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Larval and Adult Emission Spectra of Bioluminescence in Three European Firefly Species¶

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ABSTRACT

We studied the spectral characteristics of the larvae of three sympatric Belgian species of fireflies, *Lampyris noctiluca*, *Phosphaenus hemipterus* and *Lamprohiza splendidula*. An *in vivo* spectral study was performed to compare bioluminescence spectra. The emission spectrum of a laboratory reared female *L. noctiluca* was recorded by a different, more exact method. The mean peak wavelength (λ_{max} = 546 nm) and shapes of the unimodal emission spectra are visually similar for the larvae of all three species. The emission spectrum of the adult female *L. noctiluca* peaked in the same range as the larval bioluminescence between 546 and 551 nm. The bandwidth at half-maximum intensity was slightly greater for larval *L. noctiluca* (77 ± 4 nm) compared with *P. hemipterus* (70 ± 10 nm). The bandwidth of larval *L. splendidula* (77 ± 8 nm) was not different compared with the other larvae, whereas the females' bandwidth was somewhat narrower (68 nm). The ecological significance of the color of bioluminescence and conservancy of green emission in larval fireflies and other luminescent beetle larvae is discussed.

INTRODUCTION

Fireflies are one of the best-known examples of luminescent organisms, but most studies have focused on the importance of light in the adults where it is known to be used in courtship signals (1–3). Interspecific recognition is not only made possible by light organ patterns (4) or flash codes (1,2), but also by the timing and place of activity whether combined or not with the color of the emitted light (5–7). In some cases, the color of the adult light shows sexual dimorphism (7), or the color of emission appears to be evolutionarily tuned for maximum discrimination of conspecific signals from spectrally broader backgrounds, depending on the type of habitat and time-dependent ambient light conditions (5–8).

However, the larvae of all firefly species produce light as well, and fireflies spend most of their lifetimes in this stage (2–3 years *vs* ~10 days as an adult). Although firefly larvae use the same biochemical reaction of the luciferase–luciferin system to produce light, their luminescence differs from adults because of the use of isozymes (9,10), differences in location, morphology and physiology of the light organs (11,12), often resulting in a different color of bioluminescence and behavioral displays (6). Most studies that report *in vivo* bioluminescence emission spectra or describe the color of bioluminescence only deal with the adults (8,13). Exceptions are some recent reports on Brazilian fireflies (6,14) and an earlier European study using less precise photographic techniques (4), where the adult and larval colors of bioluminescence are compared.

Few spectral studies have been carried out on European fireflies in general. Apparently no *in vivo* emission spectra have been published yet, and the only descriptions found were for the common European glowworm, *Lampyris noctiluca*: *in vitro* peak emission (λ_{max}) = 555 nm (15), *in vivo* adult female λ_{max} = 550.5 ± 7.2 nm STE (standard error) and bandwidth at half-maximum emission 62.7 ± 2.5 nm STE (13). Schwalb's study (4) suggests that the spectral maxima in *Lamprohiza splendidula* and *L. noctiluca* are identical for larvae and adults of both species and lie within a bandwidth of 550–580 nm. However, to the naked eye, the color of emission seems identical among these species, but only spectrographic analysis can prove this.

The present study was originally carried out to compare the larval bioluminescence of *L. noctiluca* with the color of light-emitting diodes that were used to mimic larval glows in experiments on bioluminescent aposematism (16,17). In Central Europe, two other firefly species often live sympatrically with *L. noctiluca*. To gain insight into the ecology of spectral colors of larval bioluminescence, we also recorded the emission spectra of *Phosphaenus hemipterus* and *L. splendidula*. Quite exceptionally for firefly larvae, the bioluminescence in the genus *Lamprohiza* (and in the closely related American genus *Phausis*) comes from a variable number of light organs. In *L. splendidula* there are between two and 12 usually paired ventrolateral spots in the second to sixth abdominal segments (4). Additionally, the light from *L. splendidula* also shines through less pigmented spots of the expanded dorsal cuticle. Therefore, the light might be filtered in such a way that the color differs from ventrally shining light. The more usual situation in firefly larvae is one pair of ventral light organs in the eighth abdominal segment (18). Interspecific distinction between species may be some of the possible outcomes if the colors of larval bioluminescence differ between sympatric species. This would then offer arguments for hypotheses about intraspecific communication between larvae or between immature stages and adults (19). On the other hand, many arguments disfavor the possibility of bioluminescent communication among

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Abbreviations: λ_{max}, peak wavelength; STE, standard error; STD, standard deviation.

