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Colour vision in the glow-worm *Lampyris noctiluca* (L.) (Coleoptera: Lampyridae): evidence for a green-blue chromatic mechanism

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Summary

Male glow-worms *Lampyris noctiluca* find their bioluminescent mates at night by phototaxis. There is good evidence that location of mates by lampyrid beetles is achieved by a single spectral class of photoreceptor, whose spectral sensitivity is tuned to the bioluminescent spectrum emitted by conspecifics, and is achromatic. We ask whether glow-worm phototaxis involves interactions between two spectral classes of photoreceptor. Binary choice experiments were conducted in which males were presented with artificial light stimuli that differ in spectral composition. The normal preference for a green stimulus (λ_{max}=555 nm), corresponding to the bioluminescence wavelength produced by signalling females, was significantly reduced by adding a blue (λ_{max}=485 nm) component to the signal. This implies an antagonistic interaction between long- and short-wavelength sensitive photoreceptors, suggesting colour vision based on chromatic opponency. Cryosections showed a band of yellow filter pigment in the fronto-dorsal region of the male compound eye, which could severely constrain colour vision in the dim conditions in which the insects signal. This apparent paradox is discussed in the context of the distribution of the pigment within the eye and the photic niche of the species.

Key words: *Lampyris noctiluca*, glow-worm, colour vision, phototaxis.

Introduction

Glow-worms and fireflies (Lampyridae) are well known for using bioluminescent sexual signals. In common with many nocturnal insects they possess superposition eyes that are well adapted to vision in dim light (Horridge, 1969; Land and Nilsson, 2002)

In a classic series of neuroethological studies, Lall and colleagues (Lall, 1981; Lall et al., 1980, 1988) measured bioluminescence and visual sensitivity in a range of North American firefly species. The bioluminescence and the spectral sensitivity of the photoreceptors vary according to the time after sunset, and hence light level, when the species is normally active. Lampyrid emission spectra peak in the range 545–575 nm. Nocturnal species use shorter wavelengths than crepuscular fireflies (Lall et al., 1980), and peak spectral sensitivity of the photoreceptors matches the spectral peak of the bioluminescent emission. It is argued that this shift of spectra and spectral sensitivities probably optimises detectability of bioluminescent signals within species-specific photic niches. Possible reasons for the shift are: (i) that under relatively high ambient illumination the detectability of the bioluminescence is enhanced by their having spectra displaced from the leaf-background (Seliger et al., 1982a), whereas under low illumination contrast with the background is less important, or (ii) the communication system needs to minimise receptor noise (i.e. photon-noise plus dark-noise). Dark-noise arises from thermal isomerisation of photopigment which, at least in vertebrates, may occur at a rate that increases with wavelength (Rieke and Baylor, 2000; but see Koskelainen et al., 2000). As light intensity falls, the importance of dark-noise relative to photon-noise increases, and this will favour a blue-shift by the photopigment.

The presence of coloured filter pigments within the eyes of fireflies (Cronin et al., 2000; Lall et al., 1988) somewhat complicates the evolutionary interpretation of this communication system. These filter pigments are thought to narrow the spectral sensitivity of the receptors at the expense of light capture, a trade-off that might be expected for the dusk-active species, but is more surprising for nocturnal species, where one might expect the eyes to maximise light catch and minimise dark-noise (Warrant, 1999).

By comparison with measurements of bioluminescence and electrophysiology of the eyes there are few studies of firefly behavioural action spectra. The response of female *Photinus pyralis* is tuned to the emission spectrum of the male (Lall and Worthy, 2000), and a general implication of the work on fireflies is that the behaviour is achromatic and mediated by photoreceptors tuned to the bioluminescence. However, a striking feature of behavioural responses in fireflies and glow-worms is insensitivity to short wavelength light. Buck (1937) found that the firefly *Photinus pyralis* responded to stimuli

from 520–700 nm, but that wavelengths below 500 nm elicited no response, even when presented at 900 times the intensity of the attractive stimuli. He concluded that these insects could not perceive the shorter wavelengths. Schwalb (1961) similarly reported little response to blue light stimuli in the European glow-worm *Lampyris noctiluca*. More recently, Lall and Worthy (2000) showed that female *Photinus pyralis* fireflies failed to respond to any stimuli of wavelength less than 480 nm, even when these were presented at 100 times the intensity of longer wavelength stimuli. This characteristic sharp cut-off in the behavioural response at short wavelengths has been attributed to the effect of various long-pass filter pigments that have been described from the eyes of several firefly species (Cronin et al., 2000; Lall et al., 1988).

