveals the so-called deep pseudopupil (Franceschini, 1975; Franceschini & Kirschfeld, 1976). This shows within the main brown-red background colour, which is generated by reflections from pigment in the secondary pigment cells, a reddish ring, representing an image of the primary pigment cells (Fig. 1D and E). When dark-adapted, within the red ring a hint of yellow is seen (Fig. 1D), which upon prolonged illumination

intensifies and creates a yellow-coloured trapezoidal pattern, identical to the pattern of the R1–6 rhabdomeres (Fig. 1E). This phenomenon is due to yellow granules in the cell somata of the R1–6 photoreceptors, which migrate toward the rhabdomere when the photoreceptors are illuminated with bright light (Fig. 1A and B). The assembly of pigment granules functions in each photoreceptor as a dynamic screen, which reduces the light flux propagating in the visual pigment-containing rhabdomere, i.e. it acts in a similar way as the light-controlling pupil of vertebrate eyes; in the dark the granules migrate away from the rhabdomere (Kirschfeld & Franceschini, 1969; Franceschini & Kirschfeld, 1976; Stavenga, 1979; Satoh *et al.* 2008). The spectral sensitivity of the fly's pupil mechanism corresponds to that of the photoreceptors, meaning that light absorbed by the photoreceptor's visual pigment is the principal trigger activating the pigment migration (Bernard & Stavenga, 1979; Stavenga, 1995).

Fly visual pigment photochemistry, phototransduction and photoregeneration

Blowfly, housefly and fruitfly photoreceptors express a variety of visual pigments (Fig. 2A, Table 1). The R1–6 cells express a rhodopsin, Rh1, with main absorption band peaking at ~490 nm. Whereas the chromophore of human rhodopsin is retinal, the chromophore of fly rhodopsin is 3-hydroxy-retinal (Vogt & Kirschfeld, 1984; Vogt, 1989; Seki & Vogt, 1998). After photon absorption by the rhodopsin molecule, the 11-*cis* conformation of the chromophore changes into the all-*trans* form, which causes a transformation of the visual pigment's opsin, resulting in the metarhodopsin state. The peak wavelength of Rh1's metarhodopsin absorption spectrum is strongly bathochromic-shifted (i.e. toward longer wavelengths), to ~570 nm (Hamdorf, 1979; Hardie, 1985; Salcedo *et al.* 1999). The created metarhodopsin triggers the phototransduction process, but this occurs only temporarily, because the active metarhodopsin molecule is rapidly inactivated by binding an arrestin molecule, similar to what occurs in the phototransduction process of human photoreceptors (Dolph *et al.* 1993; Alloway & Dolph, 1999). Importantly, and very different from human metarhodopsin which rapidly decays by photolysis, fly metarhodopsin is long-term thermostable. The interesting consequence of the thermostability is that the all-*trans* chromophore re-isomerizes back into the 11-*cis* conformation when the metarhodopsin molecule absorbs a photon, which is then followed by refolding of the opsin moiety and restoration of the native rhodopsin state. Subsequently the bound arrestin dissociates and the visual pigment returns to its active state, i.e. is ready for another phototransduction round (Yau & Hardie, 2009).

This highlights a major advantage of the photochemistry of fly visual pigments over that of vertebrate

rhodopsins. To maintain sensitivity, human photoreceptors have to fully rely on a tortuous enzymatic process of metarhodopsin degradation, translation of the all-*trans* retinal to the pigment epithelium and glia (Muller cells), where the chromophore is re-isomerized and then shuttled back to the photoreceptor, where it couples to opsin to form rhodopsin again. The thermostability of fly metarhodopsin allows recovery of the native rhodopsin state by simply another photon absorption (Fig. 3).

A unique property of fly visual pigments is not only their 3-hydroxy-retinal chromophore, but also their strongly binding 3-hydroxy-retinol, the alcohol of the chromophore aldehyde, which has an absorption spectrum restricted to the ultraviolet. The latter acts as a sensitizing pigment (Fig. 2D), because the energy of the absorbed ultraviolet photons is transferred to the chromophore, which then isomerizes as usual, thereby considerably enhancing the chance of molecular transformation by UV light (Kirschfeld *et al.* 1983; Stavenga,

2004b). The intimate connection of the sensitizing pigment with the visual pigment molecule causes a fine-structured spectrum in the UV of both the rhodopsin and metarhodopsin (Fig. 2E).

The strong bathochromic-shifted absorption spectrum of the metarhodopsin with respect to the rhodopsin makes it understandable why flies have red-leaky screening pigment. The absorption spectrum of the screening pigments fully spans the wavelength range of the rhodopsin absorption spectrum, up to ~600 nm (Fig. 2D and E; fruitfly *Drosophila* and blowfly *Calliphora*), but not so well the range of the metarhodopsin absorption spectrum (Fig. 2D and E). Because long-wavelength light preferentially reconverts the metarhodopsin back to the native rhodopsin, red-stray light roaming in the eyes causes ready regeneration of the rhodopsin state, so restoring the pool of molecules that provides the photoreceptor cells with their light sensitivity (Stavenga *et al.* 1973; Stavenga, 1995; Stavenga & Hardie, 2011).

