J Comp Physiol A (2000) 186: 1–12

ORIGINAL PAPER

T. W. Cronin · M. Järvilehto
M. Weckström · A. B. Lall

Tuning of photoreceptor spectral sensitivity in fireflies (Coleoptera: Lampyridae)

Accepted: 29 September 1999

Abstract Sexual communication between male and female fireflies involves the visual detection of species-specific bioluminescent signals. Firefly species vary spectrally in both their emitted light and in the sensitivity of the eye, depending on the time when each is active. Tuning of spectral sensitivity in three firefly species that occupy different photic niches was investigated using light and electron microscopy, microspectrophotometry, and intracellular recording to characterize the location and spectral absorption of the screening pigments that filter incoming light, the visual pigments that receive this filtered light, and the visual spectral sensitivity. Twilight-active species had similar pink screening pigments, but the visual pigment of *Photinus pyralis* peaked near 545 nm, while that of *P. scintillans* had a λ_{max} near 557 nm. The night-active *Photuris versicolor* had a yellow screening pigment that was uniquely localized, while its visual pigment was similar to that of *P. pyralis*. These results show that both screening and visual pigments vary among species. Modeling of spectral tuning indicates that the combination of screening and visual pigments found in the retina of each species provides the best possible match of sensitivity to bioluminescent emission. This combination also produced model sensitivity spectra that closely resemble sensitivities measured either with electroretinographic or intracellular techniques. Vision in both species of *Photinus* appears to be evolutionarily tuned for maximum discrimination of conspecific signals from spectrally broader backgrounds. *Ph. versicolor*, on the other hand, appears to have a visual system that offers a compromise between maximum sensitivity to, and maximum discrimination of, their signals.

Key words Spectral sensitivity · Filter · Firefly · Compound eye · Vision ecology

T.W. Cronin (✉)
Department of Biological Sciences,
University of Maryland Baltimore County,
Baltimore, MD 21250, USA
e-mail: cronin@umbc.edu
Tel.: +358-410-455-3449; Fax: +358-410-455-3875

M. Järvilehto
Department of Biology, University of Oulu, Oulu, 90570 Finland

M. Weckström
Department of Physical Sciences and Department of Physiology,
University of Oulu, Oulu, 90220 Finland

A.B. Lall
Department of Biology, Howard University, Washington,
D.C. 20059, USA

Introduction

Fireflies have provided one of the great success stories in studies of vision ecology. Males emit species-specific bioluminescent flash sequences as they fly at dusk or night, locating potential mates by detecting characteristic answering flashes from females perched on vegetation. Twilight-active species tend to produce lemon-yellow to yellow-orange flashes, in contrast to nocturnal species which in general flash lime-green (Lall et al. 1980; Seliger et al. 1982a). However, there is no evidence that fireflies recognize or respond to the colors of flashes, only their patterns (Buck 1937; Worthy and Lall 1996).

Seliger et al. (1982a, b) elegantly demonstrated that the emission spectra of the various firefly species are effective under the photic conditions prevailing during the activity period of each species (see also Endler 1993). While the night-active species retain a green (and presumably ancestral) emission (Seliger et al. 1982a), twilight-active species produce yellow flashes. Visual spectral sensitivity in these latter species is narrow and peaks in yellow region of the spectrum (Lall et al. 1988), which improves the detection of bioluminescent signals against the foliage background. In these species, the emission spectra of their lanterns match their visual spectral sensitivity functions (Lall et al. 1980, 1988; Seliger et al. 1982a, b).

Seliger et al. (1982a) proposed that among twilight-active firefly species, the detection of the bioluminescent signals could be improved by reducing the environmental noise (e.g., sunlight, skylight, and reflection from green foliage) by long-pass filtering a middle-wavelength rhodopsin with an appropriate photostable pigment screen lying on top of the retina. In the terminology used by Seliger et al. (1982a), the evolutionary tuning of firefly visual sensitivity to their species-specific requirements occurs via the 'screening pigment pathway' rather than the 'opsin pathway', whereby the spectral absorption of visual pigments would be the tuned feature (Seliger et al. 1982a). If this is the case, all species of fireflies may have similar or identical visual pigments, and the variation in spectral sensitivity among species is achieved solely through selective filtering of the retinal layer by colored screening pigments. Such an evolutionary 'choice' would not come cost-free, as filtering poses a serious penalty in the form of reduced overall sensitivity, and one might expect the visual pigments of twilight-active species to be placed at longer wavelengths, more closely matching their pigment filters.

We decided to characterize the visual pigments of two twilight-active species *Photinus scintillans* (an orange-yellow flasher active early in the evening) and *P. pyralis* (which flashes lemon-yellow, and is active later in twilight), and to compare them to a night-active, green-flashing species, *Photuris versicolor*. We were thus able to examine closely the question of whether filtering alone explains their spectral sensitivity functions. Our findings demonstrate that variation occurs in both visual and filtering pigments, providing sufficient flexibility to optimize signal detection in all three species.

Materials and methods

Animals

Male specimens were collected by hand-netting at twilight and night in the vicinity of Baltimore, Maryland. Species identification was made on the basis of the flash pattern of these males. They were kept in jars and provided with paper soaked in honey water for food and humidification until use.

Anatomical and histological preparation

Electron microscopy

Dark-adapted fireflies were decapitated in fixation solution (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mol $\cdot$ l^{-1} phosphate buffer at pH 7.4), postfixed with 1% osmium tetroxide, dehydrated and embedded in epon. The embedded blocks with specimens were videographed to allow for proper orientation when blocks were trimmed. Thin sections counterstained with 2% uranyl acetate and lead citrate were examined by transmission electron microscope (JEOL TEM SCAN 100CXII, Japan).

Specimens for scanning electron microscopy were fixed in the same way to minimize tissue distortion. Standard techniques were employed for dehydration, critical point drying, and sputter coating. To expose internal structure, some specimens were broken open before coating. A field-emission scanning electron microscope (JEOL FESEM, Japan) was used to examine the samples.

Light microscopy

To determine the distribution of screening pigments in vivo, some ocular material was examined and photographed in fresh-frozen eyes, prepared and sectioned identically as for microspectrophotometry (MSP), which is described in the next section.

Microspectrophotometry

Procedure

Animals to be examined were dark-adapted overnight. Samples were prepared for MSP in dim red light. First, whole heads were removed from the dark-adapted specimens and quick-frozen using cryogenic spray (Histofreeze 2000, Fisher Scientific, USA). These were mounted in a cryostat maintained at −25 °C to −30 °C and sectioned at thicknesses of 10–14 μm. Sections were mounted in insect Ringer's (Muri and Jones 1983) between coverslips, protected by a ring of silicone grease, and placed in the microspectrophotometer.

The single-beam instrument used for this project has been described in Cronin (1985) and Cronin and Forward (1988). Samples were scanned from 400 nm to 700 nm at 1-nm intervals, using a circular spot 5 μm in diameter. The sequence of scanning was typically as follows: (1) scan the dark-adapted photoreceptor two times, using the second scan to check for stability of the preparation; (2) treat with 15 s of bright-red light (passed through a Corning CS2–61 filter) to convert most of the photopigment to the metarhodopsin state; and (3) treat with 15 s of bright-blue light (Corning CS5–56) to partially reconvert the pigment to a mixture of rhodopsin and metarhodopsin. In some cases, treatments (2) and (3) were repeated several times in sequence to test for photoreversibility of the pigment system and for the presence of only a single, bistable system. A few attempts were made to bleach the visual pigments using bright-white light, but these bleaches were not very successful (due to baseline shifts or scattering-induced changes in absorption spectral shape), and further analysis of the pigment system was carried out only using difference spectra for photoconversion.