An alternative to optical filtering to account for behavioural insensitivity to short wavelengths is that the beetles have a chromatic mechanism, involving spectral opponency between photoreceptor signals. Although it is clear that the response to the bioluminescence is mediated by the beetles' long (L) wavelength (sensitivity maximum >500 nm) receptors, there are other spectral types of receptor – as required for colour vision. Electroretinogram (ERG) data from North American (Lall et al., 1980) and Japanese fireflies (Eguchi et al., 1984) show two sensitivity peaks, corresponding to L and UV (<400 nm) receptors. In addition, a separate short (S) wavelength peak (c. 450 nm) was suggested by selective adaptation experiments on some species of *Photuris* (Lall et al., 1982, 1988) and three Japanese species including *Luciola lateralis* (Eguchi et al., 1984). To date the function of S photoreceptors has remained elusive. Lall (1993) postulates that they may be involved in the initiation of bioluminescent flashing behaviour in crepuscular fireflies, being used to detect when ambient light levels decline to twilight intensity.

This study examines the spectral selectivity of lampyrid behaviour using behavioural tests as well as investigating the distribution of coloured filter pigment in the European glow-worm *Lampyris noctiluca*. We present the results of binary choice laboratory experiments where males were presented with artificial light stimuli simulating the female bioluminescent signal. We also describe the intraoccular filter pigments from the eye of *Lampyris noctiluca*. *L. noctiluca* is a convenient subject because its signalling behaviour allows binary choice experiments. The flightless female produces a constant greenish glow from composite abdominal light organs, which attracts the winged male (Tyler, 2002). The species is active only after the end of dusk, when ambient light levels are less than 1.7 lx (Dreisig, 1971), placing them in the same nocturnal photic niche (Lall et al., 1980) as the green-emitting North American *Photuris* fireflies. There is no flash dialogue between the sexes.

We test for colour vision by a procedure first used in 1888 by Lubbock for phototaxis in *Daphnia*, and since applied to phototactic responses in various other animals (for a review, see Kelber et al., 2003). The logic is simple; first it is demonstrated that an animal is attracted to light of a given spectral composition *a* (e.g. long wavelengths), and that attractiveness increases with intensity. Next light with a different spectrum, *b* (e.g. short wavelengths) is added to the original stimulus. If the addition of *b* reduces the attractiveness of *a* we can conclude that the animal has colour vision mediated by an antagonistic interaction between the outputs of different spectral types of receptor (Kelber et al., 2003).

Materials and methods

Experimental animals

Adult male glow-worms *Lampyris noctiluca* L. were collected during July 2002 from an established colony near Lewes in East Sussex, UK, using battery powered light lures, each incorporating a green (c. 550 nm) LED. Males were kept under reversed day–night conditions so that the experiments could take place during normal working hours. The circadian clock could be resynchronised easily by delaying the onset of darkness by 12 h (following Dreisig, 1978), after which the insects were subjected to a reversed 18 h:6 h day:night cycle in an illuminated incubator. Males were housed separately in individually numbered plastic containers and kept in darkness between experimental runs. Twenty six males were used in the experiments.

Experimental arena

Behavioural experiments were conducted in a plywood arena measuring 120 cm long×80 cm deep×30 cm high, with a hinged mesh top. The floor of the arena was marked out in a 20 cm×20 cm grid to enable the insects to be tracked during each experimental run. The room was dark, but the arena was illuminated with infra-red light (>690 nm), to which most insects are insensitive (Goldsmith and Bernard, 1974). This light was provided by four 30 W luminaires, each comprising an incandescent striplight behind infra-red filter Perspex. Two were mounted externally on each of the long-axis walls of the arena such that the illumination 'windows' were flush with the interior. All cracks were sealed to exclude stray light.