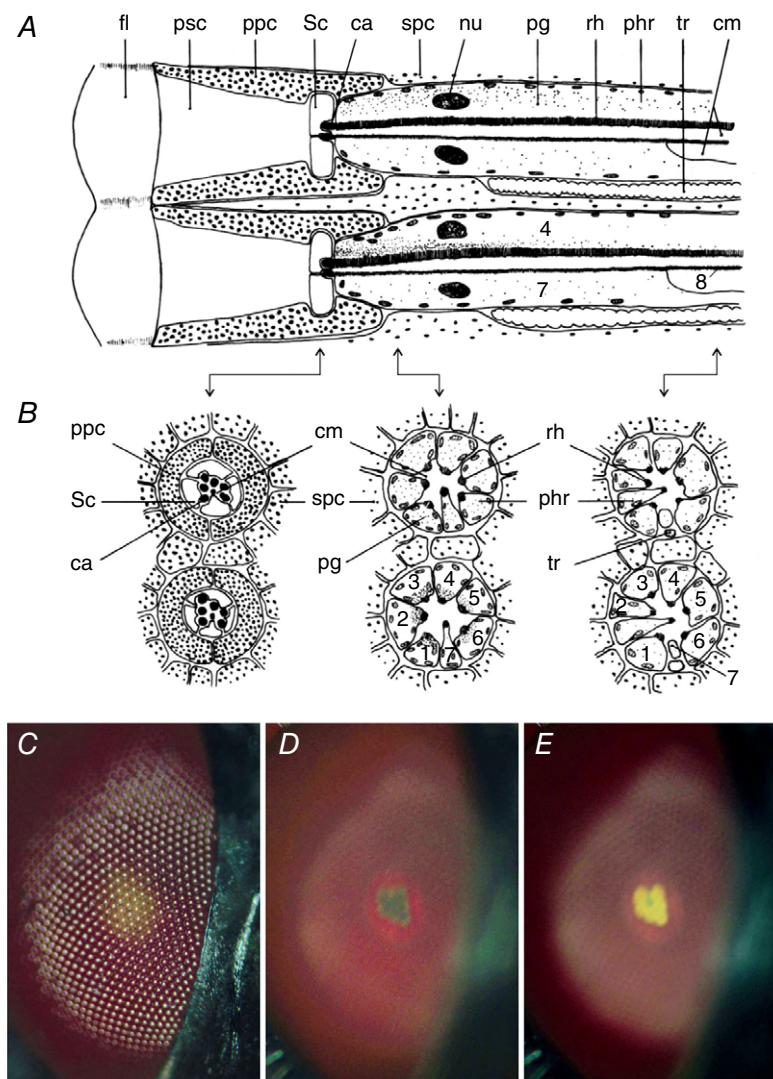


Figure 1. Fly ommatidia, screening pigments and pupil mechanism

A, diagram of two fly eye ommatidia (upper: dark adapted; lower: light adapted), with each a facet lens (fl), a pseudocone (psc), two primary pigment cells (ppc), four Semper cells (Sc), seven rhabdomere caps (ca), six secondary pigment cells (spc), eight photoreceptor cells (phr) having a rhabdomere (rh) and in the cell soma a nucleus (nu) and mobile pigment granules (pg), and furthermore there is a trachea (tr) and in between the photoreceptors a central matrix (cm). *B*, cross-sections of the diagram at locations indicated by the arrows. *C*, epi-illumination of a light-adapted, red-coloured fly eye (genus unidentified) with centrally yellowish facets seen at the level of the cornea. *D*, the eye observed at the level of the centre of curvature with the dark-adapted deep-pseudopupil. *E*, the light-adapted deep-pseudopupil.