Analysis

Photoconversion difference spectra were analyzed by fitting difference spectra with model rhodopsin/metarhodopsin photopigment pairs, using A1-based rhodopsin templates (Palacios et al. 1996), in combination with a metarhodopsin template generously provided by G. D. Bernard. The average data value from 650 nm to 700 nm was subtracted from each difference curve as a first correction for any residual baseline drift. Corrected spectra were fitted from 425 nm to 650 nm (the lower limit was chosen to limit the effect of random spectral shape changes due to alterations in scattering). A brief check indicated that the rhodopsin would have a λ_{max} near 550 nm, and would be paired with a metarhodopsin near 485 nm, at an extinction ratio near 2.0. Therefore, the test parameters were allowed to range as follows: rhodopsin λ_{max}, 500–600 nm (1-nm intervals); metarhodopsin λ_{max}, 450–550 nm (1-nm intervals); extinction ratio (ε_{max}M:ε_{max}R), 1.0–3.0 (0.05 intervals). The model spectrum with the least sum of squares of deviations from the data was taken to be the best fit.

Electrophysiology

Standard techniques were utilized for recording intracellular receptor potentials from the retinular cells in firefly *P. pyralis* males, which were shipped from Baltimore, MD to Oulu, Finland during the summer months of 1993, 1994, and 1995. The details of the method are given in Weckström et al. (1991). A short summary is presented below.

For electrical recording, live fireflies were fixed with beeswax onto a platform placed at the center of the rotation of a Cardan

arm. The arm moved around the eye at a constant distance of 45 mm. For impaling photoreceptors with microelectrodes, a small hole was made in the cornea. This hole was filled with silicon grease to prevent dehydration of the ocular tissue. Microelectrodes were made with 1.5 mm diameter glass capillary tubing (Clark Electromedical, UK), pulled on a horizontal pipette puller (P-80 PC, Sutter Instruments, USA) and filled with 2 $mol \cdot l^{-1}$ KCl solution. Their tip resistance varied from 90–140 MΩ. The recording microelectrode was moved with a piezoelectric micromover (Burleigh Inchworm PZ-550, USA) into the retina through the small hole made in the cornea. The intracellular potentials were recorded by a high-impedance preamplifier (SEC-05L, npi Electronics, Germany) filtered at 1 kHz and further amplified by a low-pass dual channel elliptical filter (VBF-23, KEMO, UK) and displayed on an oscilloscope (Tektronix, USA). The signal recording, analysis, and storage was performed using a personal computer running an ASYST-based (Keithley, USA) program (Juusola et al. 1994).

A 150-W xenon light source in conjunction with a series of 11 interference filters (half-band width ~10 nm) from 341 nm to 620 nm provided the photic stimuli. The light stimulated the eye via a Y-shaped quartz fiber-optic fixed to the Cardan arm system. The intensity of the stimulus was calibrated by a PIN photodiode and a radiometer (80-X Opt-O-Meter, United Detector Technology, USA). Stimulus intensity was controlled with a series of Wratten neutral-density filters (Kodak, USA) such that similar numbers of photons were delivered at various wavelengths across the spectrum. The stimulus duration was 10 ms. Spectral sensitivity was computed from the amplitude of the intracellular response at each wavelength after first obtaining the V-log I function, permitting millivolt response values to be converted into relative sensitivity functions.

Results

Structure of firefly eyes

Fireflies have refracting superposition compound eyes (Exner 1891; see also Land 1980). In *P. pyralis* they are about 1 mm in diameter, and each provides nearly a spherical field of view (Fig. 1A, B). However, the eyes are located under a shelf-like pronotum, which acts like a baseball cap to block the visual field at elevations above about 45E whenever the head is retracted (Fig. 1A). Horridge (1968, 1969) has already provided a general description of firefly eyes of the genus *Photuris*, and our electron microscopy observations on *P. pyralis* are generally consistent with his findings.

In *P. pyralis*, each ommatidium has a corneal lens about 18–20 μm in diameter and about 10 μm thick, which grades seamlessly into a solid crystalline cone. The entire structure of cornea plus cone is about 60 μm long. The outer surface of the cone has longitudinal grooves separated by about 1 μm, and the cone's structure becomes quite complex near its borders due to the co-occurrence of the radial density gradients and the longitudinal grooves. The cone is surrounded by a dense layer of numerous pigment cells per ommatidium.

The clear-zone region separating the tips of the cones from the distal receptor layer is about 100 μm across. It is divided into two layers, and is filled with a diversity of cell types, of which the most numerous are the accessory pigment cells. These contain most of the dark screening pigments, which vary in position with the adaptational state of the eye. Accessory pigment cells lack any detailed internal structure, but are electrodense with internal pigment granules. The proximal layer of the clear zone, about 60 μm thick, does not contain pigment granules, but does show some differential staining and is generally electron dense.

The eye has heavy screening pigmentation in three different zones: (1) in the distal zone between the crystalline cones, (2) in the proximal part of the clear zone, and (3) in the basal part of the actual retina. Typical pigment granules are about 0.5 μm in diameter and are often arranged in queues supported by a thin thread-like structure. While the clear zone is filled with these granules in light-adapted eyes, during dark adaptation they are retracted into the top of the retina – exposing the tips of the rhabdoms – and into the spaces between the bullet-shaped crystalline cones. In addition to the pigmentation, each ommatidium has a crystalline tract constructed by four cells, which separates at the top of the retina into four separate units. The retina itself is divided into two distinct layers, which we call the outer and inner retina (Fig. 1B).

Surprisingly, there is little direct evidence in electron micrographs of how the colored screening (filtering) pigments are localized, although they may be placed in the large balloon-like structures located in the filter zone (Fig. 1C). Nevertheless, these pigments are easily visualized in fresh-frozen sections of eyes (Fig. 2). The day-active species, *Lucidota atra* (which is not bioluminescent), has no filter screen; its retina probably resembles that of the ancestors of bioluminescent fireflies, and we include an image of its retina for comparison with the dusk- and night-active species. Filter material in *P. pyralis* appears reddish in color and is spread diffusely throughout the inner retina. However, its color is most intense at the junction with the outer retina and gradually fades away towards the bases of the rhabdoms (Fig. 2). *P. scintillans* (not shown) has a smaller eye than does *P. pyralis*, but its ocular structure and screening pigment distributions are very similar to those of the latter species. In *Ph. versicolor*, on the other hand, there is a clearly demarcated band of dense yellow material in the region where the outer and inner retinas join (Fig. 2).

Microspectrophotometry

The best data were obtained in all species when cross-sections of photoreceptors near the base of the retina were scanned, probably because in this region the basal screening pigment projects between the rhabdoms, making their positions obvious. In the outer layer of the retina (occupied by single large rhabdoms), we found no evidence of a light-sensitive pigment responding in any species in the scanned spectral range (400–700 nm). When the dark-adapted inner retina is given a saturating exposure to long-wavelength light (the 50% transmission point of the red filter is 619 nm), there is a loss in