Experimental light stimuli were provided using two identical units mounted at one end of the arena. These were spaced 50 cm apart and 5 cm above the arena floor. The light sources each comprised a composite three-colour LED component with blue (peak 485 nm), green (peak 555 nm) and red (peak 640 nm) diodes focused into a common diffuser. Rotary controls controlled the intensity of each LED individually. The light output of each source was measured at 2 mm from the diffuser using a calibrated spectroradiometer (USB2000, Ocean Optics, BD, Duiven, The Netherlands).

Movements of the experimental insects within the arena were monitored with a gantry-mounted Sony AVC-D7CE CCD camera 1.2 m above the arena floor and facing downwards. A VCR (Panasonic) recorded all experimental runs.

Design of choice experiments

Individual male glow-worms were presented with a choice between two different light stimuli. Animals were introduced singly into the arena through a small aperture at the opposite

end to, and facing, the two light stimuli. At this point of entry these stimuli were equidistant from the insect. Each choice test was replicated using separate runs with different males, with the positions of the stimuli being set randomly to one of the two alternative arrangements for each run. Departure of choices from an expected 1:1 ratio were tested using two-tailed binomial tests (Zar, 1984). Deliberately stringent criteria were applied in assessing the males' responses. Only those runs in which the male made close contact with a light source, or very definite and repeated attempts to do so, were scored as a choice for that particular signal. The run was aborted if there was no response within 2 min. All experiments took place within 1.5 h of the onset of the dark period, since we found that the insects tended to become very unresponsive after this, an observation that seems to reflect their normal behaviour in the field (Schwalb, 1961).

Cryosection and microscopy

A small number of male glow-worms were chloroformed and their heads detached. As much of the hydrophobic cuticle as possible was removed from around the eyes. The preparations were fixed for 1 h at room temperature in 4% paraformaldehyde (in 0.2 mol l^{-1} phosphate-buffered saline at pH 7.2) under gentle agitation, and then cryoprotected by infusion in 30% sucrose solution overnight in darkness at 5°C. The prepared eyes were then quickly frozen and transferred to a Leica Microsystems (Milton Keynes, UK) CN3000 cryostat at –25°C, where 16 nm sections were taken. Freshly cut sections were transferred immediately (within 5 min of cutting) to a Zeiss (Welwyn Garden City, UK) Axiophot microscope for inspection and photography.

Results

Choice experiments

We tested first whether the insects discriminated between identical-wavelength stimuli of differing intensity (Fig. 1A). Animals had a choice of two green (λ_{max}=555 nm) stimuli, G1 and G2, set at 2.52 cd m^{-2} and 5.11 cd m^{-2} (at 555 nm), respectively. The intensities and spectral composition of the lights resemble the emission measured from a live female (3.8 cd m^{-2} at 555 nm, N=3; average luminescent area 6.5 mm^2). The males preferred the brighter stimulus (3:18, G1:G2; P=0.0015), confirming the results obtained by Schwalb (1961).

Next we tested the effect of adding blue (λ_{max}=485 nm) light to G2, leaving the dimmer green, G1, unchanged (Fig. 1B). The addition of blue reduced the attractiveness of G2 below that of G1 (20:3, G1:G2+blue; P=0.0005).

However, when a red component (λ_{max}=640 nm) was added to G2, with the green stimulus intensity kept constant (Fig. 1C), there was no aversive effect (0:9, G1:G2+red; P=0.004).

Filter pigments in the eye

Male *L. noctiluca* have a band of bright yellow filter pigment present in the frontal and dorsal regions of the compound eye. This pigment appears to lie between the inner and outer parts of the retina (i.e. the crystalline cones and the photoreceptor microvilli), much like that of the nocturnal American firefly *Photuris versicolor* (Cronin et al., 2000). The filter pigment was absent from ventral regions of the eye. Fig. 2A shows a single vertical section taken from the lateral part of the eye, while the yellow pigment is similarly absent from frontal ventral regions (Fig. 2B).