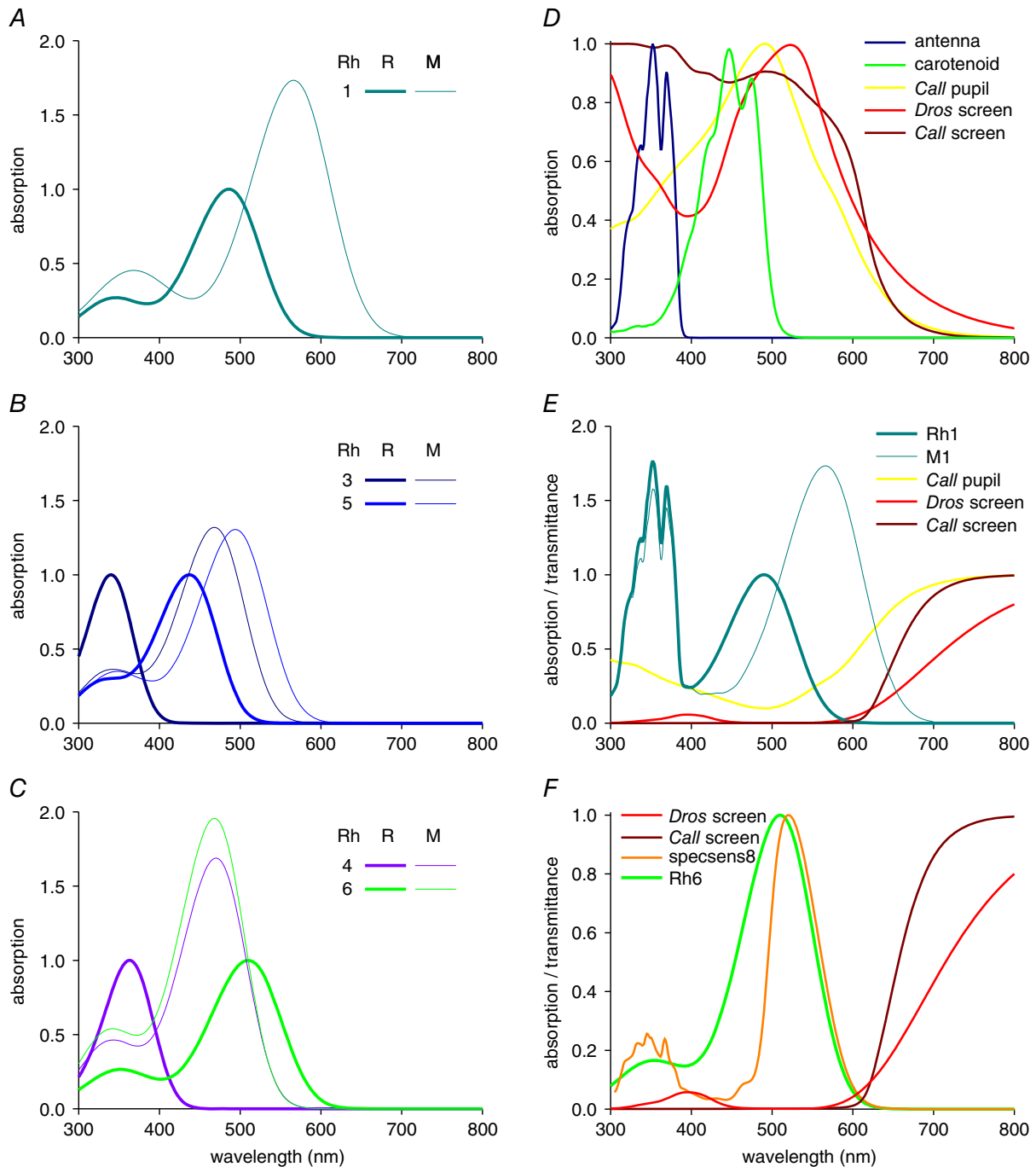


Figure 2. Pigments of a fly compound eye

A–C, absorption spectra of visual pigments Rh1 and Rh3–6, of their rhodopsin state R and their metarhodopsin state M, calculated with template formulae (Stavenga, 2010). D, absorption spectra of sensitizing (antenna) pigment, carotenoid, pupillary pigment of the blowfly *Calliphora* (Stavenga & Hardie, 2011), and the red screening pigments of *Drosophila* (Strother & Casella, 1972) and *Calliphora* (Stavenga & Hardie, 2011). E, Absorption spectra of the rhodopsin (Rh1) and metarhodopsin (M1) of blowfly R1–6 photoreceptors with bound sensitizing pigment, and transmittance spectra of the pupillary pigment of *Calliphora*, assuming a peak absorbance of 1 log unit, and the screening pigments of *Drosophila* and *Calliphora*, assuming a peak absorbance of 3 log units. F, the spectral sensitivity of the photoreceptor cell R8y and the absorption spectrum of its visual pigment Rh6, which is modified by the carotenoid filter in R7y and a UV-absorbing sensitizing pigment, and the transmittance spectra of the screening pigments of *Drosophila* and *Calliphora*.

Table 1. Visual pigment peak wavelengths of *Drosophila* and *Musca*

Rh	<i>Drosophila</i>			<i>Musca</i>
	R/M [1]	R [2]	R/M [3]	R [4]
1	486 / 566	478	486 / 566	490
3	331 / 468	345	340 / 468	335
4	355 / 470	375	363 / 470	430
5	442 / 494	437	437 / 494	460
6	515 / 468	508	510 / 468	520

Peak wavelengths (in nm) of the absorption spectra of the rhodopsin (R) and metarhodopsin (M) states of visual pigments Rh1, 3–6 according to references [1], Salcedo *et al.* (1999); [2], Yamaguchi *et al.* (2010); [3], those used in Fig. 2; and [4], the values for *Musca* (Hardie, 1985).

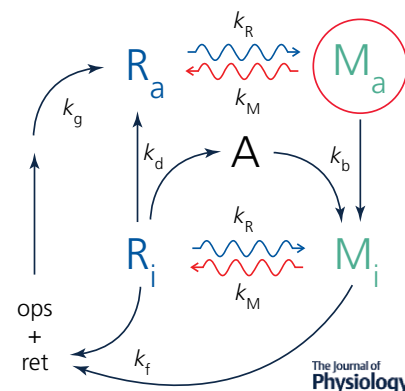
In fact, the pigment of the mobile, pupillary granules inside the photoreceptors plays a similar role. It absorbs mostly at short-wavelengths and thus a light-adapted, closed pupil favours metarhodopsin conversion (Stavenga *et al.* 1973; Stavenga, 1995; Stavenga & Hardie, 2011). Furthermore, it is interesting to note that whereas the red screening pigments in the primary and secondary pigment cells create a sharp long-pass filter, with little absorption only at those wavelengths where rhodopsin absorption is negligible, so to preserve a narrow angular sensitivity of the photoreceptors and thus optimal spatial resolution of the visual system, the pupil filter can be less fastidious. It plays at most a minor role in blocking stray light, since the pupillary granules are located within the photoreceptors where they affect the light flux propagating in the rhabdomere. Nevertheless, like the screening pigments, the pupil does affect the angular sensitivity, because when it closes, it decreases the photoreceptor acceptance angle, due to waveguide effects (Hardie, 1979; Smakman *et al.* 1984; Stavenga, 2004a).

Visual pigment cycle and turnover

Continuous light exposure of photoreceptors causes rhodopsin conversion, followed by visual pigment degradation and thus a decrease in light sensitivity, which is counteracted by a continuous cycle of restoration. As stated above, the latter occurs in vertebrates by an elaborate enzymatic process. Prolonged illumination of fly photoreceptors creates a photosteady state (also called photo-equilibrium), where the distribution of the visual pigment molecules over the two thermostable states, rhodopsin and metarhodopsin, critically depends on their respective absorption spectra as well as the spectral content of the illumination. For instance, bright blue light rapidly creates in the fly R1–6 photoreceptors a photosteady state with a high metarhodopsin content, while

red light results in a low metarhodopsin concentration (Stavenga *et al.* 1973; Hamdorf, 1979; Belušič *et al.* 2010). Nevertheless, visual pigment degradation also occurs in insect eyes. The presence of an enzymatic visual pigment cycle was demonstrated in *Calliphora* blowfly eyes (Schwemer, 1979). Strikingly, also this cycle involves both the photoreceptors and the pigment cells. First, the chromophore of the metarhodopsin molecules, all-*trans*-3-hydroxy-retinal, is liberated from the opsin part, which is further broken down. The all-*trans*-retinal is transported by retinal-binding protein to the pigment cells, where a photoisomerase assists in the reversion of the visual pigment chromophore by blue light to the 11-*cis* conformation. Retinoid-binding protein then translates it back to the photoreceptors where it is reinserted into native opsin, i.e. resulting in the biosynthesis of new rhodopsin (Schwemer, 1984, 1989). A similar visual pigment cycle exists also in *Drosophila* (Wang *et al.* 2010). Rhodopsin regeneration through an enzymatic cycle hence occurs universally. Vertebrate visual systems are different mainly because the metarhodopsins rapidly decay, while the metarhodopsins of invertebrates are much more thermostable (Yau & Hardie, 2009).