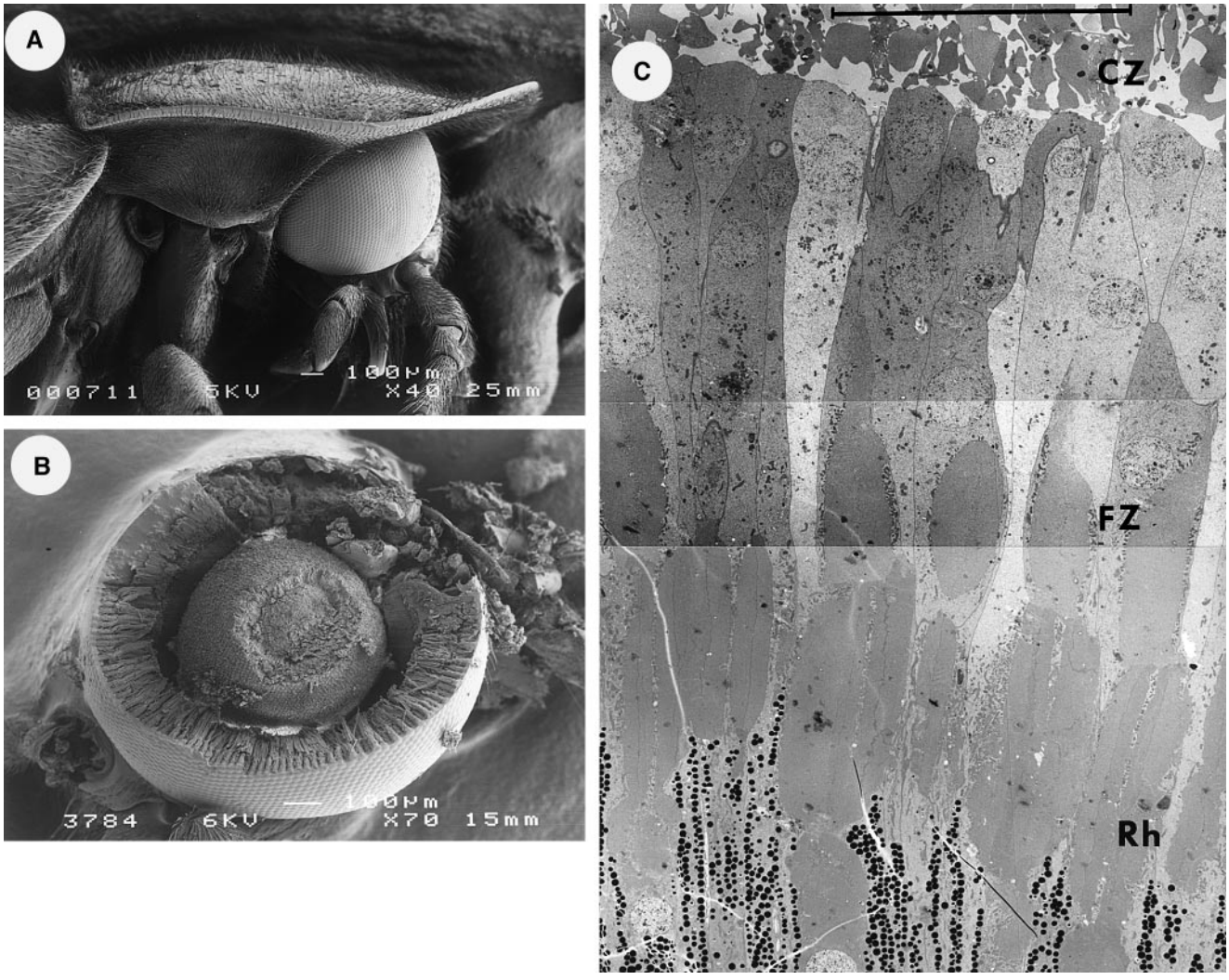

Fig. 1A–C Scanning and transmission electron micrographs (EMs) of eyes of male *Photinus pyralis*. **A** General lateral view of the right anterior region, showing the prominent spherical compound eye and the overlying, dorsally placed pronotum. Scale bar: 100 μm. **B** View of broken compound eye, showing the outer region of crystalline cones, the surface of the outer retina, with a small amount of the inner retina exposed to show the area where the colored screening pigment is located. Scale bar: 100 μm. **C** Transmission EM of the retina, showing the inner part of the clear zone (*CZ*) occupied by crystalline cones and the adjacent region of the outer retina, the filter zone where color pigments are typically found (*FZ*), and the inner retinal region where rhabdoms were scanned in this study (*Rh*). Scale bar: 50 μm

absorption beyond about 525 nm, balanced by a somewhat larger gain below this wavelength (Fig. 3). This photoconversion is partially reversible to a new photosteady-state mixture, using blue light, and from this mixture the system can be reversibly flip-flopped indefinitely by alternating saturating treatments of red and blue actinic light.

In all three firefly species, these observations are fully consistent with the presence of a bistable pigment system, interconverting between rhodopsin and metarhodopsin. The treatment of dark-adapted photoreceptors causes the greatest change, as the photopigment in the retina is almost completely in the rhodopsin state at this time (Fig. 3). Subsequent reversals after red and blue actinic exposures switch between two photosteady-state mixtures of rhodopsin and metarhodopsin, and hence produce smaller differences. Difference spectra were analyzed as described in Materials and methods, and the results are summarized in Table 1. All three treatments (dark-to-red, red-to-blue, and blue-to-red) provide similar estimates for the rhodopsin λ_{max}, mainly because this pigment is the only absorbing entity at longer wavelengths. (Both the rhodopsin and the metarhodopsin absorb near the peak for metarhodopsin, making its position more variable depending on the extinction ratio.) The visual pigment systems of *P. pyralis* and *Ph. versicolor* are very similar indeed, consisting of a rhodopsin with $\lambda_{max} = 545$ nm interconverting with a metarhodopsin with $\lambda_{max} = 480$ nm and peak absorbance about twice that of the rhodopsin. *P. scintillans* is clearly different; it has a rhodopsin peaking near 557 nm paired with a 485-nm metarhodopsin. No photoreceptors containing blue- or UV-absorbing visual pigment were ever located in any species.

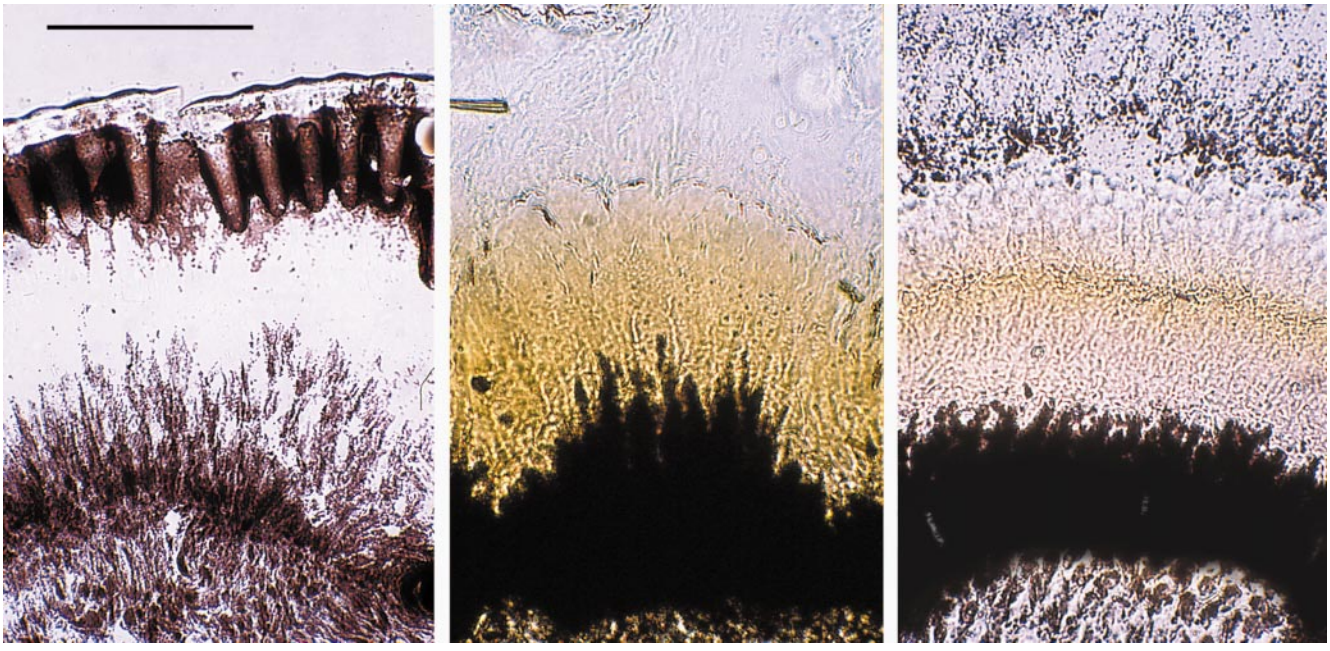

Fig. 2 Color images of fresh-frozen sections of live retina taken through the center of the eye, showing locations of colored screening pigments in eyes of three firefly species. (Colors in these photographic reproductions differ slightly from their appearance to the eye.) The scale bar in the left panel represents 100 μm and applies to all three panels. *Left* Eye of a day-active species, *Lucidota atra*, which has a relatively small compound eye. The cornea and bullet-shaped crystalline cones (that form the refracting superposition image) lie on and under the surface of the eye, and above the clear zone; the retina with strings of dark screening pigment forms a layer about halfway out from the center of the eye. No filtering pigment is seen. *Center* Retina of a dusk-active species, *Photinus pyralis*. The inner edge of the clear zone just appears at the top, and the top third of the panel is occupied by the outer retina. The reddish filter pigment appears throughout the inner retina, although its concentration is less at the bases of the rhabdoms (not visible within the dark screening pigments sheaths in this radial section). *Right* Retina of *Photuris versicolor*, a night-active firefly. The inner part of the clear zone is occupied by strands of dark screening pigment in this section, clearly marking the junction with the outer retina. The yellow filtering pigment occurs in a band at the border of the inner and outer retinas

Absorption spectra of filtering pigments were taken directly by scanning in the pigment layer, and are plotted as dotted traces in the left panels of Fig. 5. These spectra compare favorably with spectra measured in droplets of colored screening pigment that have exuded from the retina (see Lall et al. 1988).