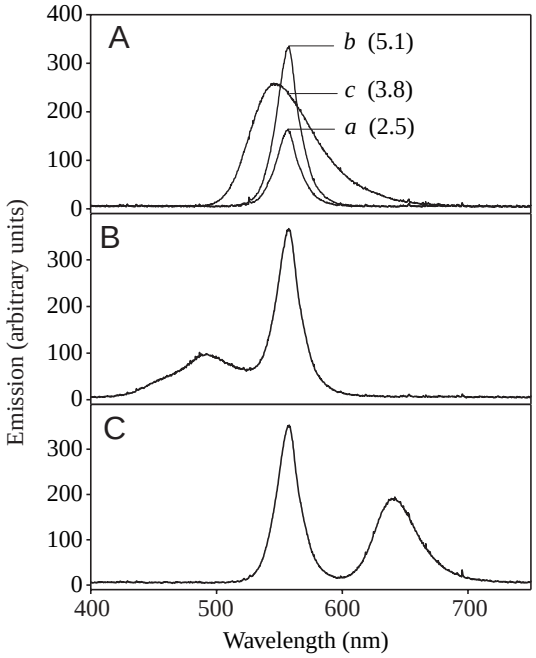

Fig. 1. Emission spectra (with arbitrary spectroradiometer photon units on *y* axis) of light stimuli used in behavioural experiments, with absolute values (cd m^{-2} at 555 nm) for comparison. (A) Green LED stimulus G1 (*a*), green LED stimulus G2 (*b*) and female bioluminescent emission (*c*). (B) Green (G2) with blue. (C) Green (G2) with red.

Discussion

We define colour vision as the ability to distinguish between lights on the basis of their spectral composition, regardless of their relative intensities (Kelber et al., 2003). This entails comparison between the outputs of photoreceptors with differing spectral sensitivity. Our behavioural experiments imply that an antagonistic interaction between the long- (L; λ_{max} >500 nm) and short- (S; λ_{max} <500 nm) wavelength-sensitive photoreceptors mediates the response of male *L. noctiluca* towards female bioluminescence. This finding explains the lack of response to bright short-wavelength light in previous studies on this and other lampyrids (Buck, 1937; Lall and Worthy, 2000; Schwalb, 1961), and contrasts with the suggestion (Cronin et al., 2000; Lall et al., 2000) that such signal detection in the Lampyridae is achromatic.