Curiously, the thermostability of invertebrate metarhodopsins and the possibility to regain the rhodopsin state by photoconversion has led some researchers of *Drosophila* photoreceptors to adhere to ‘the dogma that the visual cycle in invertebrates does not exist’ (Arshavsky, 2010; Wang *et al.* 2010). A possible reason for this erroneous belief may have been the

**Figure 3. Visual pigment cycle in fly photoreceptors**

Photoconversion of active rhodopsin (R_a) creates active metarhodopsin (M_a) which triggers the phototransduction process (red circle). Upon binding arrestin (A) it becomes inactive metarhodopsin (M_i). Photoconversion of M_i creates inactive rhodopsin (R_i), which upon arrestin release converts into the native rhodopsin (R_a). The light-induced conversion processes have rate constants k_R and k_M , and the rate constants of arrestin binding and dissociation are k_b and k_d . R_i and M_i are degraded with rate constant k_f , resulting in release of the chromophore (ret) from its opsin (ops), which then is further degraded. R_a is regenerated with rate constant k_g .

observation of an extremely long-lasting prolonged depolarizing afterpotential (PDA) that can be created in *Drosophila* photoreceptors (Wright & Cosens, 1977; Pak, 1995). The PDA phenomenon occurs generally in insect photoreceptors when a very bright flash converts an excessive amount of rhodopsin molecules, thus suddenly creating a large number of active metarhodopsin molecules that trigger the phototransduction process. Arresting all active metarhodopsins after the flash then takes some time, resulting in a prolonged afterpotential, the PDA. The PDA's duration strongly depends on the illumination conditions. In *Drosophila* photoreceptors, bright blue light results in an unabated PDA, because the number of available arrestins is less than the number of created metarhodopsin molecules, so that the surplus of active metarhodopsins continues to trigger the phototransduction process for many hours (Liu *et al.* 2008; Satoh *et al.* 2010). In blowflies, the time course of the PDA lasts at most a few minutes (Hamdorf, 1979), suggesting that here the photoreceptors contain a larger amount of arrestin molecules. Here, the time course of the PDA decay probably reflects translocation of arrestin molecules from the cytosol to the rhabdomere (Satoh *et al.* 2010; Stavenga & Hardie, 2011).

In early electrophysiological experiments on fruitfly eyes, distinct, long-lasting changes in the ERG responsivity were found when bright blue light converted a majority of rhodopsin to the metarhodopsin state (Cosens & Briscoe, 1972; Pak & Lidington, 1974; Wright & Cosens, 1977). These changes, intimately connected to the PDA elicited in the photoreceptors, had a duration of many hours, which until recently has been interpreted as an inability of photoreceptors of invertebrates, unlike the vertebrate photoreceptors, to regain the original sensitivity via enzymatic means (Arshavsky, 2010; Wang *et al.* 2010).

It is now clear that both photochemical and enzymatic pathways can govern the recovery of sensitivity through rhodopsin regeneration. The speed of enzymatic turnover can be very different between species. For instance, metarhodopsin breakdown occurs in butterflies within minutes and rhodopsin recovery within an hour, of course dependent on temperature (Bernard, 1983; Vanhoutte & Stavenga, 2005). However, in the principal R1–6 fly photoreceptors, metarhodopsin persists for several hours in the dark, which indicates that the enzymatic cycle has a low efficiency, presumably due to a low concentration of the metarhodopsin-degrading enzymes and/or of the other members of the enzymatic cycle. Photoreconversion of metarhodopsin via red stray light is apparently largely sufficient for maintenance of the rhodopsin content (Hardie & Postma, 2008).

Deleterious effects of red-transparent screening pigment on the R1–6 photoreceptors

An important point to consider now is that the stray light not only can photoreconvert metarhodopsin molecules, but can also be absorbed by rhodopsins, as demonstrated by the artefactually encountered red-receptor and the tail in the angular sensitivity functions of the R1–6 fly photoreceptors when tested with red light stimuli (Streck, 1972). Although the spectral overlap of the absorption spectrum of the R1–6 rhodopsin and the transmittance spectrum of the screening pigment is minor (Fig. 2E), red stray light will inevitably create a noisy background, degrading the photoreceptor's contrast signal, especially in red-eyed flies exposed to bright sunlight. This must have a negative effect on spatial resolution and signal quality, opposing the benefit of sensitivity gained by rhodopsin photoregeneration. Although it is generally assumed that the signal-degrading effect in normal environmental conditions must be negligible, it has not yet been unequivocally demonstrated. Flies are well known for their excellent visual capacities, and several studies have shown optimization of signal-to-noise in fly photoreceptors (e.g. Burton *et al.* 2001; Juusola & Hardie, 2001; Niven *et al.* 2007; Song *et al.* 2016). We may therefore expect that flies do not suffer from noise contamination and contrast loss of their visual signals by stray light, but explicit experimental studies demonstrating that indeed fly vision is improved by red-transparent screening pigments have not yet been undertaken.

If the function of the red screening pigments of flies is indeed to assist the photoconversion of metarhodopsin, it will only work when the metarhodopsin spectrum is bathochromically shifted and well separated from its rhodopsin spectrum. This is, however, only the case for rhodopsins with absorption peak wavelength below 500 nm (Stavenga, 1992, 1995). The main rhodopsins of virtually all insects apart from higher Diptera are green-absorbing, with peak wavelength above 500 nm (Table 2), and their metarhodopsins absorb maximally in the blue, i.e. have a hypsochromic-shifted (i.e. toward shorter wavelengths) absorption spectrum. Virtually all insect eyes therefore have very dense pigments with broad-wavelength absorption spectra, because red-transparent screening pigments will result in red stray light, preferentially converting the green rhodopsin instead of the blue-peaking metarhodopsin, thus severely reducing sensitivity (Stavenga & Hardie, 2011). This immediately raises another question, also needing further study, namely whether the red-transparency of the screening pigments of red-eyed flies acts beneficially for all photoreceptors. We will further discuss this below.