Intracellular electrophysiological recording

Obtaining intracellular recordings from firefly retinular cells is very difficult, as has been noted before (Dr. Eric Warrant, personal communication). The difficulty is due both to the hardness of the cornea and head capsule of the insect and to the location and size of the arhabdomeric regions of retinular cells. Once past the corneal surface, the electrode has to traverse about 200 μm of the clear zone to reach the rhabdomeric region. The hypertrophied retinular cells (R1–6) are about 8 μm by 32 μm; R7 and R8 are too small for penetration. The retinular cells are completely filled with crystalline rhabdomeres containing the visual pigment, leaving hardly any place for the electrode tip to seal itself in an impalement. Out of more than one hundred trials successful intracellular recordings, in which response amplitude versus stimulus intensity functions and spectral characteristics of the retinular cells could be measured, were obtained only in 11 instances. Figure 4A presents an intracellular recording from one retinular cell (resting membrane potential about −55 mV) in response to a 10-ms light flash. This cell responded over 2.5 log units of stimulus intensity, and its intensity-response function is presented in Fig. 4B.

One major problem in recording from firefly retina was the presence of large (up to 25 mV) responses, elicited by light stimulation, which were apparent when the electrode was placed in the extracellular space. We were able to discriminate the intracellular potentials of retinular cells from extracellular potentials by supplying depolarizing and hyperpolarizing current injections in the dark. With depolarizing pulses the photoreceptor showed the rectification typical of insect photoreceptors (Weckström and Laughlin 1995). With hyperpolarizing current pulses the membrane showed passive charging properties with input resistance of about 50 MΩ near the resting membrane potential.

The spectral sensitivity functions obtained in three photoreceptors from different specimens were similar. In two of these cases, the responses to spectral light stimuli were small, and a spectral sensitivity function over the full spectral range of available light stimulation could not be obtained. The spectral sensitivity function from the

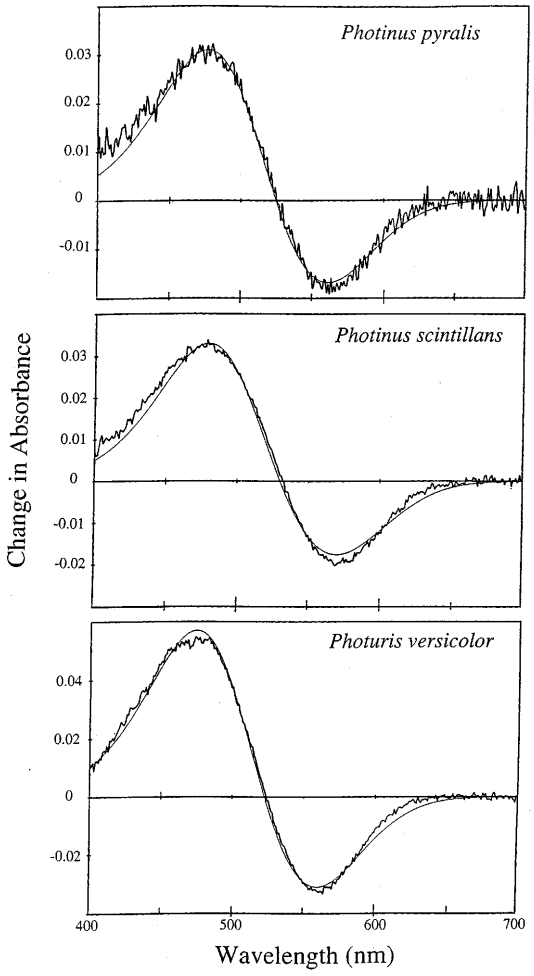

Fig. 3 Difference spectrum for photoconversion of visual pigment in inner regions of rhabdoms in the retinas of *P. pyralis* (*top*), *P. scintillans* (*middle*), and *Ph. versicolor* (*bottom*). In each panel, the *dark jagged trace* is the difference spectrum averaged from five to seven saturating photoconversions, produced by treating a dark-adapted rhabdom with 5–15 s of bright red light (Corning CS2–61 filter). This exposure converts a rhodopsin absorbing at longer wavelengths to a shorter-wavelength metarhodopsin. The *smooth curve* is the best-fit difference spectrum, obtained using templates of rhodopsin and metarhodopsin with appropriate λ_{max} values and relative extinctions (see Table 1)

cell with the best signal-to-noise ratio is given in Fig. 4C, which shows a relatively narrow peak between 525 nm and 625 nm and a small secondary peak around 450 nm.

Discussion

Spectral tuning in firefly retinas

While fireflies are essentially nocturnal beetles, some species occupy the twilight photic niche and a few are active even during the day. The visual systems of fireflies are highly specialized for nocturnal vision, and they are no doubt used for tasks like those required by any flying insect: flight control, navigation, avoiding obstacles, and so forth. It seems likely that when bioluminescence first assumed a role in firefly sexual communication, the luciferase enzyme was selected to produce green bioluminescence emission to coincide with the spectral sensitivity of the species' visual system (Lall et al. 1980, 1982; Seliger et al. 1982a, b). In modern fireflies, screening pigments tune sensitivity in both nocturnal and twilight species. But the latter have unusually narrow visual sensitivity spectra because their screening pigments severely attenuate sensitivity in the green region of the spectrum, producing a match between the shapes of the visual sensitivity and the yellowish bioluminescent emission spectra (Lall et al. 1980, 1988; see Fig. 5).

To examine the interaction of visual and screening pigments in firefly eyes, we modeled the expected spectral sensitivity functions of photoreceptors containing a rhodopsin with λ_{max} equal to 545 nm (*P. pyralis* or *Ph. versicolor*) or 557 nm (*P. scintillans*), as found in our analyses of difference spectra (see Table 1), stimulated by light transitting the pink or yellow pigment found in the outer regions of their retinas (e.g., Fig. 2). Our model is based on photoreceptor cells 60 μm in length containing a rhodopsin at an axial density of 0.008 μm^{-1} (see Cronin and Forward 1988), overlain by a layer of the appropriate colored screening pigment (e.g., Fig. 2) ranging in

Table 1 Results of analyses of visual pigment photoconversion data from retinas of species emitting yellow-orange, yellow, or green bioluminescent flashes. Data were fit with difference spectra constructed from rhodopsin and metarhodopsin templates, as described in the text. "Red after dark" refers to the difference spectrum generated by treating dark-adapted photopigment with saturating red light; "Blue after red" and "Red after blue" refer to difference spectra formed after blue-light saturating exposures of rhabdoms that had previously treated with red light, and vice versa

Species	Phototreatment	*n*	Rhodopsin λ_{max} (nm)	Metarhodopsin λ_{max} (nm)	Extinction ratio $\varepsilon_{max}M/\varepsilon_{max}R$
Photinus pyralis	Red after dark	5	546	480	1.90
	Blue after red	6	543	490	1.20
	Red after blue	9	542	481	1.55
Photinus scintillans	Red after dark	7	558	482	2.00
	Blue after red	7	556	484	1.80
	Red after blue	7	555	488	1.60
Photuris versicolor	Red after dark	5	544	478	1.90
	Blue after red	2	545	492	1.15
	Red after blue	2	541	480	2.75

