Steady-state and time-resolved bioluminescence of the firefly *Asymmetricata circumdata* (Motschulsky)

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After *Luciola praeusta* Kiesenwetter 1874, *Asymmetricata circumdata* (Motschulsky) is the second Indian species of firefly identified recently. Here we present steady-state and time-resolved measurements of bioluminescence emissions of male specimens of this new-found species. Steady-state spectra recorded in a high-resolution spectrometer show the peak wavelength at 570 nm, while the same on a colour film in a glass spectrograph show the peak at 579 nm between green and red bands, which prompts speculation that a sharp, laser-like line might exist in the emission spectrum of this species just as the one in *L. praeusta*. The diffraction pattern produced by a grating consolidates this proposition. Flashes recorded in an oscilloscope reveal the appearance of a small pulse in combination with the main one, which becomes prominent both at low and high temperatures.

Keywords: *Asymmetricata circumdata* (Motsch.), bimodal flash, emission spectrum, peak wavelength.

BIOLUMINESCENCE is the production and emission of light by living organisms. Fireflies, along with glowworms, are the best known forms of land bioluminescence. Bioluminescence is a form of 'cold' light emission: less than 20% of the light generates thermal radiation. The process of light production occurs in specialized light-emitting organs, usually in the lower abdomen of the fireflies. The enzyme luciferase acts on the luciferin, in the presence of magnesium ions, ATP and oxygen to produce light.

Numerous studies have been carried out on the spectral distribution of the firefly bioluminescence. Existence of distinct groups of bands in a few species of firefly has also been reported[1–3]. In the emission spectrum of the Indian species of firefly *Luciola praeusta*, it has been shown that the peak wavelength lies at 562 nm, with the full-width-at-half-maximum (FWHM) spreading from 537 to 592 nm (ref. 4). This 55 nm half width is amongst the narrowest reported – barring the 33.3 and 46.7 nm measured for the species *Photinus pyralis* and *Photinus consanguineus* more than a century ago[5]. A study on the structural basis for the spectral difference in luciferase bioluminescence has indicated that the degree of molecular rigidity of the excited state of oxyluciferin, which is controlled by a transient movement of Ile 288, determines the colour of bioluminescence during the emission reaction[6]. Emission spectra recorded on colour films have revealed three colours: green, yellow and red, of which the red is not observable to the naked eye under usual conditions; in that communication[7], it has been inferred that the firefly emission has a tendency for spectral narrowing within the narrow yellow sector. In a recent paper, existence of a sharp intense line at 591 nm has been shown in the emission spectrum of *L. praeusta*, and a hypothesis put forward that the emission mechanism is akin to that of a random laser[8]. Very recently, this has been consolidated by presenting the diffraction pattern in a grating, where the central principal maximum comes out as predominantly yellow, whereas in the other two orders this colour-sector shrink considerably[9]. In a quantitative characterization of the bioluminescence of the North American firefly *Photinus pyralis*, the intensity of the green component has been found to be the only temperature-sensitive quantity that linearly decreased as the temperature increased at pH 7.0 and 8.0. The robustness of the red and orange components has indicated that they had been derived from one excited state of the luciferin–luciferase complex, whose generation and luminescence yield are insensitive to environments[10].

There have been quite a few studies on different aspects of the flashing of fireflies. Measurements on a single flash have shown that the duration varies from about 70 ms (ref. 11) to a few hundred milliseconds[12–15] up to a couple of seconds[16]. It has been shown that the pulses produced by the firefly are manifestations of an oscillating chemical reaction, like the B–Z reaction[17], and that the continuous train of triangular pulses exhibits both pulse amplitude modulation (PAM) as well as pulse width modulation (PWM)[18]. Females of a firefly species have been shown to discriminate between males on the basis of variation in the flash rate of male patterns[11]. It has been found that female *P. pyralis* fireflies prefer flashes of greater intensity and precedence, which suggests that flash 'synchronization' is a competitive display[19]. Regarding the mechanism of flash regulation, it has been concluded that the flash of the adult firefly is controlled by gating of oxygen to the photocytes, and demonstrated that this control mechanism is likely to act by modulating the levels of fluid in the tracheoles supplying photocytes, providing a variable barrier to oxygen diffusion[20]. Nitric oxide (NO), a ubiquitous signalling molecule, has been found to play a fundamental and novel role in controlling firefly flash; it has been proposed that the role of NO is to transiently inhibit mitochondrial respiration in photocytes and thereby increase