Table 2. Peak wavelengths (λ_{\max}) of the main photoreceptors and eye colour of Diptera

Family	Genus, species	λ_{\max} (nm)	SP	Sex	Region	Eye colour	Method	Ref
Culicidae	<i>Aedes aegypti</i>	523		F	D,V	B	E	1
Culicidae	<i>Aedes albopictus</i>	515	+	M	D	B	E	2
Psychodidae	<i>Lutzomyia longipalpis</i>	520		F		B	E	3
Psychodidae	<i>Lutzomyia longipalpis</i>	546		M		B	E	3
Simuliidae	<i>Simulium</i> sp.	430	+	M	D	B	I, M	4
Keroplatidae	<i>Arachnocampa luminosa</i>	540		M		B	E	5
Bibionidae	<i>Biblio</i> sp.	440	+	M	D	B	E	6
Bibionidae	<i>Biblio marci</i>	520		M	V	B	E	7
Bibionidae	<i>Biblio marci</i>	440		M	D	B	E	7
Bibionidae	<i>Biblio marci</i>	520		F	D,V	B	E	7
Tabanidae	<i>Tabanus bromius</i>	525		F	D,V	B	I	2
Tabanidae	<i>Tabanus bromius</i>	525		M	V	B	I	2
Tabanidae	<i>Tabanus bromius</i>	440	+	M	D	B	I	2
Tabanidae	<i>Haematopota</i> sp.	530				B		6
Stratiomyidae	<i>Hermetia illucens</i>	504		M,F	D	B	I	8
Stratiomyidae	<i>Hermetia illucens</i>	440	+	M,F	V	B	I	8
*		—						
Dolichopodidae	<i>Condylostylus japonicus</i>	480	+	M,F	D,V	B	E	9
Syrphidae	<i>Allograpta obliqua</i>	480		F,M	D	RB	P	10
Syrphidae	<i>Allograpta obliqua</i>	455		F	V	RB	P	10
Syrphidae	<i>Eristalis arbustorum</i>	450		F	D	RB	P	10
Syrphidae	<i>Eristalis tenax</i>	450	+	F, M		RB	I, P, M	11, 12
Syrphidae	<i>Syrphus balteatus</i>	450		M	V	RB	M	13
Syrphidae	<i>Syrphus balteatus</i>	490		M	D	RB	M	13
Syrphidae	<i>Syrphus balteatus</i>	450		F	D,V	RB	M	13
Syrphidae	<i>Syrphus</i> sp.	455		M	V	RB	P	10
Syrphidae	<i>Toxomerus marginatus</i>	450		M,F	V	RB	P	10
Glossinidae	<i>Glossina morsitans</i>	500	+			RB	I	14
Calliphoridae	<i>Lucilia (Phaenicia) sericata</i>	480	+		V	RB	I	15
Calliphoridae	<i>Calliphora vicina</i>	490	+	F, M		RB	E, I, P	10, 16
Muscidae	<i>Musca domestica</i>	490	+			RB	I	17
Anthomyiidae	<i>Delia radicum</i>	490				RB	E	18
Tephritidae	<i>Dacus oleae</i>	490				RB	E	19
Diopsidae	<i>Cyrtodiopsis dalmanni</i>	490	+			RB	E	20
Chloropidae	<i>Chlorops</i> sp.	480		F, M	E	RB	P	10
Drosophilidae	<i>Drosophila melanogaster</i>	480	+	F, M	V	R	P, M	10, 21
Ephydridae	<i>Dimecoenia spinose</i>	480		M	D	RB	P	10

SP, sensitizing pigment; +: presence. Sex: F, female; M, male. Region: D, dorsal; E, equatorial; V, ventral. Eye colour: B, black-brown; RB, red-brown; R, red. Method applied for determining the photoreceptor sensitivity spectra: E, electroretinography (ERG); I, intracellular recordings; P, pupillary recordings. For the visual pigments: M, microspectrophotometry (MSP). Ref, references: 1, Muir *et al.* 1992; 2, G. Belušič, in prep.; 3, Mellor *et al.* 1996; 4, Kirschfeld & Vogt, 1986; 5, Meyer-Rochow & Eguchi, 1984; 6, Kirschfeld, 1986; 7, Burkhardt & De la Motte, 1972; 8, Ooninx *et al.* 2016; 9, Stavenga *et al.* 2017; 10, Bernard & Stavenga, 1979; 11, Horridge *et al.* 1975; 12, Stavenga, 1976; 13, Stavenga, 1979; 14, Hardie *et al.* 1989; 15, McCann & Arnett, 1972; 16, Paul *et al.* 1986; 17, Hardie, 1986; 18, Brown & Anderson, 1996; 19, Remund *et al.* 1981; 20, Burkhardt, 1972; 21, Salcedo *et al.* 1999; The asterisk corresponds to the one in Fig. 5.

Visual and screening pigments of the central photoreceptors

Whereas the ommatidia of fly eyes are homogeneous concerning the R1–6 photoreceptors, which all express rhodopsin Rh1, absorbing maximally in the blue-green, at ~490 nm (Fig. 2A, Table 1), the ommatidia are heterogeneous concerning the properties of the R7

and R8 photoreceptors. As was demonstrated in the housefly *Musca*, in two-thirds of the ommatidia, the R7 rhabdomeres contain, in addition to their visual pigment, a blue-absorbing carotenoid pigment (Fig. 2D), causing a yellow colour of their rhabdomeres when observed in transmitted light (Kirschfeld *et al.* 1978); the central photoreceptors hence are called R7y and R8y. The other one-third of the ommatidia have pale central

rhabdomeres, and thus these photoreceptors are called R7p and R8p. R7p and R7y express the UV-absorbing Rh3 and Rh4, while R8p and R8y express the blue-absorbing Rh5 and blue-green-absorbing Rh6, respectively (Fig. 2B and C, Table 1).