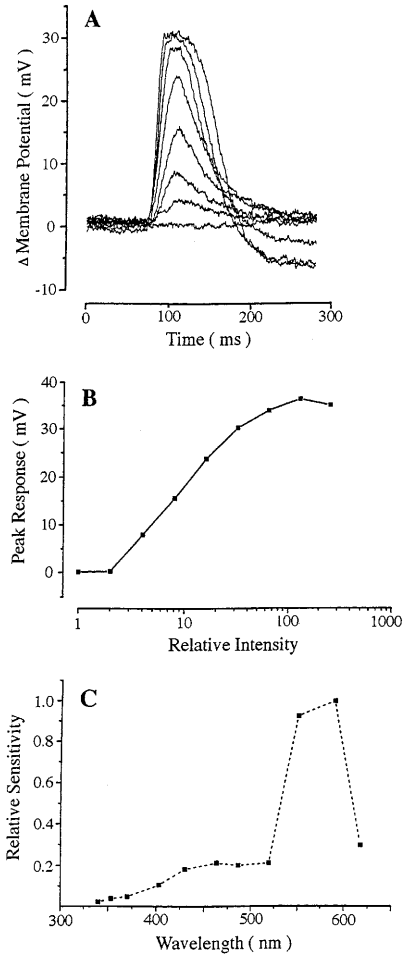

Fig. 4A–C Electrophysiology of single retinular cells in *P. pyralis*. **A** Receptor potentials recorded from one retinular cell to 10 ms of photic stimuli over a range of 2 log units in intensity. **B** *V*/log *I* curve for the potentials shown in **A**. **C** The spectral sensitivity of the intracellular response from three retinular cells in *P. pyralis*

peak optical density from 1.0 to 2.0 (in situ, the filtering pigment typically had peak absorbances of 0.03–0.04 per μm, so this corresponds to a path length of about 25–50 μm in the eye). The spectra of the rhodopsin, colored screening pigment, and computed sensitivities are illustrated in Fig. 5. We found the shape of the sensitivity spectrum to vary only slightly with reasonable estimates of rhodopsin absorbance or with filtering pigment densities greater than 1.0; in the case of Fig. 5, the peak density of the filtering pigment was 2.0. Figure 5 also shows a comparison of the modeled sensitivity spectra with spectral sensitivity measured in the various species using electrophysiological techniques. All curves are remarkably concordant, although in *P. scintillans* the model spectrum has a somewhat larger bandwidth than the electrophysiological measurements, and in *Ph. versicolor* there is electrophysiological evidence of a short-wavelength increase as well (Lall 1981). Overall, our results are fully consistent with the concept that spectral sensitivity is narrowly tuned in these species, and presumably in other fireflies, by combining particular rhodopsins with species-appropriate colored screening pigments that act as long-pass filters.

Where are the blue- and ultraviolet-sensitive photoreceptor classes?

Our microspectrophotometric explorations of retinas in all species yielded only a single photoreceptor class, containing the middle-wavelength rhodopsin. Where are the other receptor types that are indicated in electrophysiological sensitivity measurements (Lall et al. 1980, 1988)? We presume they exist in retinular cell classes that overlie retinular cells 1–6 (see Horridge 1969), in a location where we found no evidence of middle-wavelength photosensitivity (our scans were taken only from 400 nm to 700 nm, but blue-light actinic treatment would normally have revealed the presence of a bistable visual pigment system that absorbs blue or ultraviolet (UV) light. UV photoreceptors seem to be ubiquitous in insect eyes (Peitsch et al. 1992; Chittka 1996), and since they generally form a minority population of receptor cells in insect ommatidia, it is very likely that this outer rhabdomere contains a UV-absorbing photopigment. Placing short-wavelength receptors above the filtering pigment would avoid the problem of severe attenuation of short-wavelength light by filtering in the screen, as well. Since twilight skies are rich in UV, near-UV, and blue light (Munz and McFarland 1977; Seliger et al. 1982b), UV and/or blue sensitivity would be useful for flight orientation during the twilight peak in activity in species of *P. pyralis*, and possibly in *Ph. versicolor* as well.

The blue receptor class is thought to occur only in a restricted part of the retina (Lall et al. 1988). In species of *Photuris*, for instance, UV and middle-wavelength photosensitivity can both be adapted away, leaving a single blue peak (Lall et al. 1982), and the frontal part of the eye contains only UV and blue photoreceptors (Lall et al. 1988). In our intracellular and microspectrophotometric recordings, we evidently missed this retinal region. Since the short-wavelength receptors have no evident direct role in bioluminescent signal detection, indicated by the lack of any role of color in signal recognition (Buck 1937; Worthy and Lall 1996), they can be ignored in the analysis of spectral optimization we present here.

Optimal tuning of spectral sensitivity spectra in fireflies

Our results show that rhodopsins in the two twilight-active species (*P. pyralis* and *P. scintillans*) differ in λ_{max} by more than 10 nm, but that the rhodopsin of *P. pyralis* is similar or identical to that of the night-active *Ph. versicolor*. On the other hand, the filtering