Table 1. The mean wavelength with STD at λ_{max}, range of λ_{max} in the different scans and mean bandwidth at half-maximum emission with STD for larvae of European firefly species. N shows the total number of scans per species, n the number of tested individuals per species. The emission spectra for *L. splendidula* were measured from dorsal and from ventral views because the larval bioluminescence in this species comes from ventrolateral light organs that also shine through the dorsal cuticle

Species	*P. hemipterus*	*L. noctiluca*	*L. splendidula* (ventral)	*L. splendidula* (dorsal)
λ_{max} (nm)	546 ± 6	546 ± 3	546 ± 4	549 ± 3
Range λ_{max} (nm)	537–558	540–551	542–552	547–553
Mean bandwidth (nm)	70 ± 10	77 ± 4	77 ± 8	89 ± 2
N	19	18	4	4
n	2	2	1	1

larvae (16). In the case that the color of larval bioluminescence is similar between species, a whole array of ecological explanations can be given as well. In this report, we also present the emission spectrum from a live adult female *L. noctiluca* to compare with the larval bioluminescence.

MATERIALS AND METHODS

Collecting larvae. Larvae were collected over several nights in the autumn of 1998. *P. hemipterus* and *L. splendidula* were collected at the "Zoniënbos/Forêt de Soignes", a beech forest South of Brussels (Belgium), where they occur sympatrically. *L. noctiluca* occurs here as well but seems to be more restricted to the forest edges and is not easily found. Instead, we collected larval *L. noctiluca* at the "Hoboken polder" and the "Wijnegempark", nature areas, south and east of Antwerp (Belgium), respectively, where the species is much more abundant.

In vivo spectral study. Glowworm larvae were stimulated to glow by applying a 0.1 *M* solution of the neurotransmitter octopamine directly onto the light organs before putting them in the quartz cells ([15], A.K. Campbell, personal communication). After 15–30 min, all larvae glowed except for *L. splendidula* larvae, for which the method with octopamine failed. Fortunately, one *L. splendidula* larva, which was preparing to molt, could be stimulated easily to glow for some minutes by tapping on the cell. Specimens were immobilized in a quartz cell with some cotton wool. This was done in such a manner that the ventral light organs faced the emission slit of the spectrofluorometer (Aminco-Bowman SLM series 2 spectrometer). Bioluminescence emission was measured from 400 to 700 nm with the excitation shutter of the fluorometer closed. All emission spectra were corrected automatically for the wavelength-dependent instrument response by the internal software of the instrument.

The monochromator band pass for emission measurements was set at 4 nm. The sensitivity was optimized at 800 mV, and the scan rate was 1 nm/s. To determine the luminescence intensity, emission spectra were recorded at different intervals and at least 4 times per specimen. Because of small body movements or maybe sudden changes in luminosity, some of the recorded emission spectra showed artifacts. These data were not used in the analyses. For *L. noctiluca* and *P. hemipterus*, we picked the two brightest shining specimens to obtain a total of, respectively, 18 and 19 moderately smooth emission spectra. We recorded four emission spectra each from ventral and dorsal sides of *L. splendidula*. The maximal intensities of emission spectra were normalized. In this way, the mean relative intensities of the emission spectra were calculated to obtain an averaged emission spectrum for each species.

The emission spectrum from a live adult female *L. noctiluca* was recorded with a LOT Oriel MS127I Imaging Spectrograph with a 600 lines/mm grating and Andor Technology DV42005 CCD head giving a resolution of 0.8 nm. To reduce dark current over a 30 s integration time, the CCD head was cooled to −60°C. Apart from being automatically calibrated by internal software, this setup also allowed the recording of the entire spectrum simultaneously. In that manner, there was no chance of artifacts from the glow changing in intensity during scanning. The female was reared in captivity. A single recording was taken over the range of 450–650 nm.

RESULTS

Table 1 summarizes the number of specimens and the number of records of emission spectra used per species. The mean λ_{max} observed of ventral emission is 546 nm and is the same for all species (Table 1, Fig. 1). The mean λ_{max} viewed dorsally in *L. splendidula* lies somewhat higher at 549 nm. However, a Kruskal–Wallis test showed that the spectral emission peaks did not differ significantly between the species nor between dorsal and ventral light outputs in *L. splendidula* ($H = 3.96$; $P = 0.26$).

Bandwidths of ventral emissions at half-maximum intensity differed significantly between species ($H = 7.35$; $P = 0.03$). However, the only combination yielding a significant difference in bandwidths between species pairs was between *P. hemipterus* and *L. noctiluca* ($H = 6.99$; $P = 0.009$), whereas no differences were detected in tests between *L. splendidula* and *L. noctiluca* or between *L. splendidula* and *P. hemipterus*.