The carotenoid in the γ -type ommatidia strongly modifies the spectral sensitivity of both the R7y and R8y photoreceptors. Notably the spectral sensitivity band of R8y is narrowed and its peak is shifted, from the blue-green maximum of its Rh6 visual pigment, at 520 nm, into the green, at 540 nm (Fig. 2F; Hardie, 1985). Quite curiously, however, the spectral sensitivity of the *Musca* R8y photoreceptors also has a fine-structure in the UV, demonstrating the additional action of a sensitizing pigment (Hardie & Kirschfeld, 1983). *Musca* and *Drosophila* have similar photoreceptor expression patterns, but the visual pigment absorption spectra and hence the photoreceptor spectral sensitivities differ slightly (Hardie, 1985; see Table 1). Because the R7y rhabdomeres of *Drosophila* also contain carotenoid pigment (Feiler *et al.* 1992), the green-sensitive R8y probably has a similar spectral sensitivity as shown in Fig. 2F, but due to the shorter rhabdomere length of *Drosophila* photoreceptors, the induced spectral shift may be less than that in *Musca*.

Red-screening pigment and the central photoreceptors

It must be considered quite worrisome that the spectral overlap of the absorption spectrum of the Rh6 rhodopsin and the transmittance spectrum of the screening pigment is significant (Fig. 2F), and therefore in bright sunlight the absorption of red stray light by the green rhodopsin will no longer be minor. This will especially be the case for the very red-eyed *Drosophila* (Fig. 2F), and therefore it is interesting to note that the absorption peak wavelengths of the Rh6 rhodopsins of *Musca* and *Drosophila* are around 520 nm and 510 nm, respectively. The shift of *Drosophila*'s Rh6 absorption spectrum to shorter wavelengths may have been driven by the much less-dense screening pigment of the fruitfly compared to the housefly.

The metarhodopsin states of the visual pigments of the R7 and R8 photoreceptors all have a main absorption band peaking in the blue to blue-green wavelength range (Fig. 2B and C, Table 1). The absorption spectra of the metarhodopsins of Rh3, Rh4 and Rh5 are strongly bathochromic-shifted with respect to the rhodopsin spectra, and a long-wavelength transparent screening pigment could then in principle be beneficial. However, photoconversion of the metarhodopsins by stray light will be negligible, because their absorption spectrum is restricted to the blue wavelength range.

On the other hand, the green-absorbing Rh6 rhodopsin of the R8y photoreceptors has a hypsochromic-shifted metarhodopsin, like the other green-peaking insect

rhodopsins. Red stray light hence will cause a progressive decrease in the Rh6 rhodopsin content, and thus the beneficial effect of photoregeneration of Rh1 rhodopsin in the R1–6 cells is inevitably connected to a distinctly deleterious effect on the R8y receptors. Possibly to maintain the sensitivity of the green R8 photoreceptors, rhodopsin turnover is very high, quite different from the situation in the R1–6 cells, which may be related to the demonstrated thermal instability of the green visual pigment's metarhodopsin (Salcedo *et al.* 1999).

Visual pigment, arrestin and the turnover cycle

The visual pigment composition in the photoreceptors depends on the light conditions, the concentration of arrestin molecules, and the components of the enzymatic turnover cycle. As shown in Fig. 3, both rhodopsin and metarhodopsin can exist in an active and inactive state. The light-induced signal is proportional to the concentration of active rhodopsin, R_a , and the rate constant k_R for creation of active metarhodopsin, M_a , the trigger of the phototransduction process; k_R is proportional to the illumination intensity. The lifetime of M_a is determined by the concentration of arrestin, A , and the arrestin binding constant, k_b . Fly photoreceptors express two arrestin types, but arrestin2 is the dominant form that blocks M_a (Dolph *et al.* 1993; Hardie & Postma, 2008). Under normal light conditions, the rate constant k_M of the photochemical reconversion of M_a into R_a is negligible with respect to $k_b A$, the rate constant of M_a decay (A is the concentration of free arrestin). This is completely different for the long-lived inactive forms, M_i and R_i . Prolonged illumination will cause a photosteady state, where the concentration ratio of rhodopsin and metarhodopsin depends on the ratio of the rate constants k_R and k_M , which contain the absorption spectra of the visual pigment states and the illumination spectrum (for a formal treatise of fly visual pigment photochemistry and the arrestin cycle, see Stavenga *et al.* 2000, and Stavenga & Hardie, 2011).

The distribution of the visual pigment molecules over the four active and inactive states crucially depends on the rate constant k_f of the enzymatic degradation of the inactive states M_i and R_i and the rate constant k_g of rebuilding native, active rhodopsin R_a (Fig. 3). Two special examples are shown in Fig. 4.