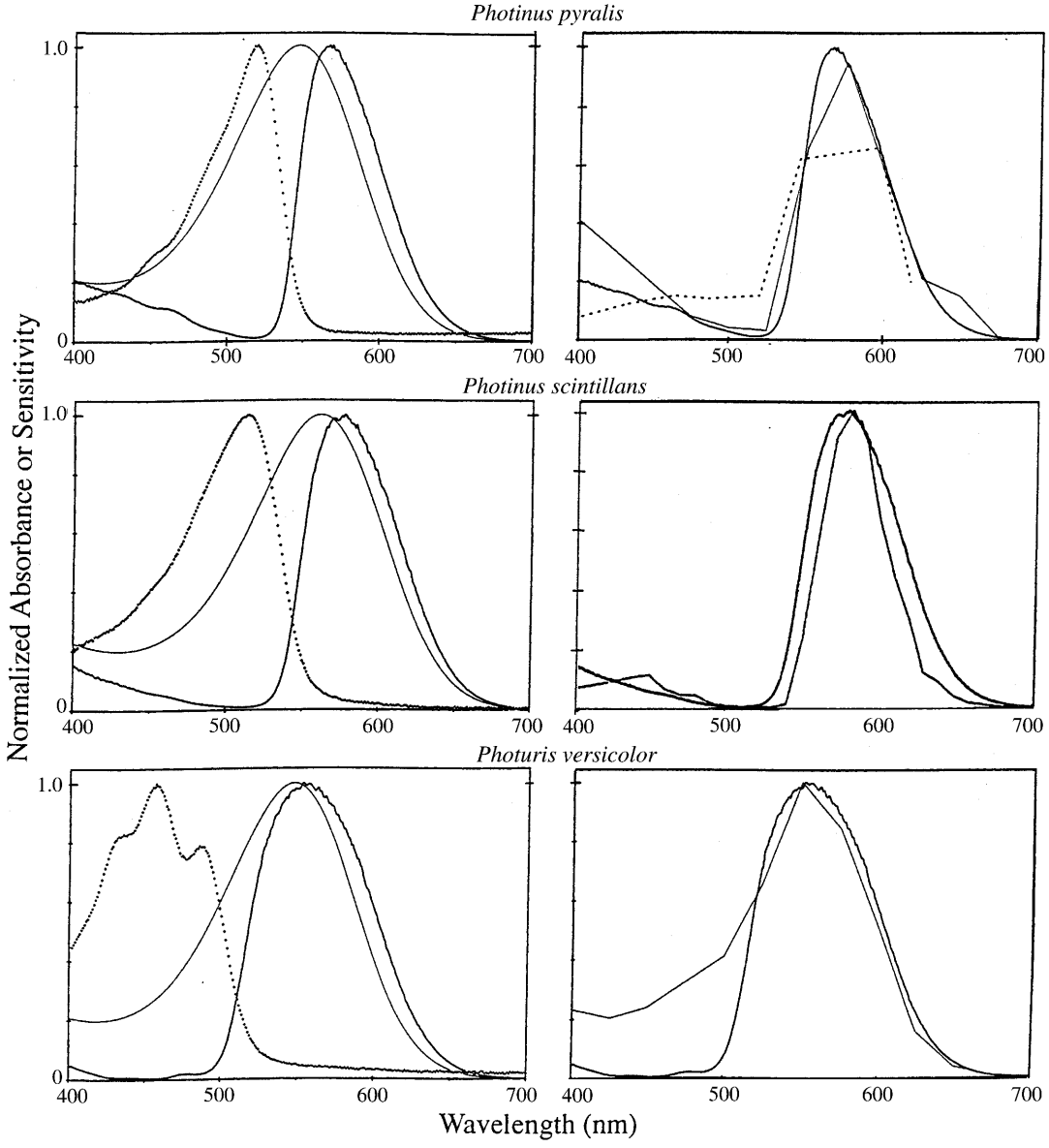

Fig. 5 Absorbance spectra of colored screening pigments and visual pigments, as well as spectral sensitivities, of the three study species of fireflies. *Left panels* Normalized absorbance spectra of the retinal filtering pigment of fireflies (*dotted curves*), and their rhodopsins (*thin traces*), together with the computed normalized sensitivity spectrum of each species (*dark traces*). Numbers of scans averaged together in the spectra of filtering pigments: *P. pyralis*, 16; *P. scintillans*, 5; *Ph. versicolor*, 2. Spectral sensitivities were computed based on a 60-μm-long photoreceptor containing the rhodopsin ($\lambda_{max} = 545$ for *P. pyralis* and *Ph. versicolor*; 560 nm for *P. scintillans*) at an axial density of 0.008 μm^{-1}, following filtering by the screening pigment at a peak density of 2.0 (*dark traces*). *Right panels* Comparisons of computed spectral sensitivities (*dark traces*) with measurements previously taken using electrophysiological techniques. Sources for electrophysiological data are as follows: *P. pyralis*, *thin trace* – electroretinographic measurement (Lall et al. 1988), *dotted trace* – single-cell recording of green receptors (this paper); *P. scintillans*, *thin trace*, electroretinographic measurement (Lall et al. 1988); *Ph. versicolor*, *thin trace* – electroretinographic measurement (Lall 1981)

pigments of the twilight species are very similar in spectral absorbance and ocular location, while the pigment screen of night-active *Ph. versicolor* is distinctive both in spectrum and in its deployment in the retina. Might these various combinations be adaptive? We investigated possible answers to this question by modeling firefly signaling systems, using the known spectra of filter and rhodopsin absorption and of bioluminescent emission. Included in our model is a simplified consideration of the photic conditions within which the signaling of each species takes place, following some of the ideas originally presented by Seliger et al. (1982a, b).

We first examined how rhodopsin λ_{max} influences the spectral sensitivity functions of *P. pyralis*, *P. scintillans*, and *Ph. versicolor*, using our previous assumptions (peak filter density, a variable that only minimally influences our analysis, is 2.0; and the rhabdom containing the middle-wavelength pigment has a length of 60 μm and an axial density of 0.008 μm^{-1}). Typical results, illustrated in Fig. 6, show some interesting features. In *P. pyralis* and *P. scintillans*,

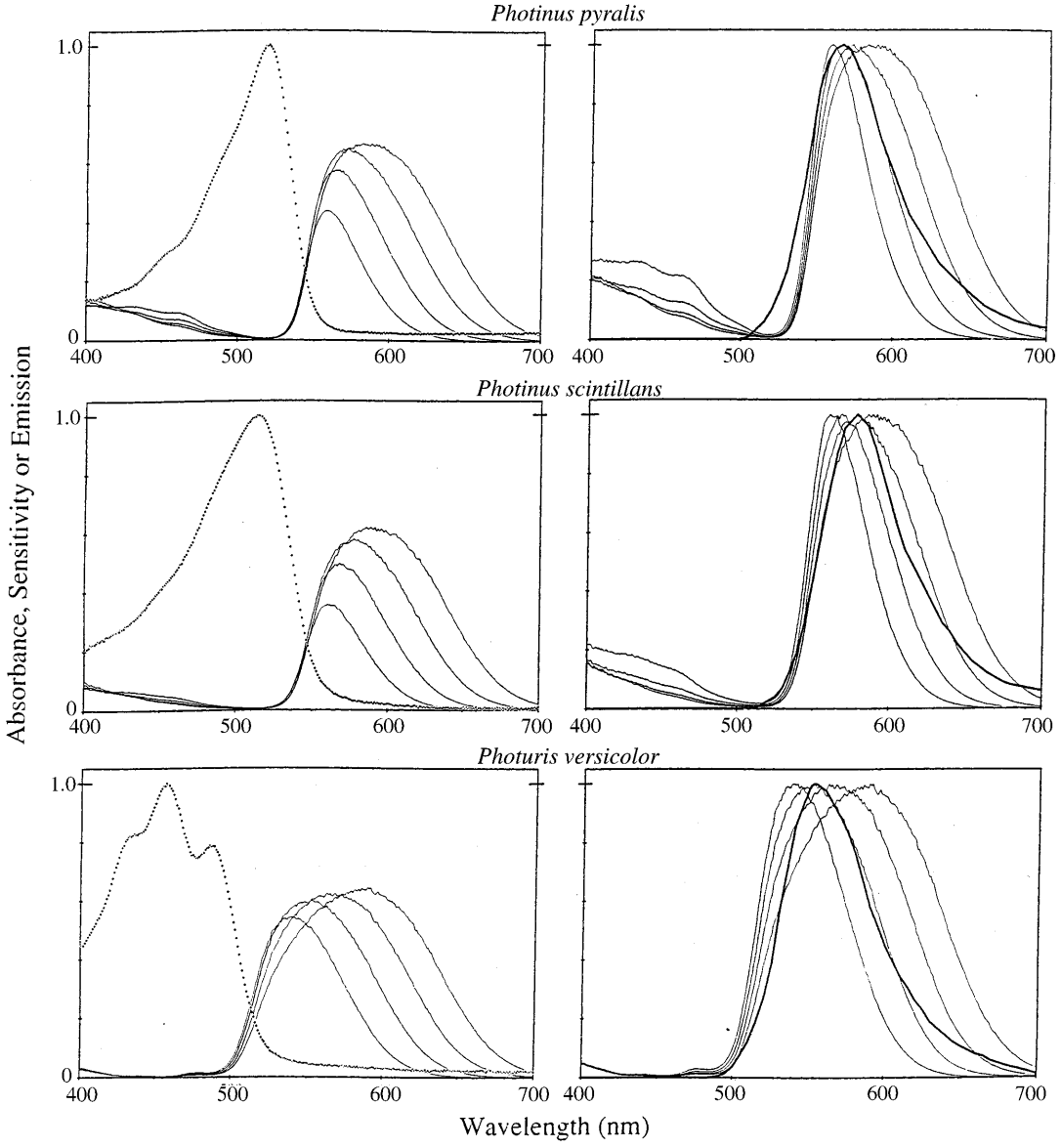

Fig. 6 Modeled spectral sensitivity functions based on different visual pigments in three firefly species. In each panel, the λ_{max} of the rhodopsin included in the model in successive curves, left to right, is 520 nm, 540 nm, 560 nm, or 580 nm. All computations assume a screening pigment density of 2.0 atop a rhabdom 60 μm long containing rhodopsin absorbing at 0.008 μm^{-1}. *Left panels* Absolute spectral sensitivity functions, plotted as proportional photon capture (a value of 1 indicates absorption of all photons at that wavelength). The normalized absorption spectrum of each corresponding screening pigment is illustrated as a *dotted trace*. *Right panels* Normalized spectral sensitivity functions (same set as in left panels) plotted together with the appropriate normalized bioluminescence emission spectrum (*dark trace*). Emission spectra are taken from Seliger et al. (1964) and Biggley et al. (1967)

where the filter cut-on wavelength is relatively long, sensitivity increases with rhodopsin λ_{max} (Fig. 6, top and middle left). This is due to both an increase in peak height (because more of the rhodopsin absorption extends beyond filter absorption) and an increase in bandwidth (because the rhodopsin's long-wavelength limb moves to longer wavelengths commensurately with λ_{max}). Nevertheless, the sensitivity spectrum which most resembles the shape of the yellowish emission spectrum is produced by a rhodopsin with a λ_{max} near 540 nm in *P. pyralis* or 560 nm in *P. scintillans* (Fig. 6, top and middle right panels). In *Ph. versicolor*, however, the height of the sensitivity peak is less dependent on rhodopsin λ_{max} because the filter cuts on at considerably shorter wavelengths, exposing more of the rhodopsin's absorption. Once again, because filtering trims the visual pigment's short-wavelength limb, bandwidth (and thus total photon capture) increases with rhodopsin λ_{max} (Fig. 6, bottom left). The best match between the shapes of the sensitivity and bioluminescent spectra again occurs for a relatively short-wavelength rhodopsin (λ_{max} near 540 nm; Fig. 6, bottom right).