Because the dorsally penetrating light in *L. splendidula* is filtered to *ca* 43% by the cuticle, we normalized it to compare with the ventrally emitted less-filtered light (Fig. 1). The dorsally penetrating light of *L. splendidula* was somewhat more yellowish in hue, attributable to a relatively greater transmission of the long wavelength component of the spectrum. This is reflected in the significantly broader bandwidth at half-maximum intensity compared with the ventral emission in *L. splendidula* ($H = 5.39$; $P = 0.02$). The same was true in comparison with the other species (*P. hemipterus*: $H = 9.57$, $P = 0.002$; *L. noctiluca*: $H = 9.66$, $P = 0.002$).

The emission spectra of the larvae correspond well with the one taken for the adult female *L. noctiluca* (Fig. 1). The latter has a similar shape as the larval spectra, and it peaks around 550 nm (range 546–551 nm). The bandwidth at half-maximum intensity is 68 nm, which suggests that the female spectrum is somewhat narrower than the larval emissions (compare with Table 1). However, the female bandwidth still falls within the range observed in ventral emissions of the larvae of each species.

DISCUSSION

Emission spectra of the studied firefly species

In view of the paucity of published spectra on European fireflies, and on larval fireflies in particular, this report may modestly contribute to the existing literature.

Our results indicate that the mean λ_{max} of light emissions is identical for larvae of the species studied and corresponds chiefly to the lime green color seen by the human eye. Although the general shapes of the emission spectra looked similar, the bandwidth at half-maximum intensity differed between species, with *P. hemipterus* having a slightly narrower emission spectrum than *L. noctiluca*. The λ_{max} as well as the bandwidth at half-maximum intensity of the female lie well within the ranges observed for the larvae. The emission spectra of the larvae closely match that of the female reported here as well as to previously

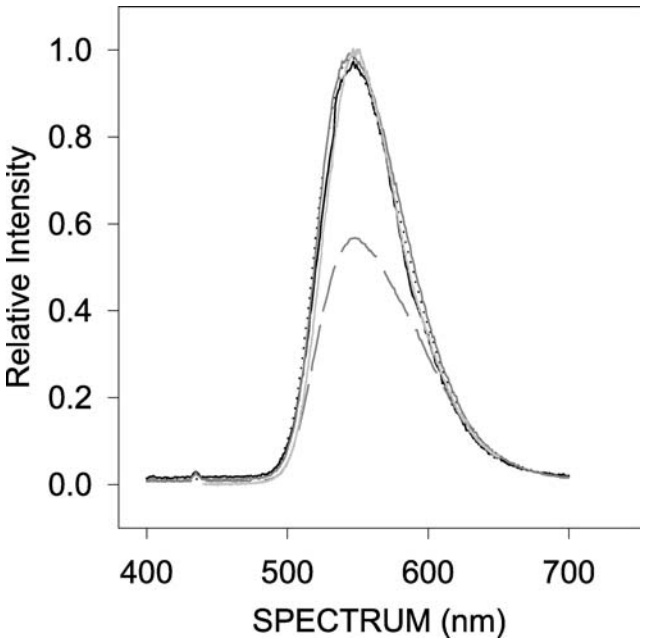

Figure 1. Bioluminescence emission spectra of larval *L. noctiluca* (dotted curve), *P. hemipterus* (solid black curve), *L. splendidula* (ventral luminescence, dark gray curve; dorsal luminescence, dashed dark gray curve) and a female *L. noctiluca* (light gray curve).

reported values (female *L. noctiluca* λ_{max} = 550.5 ± 7.2 nm STE and bandwidth at half-maximum emission 62.7 ± 2.5 nm; [13]). We have no appropriate data on the light intensities in photons per second emitted per surface, but to the eye these seem to be comparable between the species. Schwalb (4) recorded intensities of *in vivo* bioluminescence by measuring the darkening of photographic negatives and found that the maximal intensity per surface of light organ is comparable between larval (and adult) *L. splendidula* and *L. noctiluca*. Of course, the total light production is probably highest in *L. splendidula*. These larvae bear more than one pair of light organs of which several are comparable in size to the light organs in similarly sized larvae of the other species. In addition to light output, the species also differ in bioluminescent behavior. *L. splendidula* only glows when disturbed whereas *P. hemipterus* and *L. noctiluca* also glow spontaneously by emitting glow pulses while active at night (R. De Cock, unpublished). Another difference that distinguishes *L. splendidula* from the other larvae is that octopamine did not initiate glowing, at least when applied superficially. Although more research is needed, this might indicate that the neurophysiology of glowing is different in *L. splendidula*.