In the case of *Drosophila* R1–6 photoreceptors (Fig. 4A–C), each of the 30,000 rhabdomeric microvilli contains about $R_0 = 1000$ visual pigment molecules Rh1 and $A_0 = 370$ arrestin molecules, with $k_b = 0.54 \text{ s}^{-1} \text{ molecule}^{-1}$ (Hardie & Postma 2008; Liu *et al.* 2008). A broad-band illumination is assumed, which causes a photosteady state of the visual pigment with 80% rhodopsin and 20% in the metarhodopsin state. With negligible degradation, i.e. $k_f = 0$, the concentration of active metarhodopsin increases linearly with the light

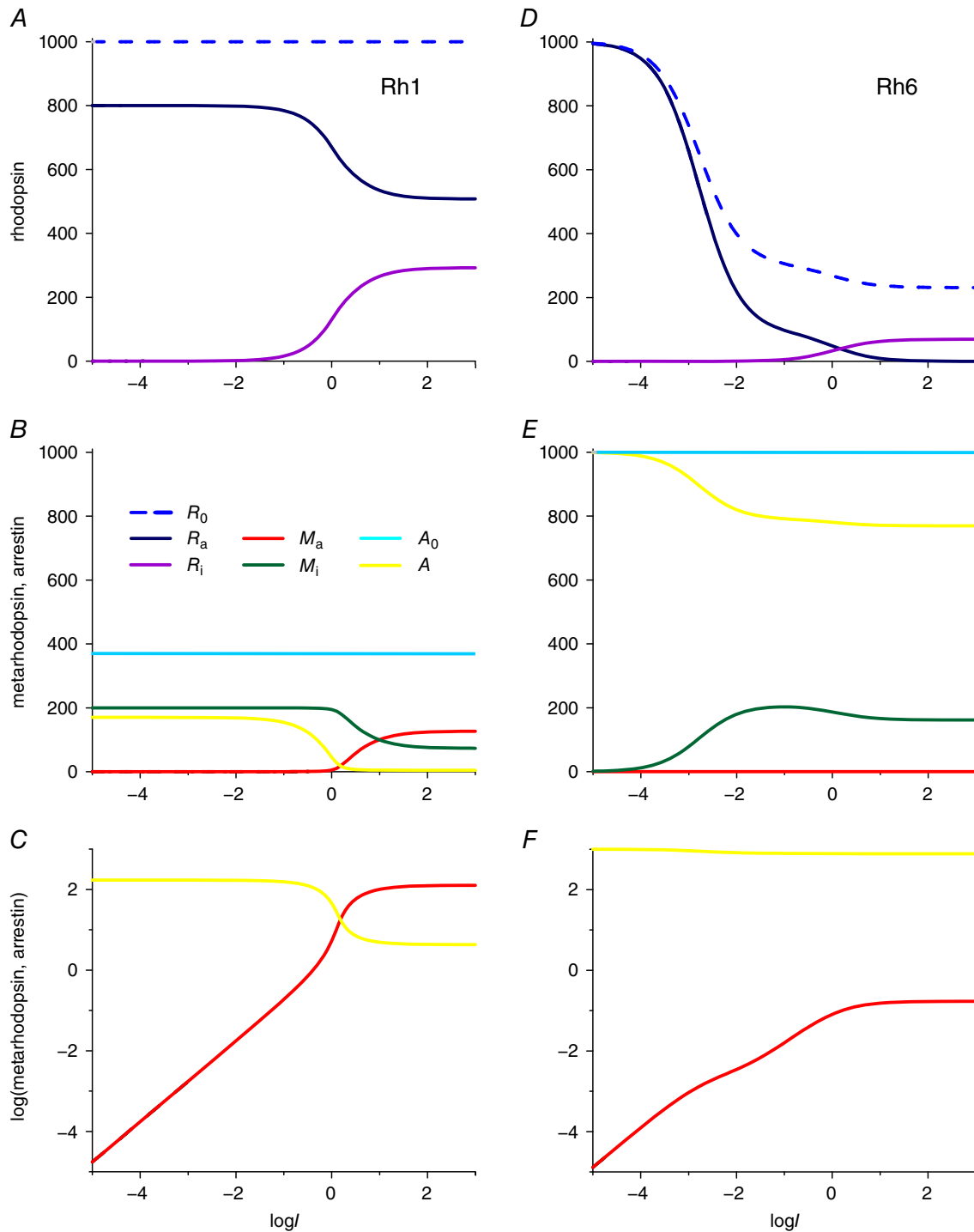


Figure 4. Visual pigments Rh1 and Rh6 in the photosteady state in a rhabdomeric microvillus with total $R_0 = 1000$ visual pigment molecules, assuming different amounts of arrestin and enzymatic rate constants, as a function of light intensity ($\log I$; see text)

A–C, population in the photosteady state of the different visual pigment states, R_a , R_i , M_a and M_i , representative for *Drosophila* R1–6 photoreceptors, where the number of arrestin molecules in a microvillus is $A_0 = 370$ of which arrestin is unbound, and turnover can be considered negligible. Illumination with broad-band white light creates a photosteady state with 800 rhodopsin and 200 metarhodopsin molecules. D–F, population of the visual pigment states representative for a green receptor with 1000 arrestin molecules per microvillus, and assuming time constants of degradation and regeneration $k_f^{-1} = 3$ min and $k_g^{-1} = 10$ min.

intensity up to about $\log I = 0$, where I is the relative photon flux normalized so that at $\log I = 0$ the sum of the photoconversion rate constants $k_R + k_M = 1 \text{ s}^{-1}$, that is, the time constant for creating a photosteady state is 1 s. At $\log I = 0$ (very bright daylight) in each microvillus about one active metarhodopsin molecule then exists. At higher intensities, metarhodopsin inactivation can no longer keep up with the creation of new active metarhodopsins, so that M_a accumulates, leading to saturation (or even blinding) of the photoreceptor. Note, however, that the light-controlling effect of the pupil has not been incorporated in Fig. 4A–C; in *Musca* photoreceptors, the reduction in light flux will stretch the high-intensity part of the graphs with about 0.8 log unit, the estimated extreme density of the pupil (Stavenga, 2004b).