Figure 7A shows how effective various rhodopsins, ranging in λ_{max} from 400 nm to 700 nm, would be for

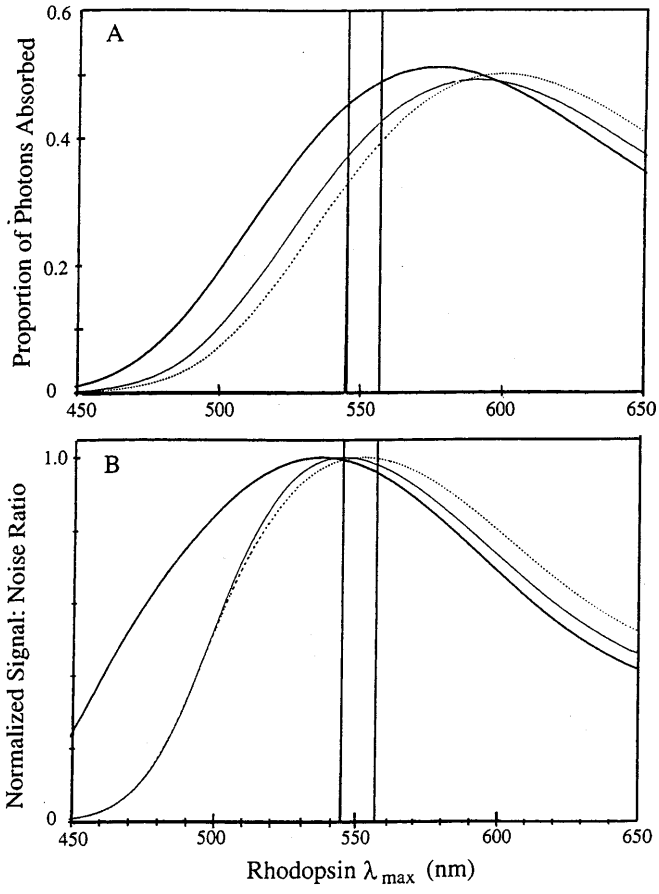

Fig. 7A, B Absorption of bioluminescence by rhodopsins of different λ_{max}. **A** Fraction of all bioluminescence from conspecific emitters absorbed by inner photoreceptors of *P. pyralis* (*thin trace*), *P. scintillans* (*dotted trace*), and *Ph. versicolor* (*thick trace*). Computations as in Fig. 5, with rhodopsin λ_{max} ranging from 450 nm to 650 nm. *Vertical lines* indicate the estimated rhodopsin λ_{max} of *P. pyralis* and *Ph. versicolor* (545 nm) and *P. scintillans* (557 nm). The graph is scaled to show the proportion absorbed by an inner rhabdom of all photons originating from conspecific bioluminescence that enter the rhabdom tip (above the filtering pigment). **B** Normalized signal:noise (S:N) ratios versus rhodopsin λ_{max} for detection of conspecific bioluminescence by *P. pyralis* (*thin trace*), *P. scintillans* (*dotted trace*), and *Ph. versicolor* (*thick trace*). S:N ratio is defined as the signal arising from the bioluminescence alone divided by the total signal arising from a 'white-noise' background having equal photon intensity at all wavelengths, 400–700 nm. The *vertical lines* indicate the estimated rhodopsin λ_{max} of the species, as in **A**

capturing photons arriving from conspecific bioluminescence in these three species. These curves were generated by dividing the product of sensitivity and normalized bioluminescence by the area under the luminescence curve (summing at 1-nm intervals from 400 nm to 700 nm), using Eq. 1:

$$P = \frac{\Sigma\, S(\lambda)E(\lambda)}{\Sigma\, E(\lambda)} \qquad (1)$$

where P is the total photon catch, and $S(\lambda)$ and $E(\lambda)$ are the absolute sensitivity and normalized emission spectral values, respectively (curves in Figs. 5, 6).

As expected, the results show that in *P. pyralis* and *P. scintillans*, species that luminesce in the yellow and whose screening pigments have longer cut-on wavelengths, more of the signal is captured using a relatively long-wavelength rhodopsin, optimally with λ_{max} near 590–600 nm. The actual rhodopsins of these species capture about 20–25% fewer photons than the maximum possible. Even in *Ph. versicolor*, the strongest signal requires a visual pigment peaking near 575 nm, although the cost of using a middle-rhodopsin (near 545 nm) is only about 10%. Because its screening pigments transmit at shorter wavelengths, *Ph. versicolor* always outperforms the twilight species in capturing a bioluminescent signal except for cases of extremely long-wavelength rhodopsins. In fact, few insects make long-wavelength rhodopsins, and if there is a loss of thermal stability or quantum efficiency with increasing λ_{max}, middle-wavelength rhodopsins may represent the best compromise between peak absorption and optimal visual function.

Thus, the rhodopsins we identified produce some decrease in potential sensitivity to conspecific flashes in *Ph. versicolor*, and in the twilight-active species of *Photinus* there is a fairly severe sensitivity penalty. *Ph. versicolor* is active late in the evening, searching for flashing mates after dark when there is very little ambient light reflected from the direction of foliage. Contamination of this signal by environmental light only becomes a serious problem when bright moonlight penetrates the overlying forest canopy, and at such times natural flashing activity is observed to decline. In contrast, both species of *Photinus* are active at twilight, and their visual systems must not only capture the light of bioluminescent flashes, but also discriminate them from the wash of light reflected from green foliage and other objects (see also Seliger et al. 1982b). Unlike *Ph. versicolor*, a primary signal-detection task in the *Photinus* species is to reject the 'noise' of light reflected from blades of grass or other objects on which females perch, maximizing the contrast of their signal against the background.

We used a very simple approach to examine contrast enhancement in *P. pyralis*, comparing the 'signal' arriving from the bioluminescence of conspecifics to the 'noise' produced by broad-band white light:

$$Contrast = \frac{\Sigma\, S(\lambda)E(\lambda)}{\Sigma\, S(\lambda)N} \qquad (2)$$

where N represents an intensity, constant at all wavelengths (400–700 nm), ranging here from 0.001 to 1.0 and representing the ratio of the background noise throughout the spectrum to the peak height of bioluminescence. The use of a flat white spectrum instead of a particular reflectance spectrum (for example, from photosynthetic pigments of leaves) provides, if anything, a pessimistic estimate of contrast, since overhead light at twilight is very blue, and the yellow emissions of *P. pyralis* and *P. scintillans* actually lie in spectral locations where the reflectance spectrum of green vegetation at twilight is at a minimum (see Seliger et al. 1982b).