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O_2 levels in the peroxisomes[21]. It has also been suggested that firefly flash could be regulated by calcium[22]. Very recently, it has been found that the oxygen consumption corresponding to mitochondria functions exceeds the maximum rate of oxygen diffusion from the tracheal system to the photocytes, and that the flashing mechanism uses a large portion of this maximum rate. Thus it has been concluded that the flashing control requires passivation of the mitochondria functions, e.g. by nitric oxide, and switching of the oxygen supply from them to photoluminescence[23].

Regarding the effects of external factors like temperature and pressure, Lloyd[13] has observed that in four *Luciola* species of fireflies of Melanesia flash periods decrease with increase in temperature. Similarly, studies on inter-flash intervals of *Luciola cruciata* at five different sites in central Japan have indicated significant negative correlation between ambient temperature and inter-flash intervals at any of the five sites[24]. In the study on the exponential decay time of the *in vitro* bioluminescence of *P. pyralis* at various temperatures[10], it has been found that the lifetime is shorter at pH 7.0 than at pH 8.0, lengthening sharply above 30°C at pH 8.0. Very recently, it has been observed that the flash duration of *L. praeusta* changes with change in temperature, and the change is substantially linear, implying that the speed of the enzyme-catalysed chemiluminescence reaction, which produces the light of the firefly, varies linearly with temperature[25]. Against a general contention that high pressure antagonizes anaesthetic actions, Moss *et al.*[26] have reported that pressure does not show any effect on the initial flash intensity of the purified lipid-free firefly luciferase in the presence or absence of anaesthetics. A study measuring the effect of high pressure on the enzyme kinetics has shown that firefly luciferase is not exceptional to other enzymes in responding to high pressure[27]. The maximum light intensity has been observed at about 22.5°C, and pressure has negligible effects on the light intensity at 20–25°C. Recently, time-resolved bioluminescence experiments have been performed for fireflies placed in pulsed and static magnetic fields, and the results prompt speculations that the magnetically induced current inside the firefly in the pulsed magnetic field affects its nervous system or the photochemical processes in the light-producing organ[28], whereas the diamagnetic torque and Lorentz forces induced by the 10 T field have inhibitory and stimulating effects respectively, on the bioluminescence system[3,16].

A few male specimens of the species *Asymmetricata circumdata* (Motschulsky) were collected from Khatkhati, near the Garo Hills in Meghalaya, about 60 km southwest of Gauhati University, India. This species was taxonomically identified by Lezley Ballantyne, Charles Sturt University, Australia, who had previously identified *L. praeusta* as well. It could be mentioned here that unlike *L. praeusta*, this species seldom comes out in the open: it is found in jungle areas. Specimens of found species (Figure 1) are approximately 10 mm in length and 5 mm in width.

Prior to recording the *in vivo* emission spectra, a high-resolution spectrometer (Ocean Optics HR4000 series) was calibrated with standard lines produced from an iron arc. An intensely flashing specimen was kept immobile using sponge and sellotape with its lantern positioned towards the glass spectrograph (Hilger and Watts). A Fuzifilm (Fuzicolor CRYSTAL X-TRA 400) was used to record the emission spectrum. The firefly was kept fitted at the slit from about 20 : 00 h in the evening to 06 : 00 h in the morning. A total of ten male specimens were used for recording the emission spectra. Most of the specimens were found to be dead in the morning; a couple of specimens were in the dying stage; the spectrum displayed here (Figure 2) is a recording from one of them. The

Figure 1. Specimens of the species *Asymmetricata circumdata* (Motschulsky).