Nonetheless, there is compelling evidence from previous

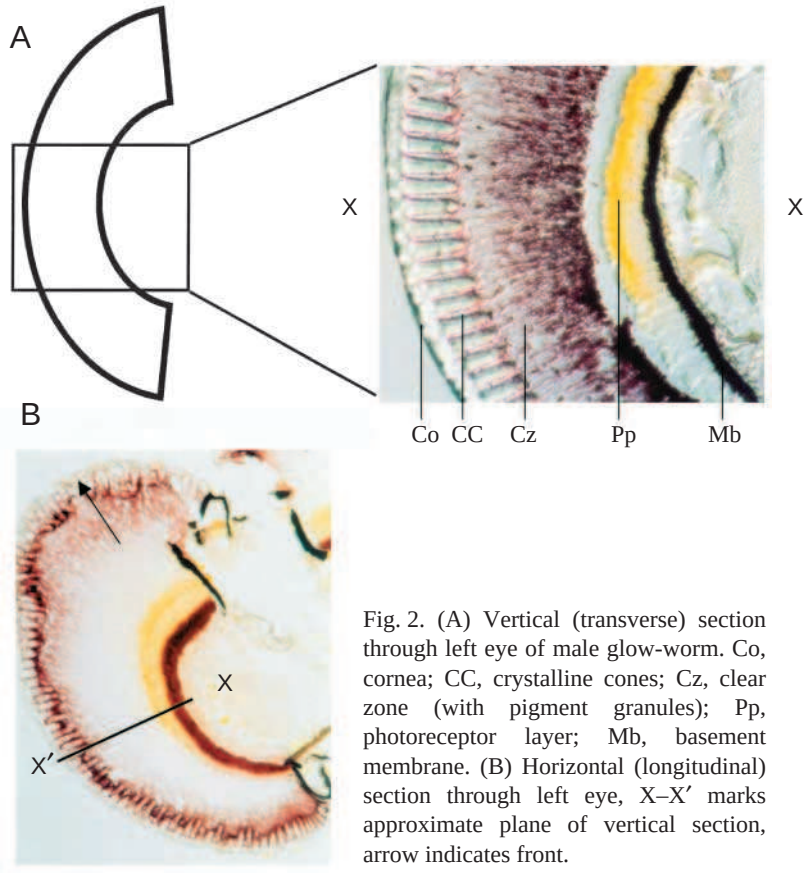

Fig. 2. (A) Vertical (transverse) section through left eye of male glow-worm. Co, cornea; CC, crystalline cones; Cz, clear zone (with pigment granules); Pp, photoreceptor layer; Mb, basement membrane. (B) Horizontal (longitudinal) section through left eye, X–X′ marks approximate plane of vertical section, arrow indicates front.

studies that the spectral tuning of visual sensitivity to bioluminescence in lampyrids is accomplished by species-specific pairings of an L photoreceptor with an overlying long-pass filter pigment (Cronin et al., 2000; Lall et al., 1988).

The use of colour vision by these North American species would not negate the significance of such systems in the visual ecology of the Lampyridae; rather we propose the existence of another component that may operate synergistically with the tuned photoreceptor/filter pigment pairs. The relative contribution of each component to sexual signalling behaviour, however, may differ between species occupying divergent photic niches.

The possibility of colour vision in the Lampyridae has been mooted (Lall et al., 1982), but until now there has been no direct evidence for chromatic opponency. There are a number of possible explanations as to why colour vision was not found. Firstly, our study animal, *L. noctiluca*, allows simple binary choice experiments that are more difficult in species that have a flash dialogue between the sexes. Moreover, few experiments have measured behavioural, rather than electrophysiological, responses to spectral lights. A notable exception was the recent measurement of behavioural action spectra in the twilight-active firefly *Photinus pyralis* (Lall and Worthy, 2000). While this experiment was not designed to test for opponency between L and S receptors, the data presented do suggest that *P. pyralis* females can perceive light at shorter wavelengths (<480 nm) but do not respond to it.

Blue photoreceptors and yellow filters: a paradox?

Male glow-worms fly at light intensities at which humans are colour blind. Colour vision in dim light is problematic due to photon-noise (Land and Osorio, 2003). The yellow (blue-absorbing) pigment apparently overlying blue photoreceptors in the glow-worm eye presents an intriguing paradox because the resultant reduction in photon catch would tend to restrict green-blue colour vision still further.

However, the yellow filter pigment in *L. noctiluca* is not evenly distributed across the visual field (Fig. 2). Pigmentation is most pronounced in the frontal-dorsal region, and is reduced, or absent, in the ventral- and dorsal-most portions. Although their precise flight patterns have not been studied, male glow-worms are believed to mate-search by flying low over the vegetation (Tyler, 2002), so the ventral retina probably plays a vital role in the initial stage of mate location. In our experiments the males were not overflying the light stimuli, and in most cases the choice between stimuli was made as the male walked along the arena floor. It is difficult to explain how the ventral-most portions of the eyes might have been used to compare the two signals in this situation.

Until we have resolved the position of the yellow pigments in the optical pathway with respect to the short-wavelength photoreceptors we cannot be certain to what extent the pigments screen the receptors, even in those ommatidia where the former occur. Lampyrids have a three-tiered retina (Hariyama et al., 1998; Horridge, 1969) in which a distal rhabdomere overlies the main bundle of rhabdomeres, with a single, small, basal rhabdomere close to the basement membrane. It is therefore conceivable that at least some rhabdomeres protrude through the filter pigment.