The results of this analysis (Fig. 7B) demonstrate convincingly that the 545-nm rhodopsin of *P. pyralis* or the 557-nm pigment of *P. scintillans*, in combination with the filtering pigment of each, indeed maximize the brightness ratio between a yellow to yellow-orange bioluminescent flash and a flat white background (and presumably other, natural backgrounds for the reasons just described). This result holds for very dim backgrounds, only 0.1% of the brightness of the peak luminescence, as well as for backgrounds at least as bright as the flash peak itself. An inspection of Fig. 6 shows why this should be so; as first demonstrated by Seliger et al. (1982a, b), matching the shape of the sensitivity function to the luminescence literally screens out virtually all environmental photons acting as 'noise' (for example, skylight or reflections from background foliage), leaving open a window that admits few photons besides those emitted within the expected luminescent flash.

In earlier analyses (Seliger et al. 1982a; Lall et al. 1988), it was assumed that both *P. pyralis* and *P. scintillans* have the same visual pigment, with $\lambda_{max} = 550$ nm. The requirement to match narrow yellow visual spectral sensitivity curves (Lall et al. 1988) to bioluminescence in these species required screening pigments of different maximum densities, estimated at 1.6 in *P. pyralis* and 2.2 in *P. scintillans*. Such a dense pigment layer would severely attenuate absolute sensitivity in *P. scintillans*, and the presence of a visual pigment with λ_{max} placed even a few nanometers towards longer wavelengths produces the desired tuning with a significant improvement in overall sensitivity.

In summary, twilight-active species of *Photinus* enhance their detection of flash signals by optimizing contrast between the yellow flash and the blue or green background, whereas night-active *Ph. versicolor* is tuned for an increased capture of photons from the luminescent signals of conspecifics. While the twilight species (*P. pyralis, P. scintillans*) fit the classic model of sensory evolution to maximize signal contrast (see also Seliger et al. 1982b), *Ph. versicolor* may have a visual system that makes a compromise between the demands of optimizing signal strength and signal contrast (see Lythgoe 1972 and McFarland and Munz 1975 for a discussion of sensitivity and contrast in vision). Its 545-nm visual pigment is placed at wavelengths a bit too short for maximal capture of bioluminescence (Fig. 7A), but a bit too long for greatest signal-to-noise ratio (Fig. 7B). Since the nocturnal photic environment within which it searches for answering flashes may be variably, and unpredictably, dark (and since there must be at least enough light to permit flight), this evolutionary trade-off seems warranted. Clearly, if *Ph. versicolor* is concerned solely with maximizing sensitivity, there would be no point in incorporating a yellow screening pigment into its retina.

In all cases, natural selection acts on these firefly species to produce the same result: the detection with high reliability the signals of potential mates under the photic conditions of their visual worlds. This is achieved by the coordinated tuning of bioluminescent emission to visual spectral sensitivity, and by the rejection of potentially contaminating environmental light. In its path to this common goal, evolution has subtly adjusted *both* the colored screening pigments of the eye *and* the middle-wavelength visual pigments placed in the photoreceptors at the base of the retina. Fireflies occupy photic environments that are relatively simple and predictable, and their long-distance signals are carried exclusively by bioluminescence that has highly consistent spectral properties. Consequently, they provide the best example we have of a visual system that is tuned almost exclusively to optimize the success of intraspecific signaling.

Acknowledgements Our warmest thanks go to Ms. Riitta Harjula, who provided valuable assistance with the microscopy. All experiments are in compliance with current animal care guidelines of the University of Maryland system. This report is based on work supported by the National Science Foundation under grants number BNS-8917183, IBN-9413357, and IBN-9724028 (to TWC), and BNS-9013076 (to ABL), and by the NIH under grant number N16MS-SO-66-MO-8016 (to ABL).

References

Biggley WH, Lloyd JE, Seliger HH (1967) The spectral distribution of firefly light. II. J Gen Physiol 50: 1681–1692

Buck JB (1937) Studies on the firefly. II. The signal system and color vision in *Photinus pyralis*. Physiol Zool 10: 412–419

Chittka L (1996) Does bee color vision predate the evolution of flower color? Naturwissenschaften 83: 136–138

Cronin TW (1985) The visual pigment of a stomatopod crustacean, *Squilla empusa*. J Comp Physiol 156: 679–687

Cronin TW, Forward RB Jr (1988) The visual pigments of crabs. I. Spectral characteristics. J Comp Physiol A 162: 463–478

Endler JA (1993) Some general comments on the evolution and design of animal communication systems. Philos Trans R Soc Lond B 340: 215–225

Exner S (1891) Die Physiologie der facettierten Augen von Krebsen und Insecten. Deuticke, Leipzig

Horridge GA (1968) Pigment movement and the crystalline threads of the firefly eye. Nature (Lond) 218: 778–779

Horridge GA (1969) The eye of the firefly *Photuris*. Proc R Soc Lond Ser B 171: 445–463

Juusola M, Kouvalainen E, Järvilehto M, Weckström M (1994). Contrast gain, signal-to-noise ratio, and linearity in light adapted blowfly photoreceptors. J Gen Physiol 104: 593–621

Lall AB (1981) Electroretinogram and the spectral sensitivity of the compound eyes in the firefly *Photuris versicolor* (Coleoptera: Lampyridae): a correspondence between green sensitivity and species bioluminescence emission. J Insect Physiol 27: 461–468

Lall AB, Seliger HH, Biggley WH, Lloyd JE (1980) Ecology of colors of firefly bioluminescence. Science 210: 560–562

Lall AB, Lord ET, Trouth CO (1982) Vision in the firefly *Photuris lucicrescens* (Coleoptera: Lampyridae). J Comp Physiol 135: 21–27

Lall AB, Strother GK, Cronin TW, Seliger HH (1988) Modification of spectral sensitivities by screening pigments in the compound eyes of twilight-active fireflies (Coleoptera: Lampyridae). J Comp Physiol A 162: 23–33

Land MF (1980) Compound eyes: old and new optical mechanisms. Nature (Lond) 287: 681–686

Lythgoe JN (1972) The adaptation of visual pigments to the photic environment. In: Dartnall HJA (ed) Handbook of sensory physiology VII/6 A. Springer, Berlin Heidelberg New York, pp 504–580

McFarland WN, Munz FW (1975) The evolution of photopic visual pigments in fish. Vision Res 15: 1071–1080

Munz FW, McFarland WN (1977) Evolutionary adaptations of fishes to the photic environment. In: Crescitelli F (ed) Handbook of sensory physiology VII/5. Springer, Berlin Heidelberg New York, pp 193–274

Muri RB, Jones GJ (1983) Microspectrophotometry of single rhabdoms in the retina of the honeybee drone (*Apis mellifera*). J Gen Physiol 82: 469–496

Palacios AG, Goldsmith TH, Bernard GD (1996) Sensitivity of cones from a cyprinid fish (*Danio aequipinnatus*) to ultraviolet and visible light. Visual Neurosci 13: 411–421

Peitsch D, Fietz A, Hertel H, Souza J de, Ventura DF, Menzel R (1992) The spectral input systems of hymenopteran insects and their receptor-based colour vision. J Comp Physiol A 170: 23–40

Seliger HH, Buck JB, Fastie WG, McElroy WD (1964) The spectral distribution of firefly light. J Gen Physiol 48: 95–104

Seliger HH, Lall AB, Lloyd JE, Biggley WH (1982a) On the colors of firefly bioluminescence. I. An optimization model. Photochem Photobiol 36: 673–680

Seliger HH, Lall AB, Lloyd JE, Biggley WH (1982a) On the colors of firefly bioluminescence. I. An optimization model. Photochem Photobiol 36: 673–680

Seliger HH, Lall AB, Lloyd JE, Biggley WH (1982b) On the colors of firefly bioluminescence. I. Experimental evidence for the optimization model. Photochem Photobiol 36: 681–688

Weckström M, Lauglin SB (1995) The ecology of voltage-gated ion channels in insect photoreceptors. Trends Neurosci 18: 17–21

Weckström M, Hardie RC, Lauglin SB (1991) Voltage-activated potassium channels in blowfly photoreceptors and their role in light adaptation. J Physiol (Lond) 440: 635–657

Worthy KM, Lall AB (1996) Action spectrum of the female response in firefly *Photinus pyralis* (Coleoptera: Lampyridae). In: Soc Neurosci Abstr 22:1145 (no. 452.20)