A very different case, a green receptor with Rh6, is treated in Fig. 4D–F. Each microvillus contains again 1000 visual pigment molecules, and a broad-band illumination is assumed to cause a photosteady state with 30% rhodopsin and 70% metarhodopsin molecules. To prevent an endless PDA now 1000 arrestin molecules per microvillus are assumed, and degradation of the inactive M_i and R_i and regeneration of active R_a occurs with time constants 3 and 10 min, respectively (see Stavenga & Hardie, 2011). At low light intensities, the active metarhodopsin content then increases again linearly, but at higher light intensities the total number of visual pigment molecules (R_0 ; Fig. 4D) decreases, because the rhodopsin decay is faster than the regeneration; in the vertebrate literature this is known as bleaching. The concomitant effect is that the number of active metarhodopsin molecules is maintained within bounds, i.e. less than one per microvillus, and this number stabilizes at light intensities above $\log I = 0$ (Fig. 4F). It also means that the photoreceptor remains within the physiological working range at very high intensities without being blinded. Further cases, with details how to calculate the concentrations of the different visual pigment states as a function of light intensities, have been discussed by Stavenga & Hardie (2011).

Visual and screening pigments in various Diptera

The Diptera (true flies) are divided into the suborders Nematocera (e.g. midges, mosquitoes) and Brachycera; the latter is subdivided into the lower Brachycera (e.g. horse flies, soldier flies, robber flies) and higher Brachycera (e.g. flower flies, fruitflies, houseflies) (Marshall, 2006). Several studies have shown that in many fly species belonging to the Brachycera, the majority of photoreceptors have a peak sensitivity at a wavelength $< 500 \text{ nm}$, in the blue-green wavelength range, at variance with the Nematocera, which have a majority of green-sensitive photoreceptors, with peak wavelength around or above 530 nm . Table 2 lists the dipteran species investigated so far, together with

the peak wavelengths of the main photoreceptors as determined by electro- or optophysiological recordings, or via spectrophotometry of the visual pigments, together with the eye colour as visually observed. The rhodopsins of the main photoreceptors of species belonging to the Nematocera have a peak wavelength $\sim 530 \text{ nm}$. This is also the case for the horse flies and soldier flies. The main photoreceptors R1–6 of the higher brachyceran species, at least the hoverflies, houseflies, blowflies and fruitflies, however, have peak wavelengths hypsochromic-shifted to the blue-green. Combining the peak wavelength of the major class of photoreceptors with the phylogeny of the dipteran families shows that the transition of the main photoreceptors' peak wavelength from above to below 500 nm has occurred around the time of separation of the Tabanidae (horse flies) and the Stratiomyidae (soldier flies) from the other Brachyceran families (Fig. 5). In some nematoceran and lower brachyceran species, the main photoreceptors in the male dorsal area have peak sensitivities in the blue wavelength range, distinctly different from the main green receptors in the ventral retina (*Similium*, *Bibio*, *Tabanus*). Presumably the dorsal short-wavelength receptors serve for spotting potential female partners against the blue sky. Funnily, the situation in soldier flies is the opposite: in both the male and female, the main class of photoreceptors is dorsally green sensitive and ventrally blue sensitive (Table 2).

The screening pigments of the Nematocera and lower Brachycera have a dense, dark-brown to black colour, but those of the higher brachycerans are rather

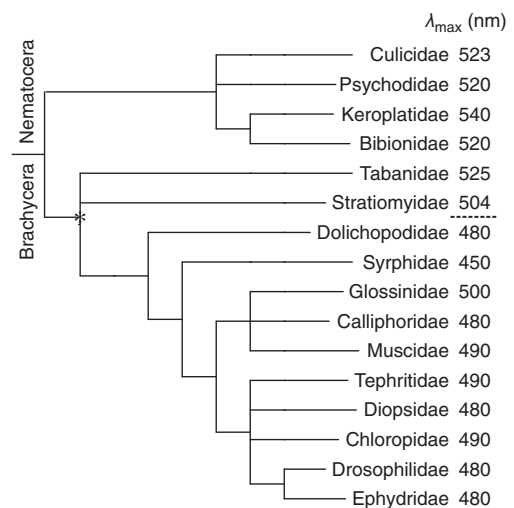


Figure 5. Phylogeny of Diptera and peak wavelengths of the main class of photoreceptors

The asterisk indicates the event where the rhodopsin absorption spectrum of the main photoreceptors (R1–6) has become bathochromic shifted from $> 500 \text{ nm}$ to $< 500 \text{ nm}$. The tree was created with phyloT, which is based on NCBI taxonomy, and visualized with iTOL (Letunic & Bork, 2016).

reddish-brown (Table 2). The close correlation between the spectral properties of the main class of photoreceptors and the screening pigments strongly suggests an intimate, functional relationship. As argued above, we nevertheless may have to be cautious in concluding that the red-transparency is a clever, beneficial solution to cheaply regenerate visual pigment. Quite possibly this is a tightrope strategy, a balancing act between optimization of the function of the R1–6 receptors and restricted degradation of the function of the R8y receptors.

An indication of the subtle tuning of the screening pigment properties may be gleaned from the difference in the pigments of the primary and secondary pigment cells. As can be deduced from Fig. 1, the primary and secondary pigment cells have a red and brown colour, respectively. Microspectrophotometry studies on squash preparations of blowfly (*Calliphora*) eyes revealed different types of pigment granules, red and brown, due to different redox states of xanthommatin pigment (Langer, 1967, 1975). The concentration of redder pigment in the primary pigment cells may be functionally significant, because the increased red transparency means a higher efficiency for reconversion of Rh1 metarhodopsin into rhodopsin. Incident red light entering an ommatidium only slightly off-axis will traverse the primary pigment cells and hence only causes a slightly increased acceptance angle. Incident light entering at larger angles must be largely blocked by the pigment in the secondary pigment cells, which has a broader absorption band than the pigment in the primary pigment cells.

As a final remark, we may note that the intimate relationship between screening pigment and the photochemical properties of the visual pigments can be recognized also in a number of special cases of insect compound eyes. Striking examples are the regionally specialized dorsal eyes of dragonflies, owlflies, mayflies, male horse flies, and even of the nematoceran simuliids and bionids (Table 2), where screening pigments only guard the rhodopsins of purely UV- or blue-sensitive photoreceptors and not the bathochromic-shifted metarhodopsins (for a review, Stavenga, 2002).

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Additional information

Competing interests

None declared.

Author contributions

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