Variation in the color of beetle bioluminescence

To describe the color of bioluminescence, we follow Viviani (6), who defined the range 540–557 nm as green, 558–568 nm as yellowish green, and 569–580 nm as yellow. Table 2 compares colors and λ_{max} of beetle larvae cited in the literature and those found in this study. Except for one species, lampyrid larvae typically show green bioluminescence. In relation to the adult stage, the luminescence color in the larval stage often seems greenshifted (6). Yellow luminescence is unknown from lampyrid larvae (14), whereas, depending on the species, firefly adults show green (548–551 nm) to yellow-orange light (570 nm) (6,7,20).

Table 2. λ_{max} and color ranges of *in vivo* bioluminescence of beetle larvae

Taxonomic group	λ_{max} (nm)	Color*	Reference
Lampyridae (fireflies)			
L. noctiluca	546	g	This study
L. splendidula	546–549	g	This study
P. hemipterus	546	g	This study
Bicellonychia spp.	540–557	g	6
Pyrogaster spp.	540–557	g	6
Cratomorphus spp.	548–550	g	14
Aspisoma spp.	558–560	y-g	14
Elateridae (click beetles)			
Pyrophorus spp.	524–560	g, y-g	21
Pyrearinus termitilluminans	537	g	21
Unidentified elaterid wireworm	?	r	22
Phengodidae (Railroad worms)	535–636	g, y-g, y, o, r	24
Rhagophtalmidae	555	g	25
Staphilinidae, unknown sp.	568	y-g	30

*g = green, y-g = yellow-green, y = yellow, o = orange, r = red.

Interestingly, the bioluminescence spectra distribution in click beetles (Elateridae) also shifts from the green to the red region when the insect develops from egg to adult (21). However, elaterid larvae emit only one color of light in the green to greenish yellow range [(21), Table 2], except that of an unidentified red glowing elaterid wireworm from South America (8,22,23). Depending on the species, luminescence in elaterid larvae is either produced constantly following a circadian rhythm or elicited upon disturbance. Light emission by elaterid larvae is used in defensive strategies (21), and in the case of *Pyrearinus termitilluminans*, it functions to attract prey at the same time (24).

Phengodid larvae show the most variation in colors ranging from green through yellow and orange to red (Table 2). In this group, even larvae often emit light of different colors simultaneously, with yellow, orange or red emission from the head in combination with green abdominal lights. In *Phrixothrix* spp., the red headlights shine continuously, and a preliminary electroretinogram study revealed redshifted vision in these larvae, implying that they might use a visual channel not used by their prey (25). Conversely, the green body lights are only lit when disturbed, which suggests a defensive function (25). The color of emission in larval and adult *Rhagophtalmus ohbai*, an Asian relative of the American Phengodidae, is green as well (26).

The intra- and interspecific color differences in luminescence have been shown to reflect differences in the enzymology of bioluminescence. The existence of isozymes in beetle luciferases has already been shown by previous studies (9,10). The natural color polymorphism in beetle bioluminescence seems to be a direct result from substitutions of single amino acids in the primary structures of the luciferases (27–29). In our study, the color of bioluminescence was identical between adult and larval *L. noctiluca*. The seemingly narrower bandwidth at half-maximum intensity in the adult might indicate that stage-dependent luciferase isozymes are also present in *L. noctiluca*.

Conservancy of green bioluminescence in beetle larvae

The conservancy of green emission in beetle larvae opposed to adults agrees with the lack of an intraspecific function (mating) in larvae and the increased importance of an interspecific function such as defense (6). The fact that the dorsally filtered light in

L. splendidula conserves the principal color of the ventrally emitted luminescence also supports this viewpoint. The trend of lampyrid larvae, and most other bioluminescent beetle larvae, to emit similarly colored light, together with the fact that many of these larvae use light as a defense (6,14,16,17,30), allows the possibility that Batesian or Müllerian mimicry could have evolved within and between these taxonomic groups. Displaying green light may not only be adaptive for reasons of mimicry but also to be as conspicuous as possible. Indeed, many vertebrate and arthropod eyes show their highest spectral sensitivity in the green region of the light spectrum (32–37). Furthermore, Lloyd (3), basing his arguments upon the color sensitivity of superposition insect eyes in general, argues that green (adult) luminescence, being the original, plesiomorphic color, originated at some basic level of lampyrid evolution (8,38,39).

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