Lall et al. (1988) found that in the nocturnal firefly *Photuris frontalis* near-UV and blue sensitivity was most acute in the dorsal-frontal region of the eye, which is where our results suggest the yellow pigment is most dense. Further, this species did not exhibit the expected attenuation of spectral sensitivity ($S\lambda$) below 500 nm, which is expected from the spectral properties of the yellow filter pigments. Given that the short-wavelength photoreceptors almost certainly play a part in flight orientation (Kelber, 1999; Lall, 1993, 1994), the primary function of these filter pigments in nocturnal lampyrids may be more concerned with shielding the sensitive eye from skylight than with signal discrimination. In this regard the distinction should be made between nocturnal species, such as *L. noctiluca*, and the crepuscular North American fireflies: the latter possess filter pigments that are quite different in both spectral absorbance properties and location within the eye (Cronin et al., 2000), and it has already been suggested that the

role played by filter pigments may differ between nocturnal and crepuscular species (Lall et al., 1988).

Nocturnal colour vision and detection of bioluminescence

Until recently there has been little work on colour vision of insects at low light intensities (but see Menzel, 1981; Rose and Menzel, 1981). Photon noise limits vision at low intensities (Land and Nilson, 2002), and because colour vision is based on a comparison of two receptor signals, and these differences are relatively small, one might expect colour to be of little use at night. However recent work demonstrates that the nocturnal hawk moth *Deilephila elpenor* (Kelber et al., 2002), sees colour of (model) flowers at starlight intensities (10^{-3}–10^{-4} cd m^{-2}). *Lampyris noctiluca* is also strictly nocturnal, but the problems of locating flowers by reflected starlight and bioluminescence are different. The bioluminescent emission of the female glow-worm (3.8 cd m^{-2} at 555 nm) is a brighter target. Under such conditions one might assume that colour vision is less limited by noise than by reflected light. The female's colour is indeed visible to humans from several metres (Tyler, 2002), as were the test stimuli used in our experiments. However this fact alone does not explain why *L. noctiluca* uses colour vision. First, the female's light is likely to subtend a smaller angle than the receptive field of a single photoreceptor, so its effective intensity is lower than 3.8 cd m^{-2}. At a range of 1 m a 3 mm spot subtends c. 0.35° whereas the acceptance angle ($\Delta\rho$) of the photoreceptors (at 50% max sensitivity) is at least 2°. Assuming $\Delta\rho$=2°, the effective brightness of the female is <10% (7.5%) that of an extended source, although this disadvantage is offset by the relatively low *f*-number of beetle superposition eyes (Land and Nilsson, 2002). More important, photon noise will be higher in the S-receptor than the L-receptor. Thus S-receptor noise will dominate any L–S chromatic signal, in effect giving colour vision performance expected for effective intensity experienced by the S-receptor *not* the L-receptor.

Conclusions

The matching of visual sensitivity and bioluminescent signalling to ecological constraints in the Lampyridae remains an excellent case study of the coevolution of signals and sensors. There is convincing evidence that interspecific differences in signal wavelength are adaptations to different light environments or 'photic niches' (Cronin et al., 2000; Lall et al., 1980; Seliger et al., 1982b). Coloured filter pigments have been found in every species so far examined, with spectral absorbance properties that imply a role in tuning spectral sensitivity to match the species' bioluminescence (Cronin et al., 2000; Lall et al., 1988). Our results show that in addition to possessing intraocular filter pigments, *L. noctiluca* is able to distinguish light signals on the basis of chromaticity.

Glow-worm colour vision is probably attributable to an opponent interaction between long and short-wavelength photoreceptors. The use of colour vision is not consistent with the notion that under low ambient illumination photon capture alone limits detection of bioluminescent signals by lampyrids (Seliger et al., 1982b). Were this the case an achromatic mechanism would be best. The presence of colour vision implies that even in their nocturnal photic niche the insects need to discriminate between bioluminescence and background noise (such as reflections of moonlight from water droplets or wet leaves), or possibly between species (although in England there is only one).

Further work is required to understand the visual ecology of luminescent lampyrids, and to establish the extent to which colour vision is used for bioluminescent signal discrimination in other members of the Lampyridae. For example, studies are needed to establish whether the balance between colour vision and achromatic contrast-enhancement strategies differs between the nocturnal and crepuscular fireflies. A key question is whether glow-worm phototaxis is based purely on a chromatic signal, or if there is also an achromatic component to this behaviour.

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