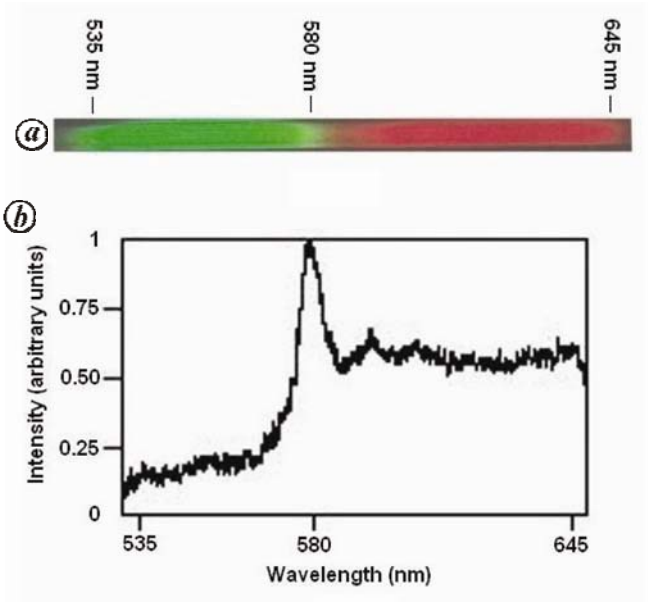

Figure 2. Coloured emission spectrum of firefly *A. circumdata* recorded in the glass spectrograph (***a***) and intensity profile (***b***). The peak wavelength appears at 579 nm – in the narrow yellow sector.

average temperature in the laboratory at Gauhati University during this experiment was 30°C. For reference, a spectrum from an iron arc was recorded above this one on the film. The developed positive of the coloured film was scanned (HP Deskjet Ink Advantage 3545) at 1200 DPI. Intensity profile of the scanned spectrum was plotted using the software ImageJ. For recording the diffraction pattern of the light of the firefly, an analytical grating (Hilger) of 15,000 lines/inch was used. Distance of the grating from the light-emitting organ of the firefly was approximately 1 cm. A camera Sony Cyber-shot DSC-H7S was used to photograph the diffracted light. Distance of the lens of the camera from the grating was approximately 2 cm. Experiments with the grating were conducted just after sunset from 1900 to 2100 h IST.

For recording flashes of the firefly, a single flashing specimen was fixed in a thick piece of sponge using sellotape with its lantern positioned in front of a photomultiplier tube (Hamamatsu H10722 with power supply C10709). The control voltage applied in the photomultiplier tube was 0.24 V. The waveforms were observed using digital storage oscilloscope (Tektronix TDS 2022C), and saved with .CSV file extensions in an external devise HP USB. The experiment was performed at the normal laboratory temperature of 28°C, and then both at lower and higher temperatures of 22°C and 38°C respectively. The low temperature was realized with the help of the air-conditioner, and the high temperature was produced by a heater. For noting down the temperatures, a digital thermometer was made by using IC LM35 connected to a multilmeter (MASTECH MAS 83L), and placed adjacent to the fixed firefly. The resolution of this thermosensor was 0.5°C. Fifty pulses of ten specimens were recorded.

Figure 3 shows an emission spectrum of the firefly recorded in the high-resolution spectrometer. The peak wavelength appears at 570 nm, and FWHM is measured as 57 nm, spreading from 545 to 602 nm. These values are constants for all the specimens used in the experiment, and compare well with those obtained in our earlier measurements of *Luciola praeusta*[4]. The spectra are quite similar in appearance as well. It has been hypothesized that different species of firefly emit in different wavelength regions because of slight differences in their enzyme structures.

Figure 2 shows the spectrum recorded on a colour film in the glass spectrograph with its intensity profile. Similar to the one for *L. praeusta*[7,8], this spectrum also reveals three colour sectors: two broad sectors of green and red, and one narrow sector of yellow. It is also apparent that the yellow photons deviate less than the other two colours. The position of the peak in this spectrum is determined at 579 nm. As the calibration of the spectrometer was nearly perfect, and determination of wavelengths in the spectrum recorded in the spectrograph was done with the help of standard lines of iron, the obvious speculation is that a very sharp line is likely to exist at 579 nm. A similar paradox in the case of *L. praeusta* has recently resulted in the observation of a strong narrow yellow line in its emission spectrum[8]. In the present case, the reason for non-observation of the 579 nm line could be the non-response of the spectrometer precisely at this wavelength. The integration or exposure time given in the spectrometer was 1s, while that in the spectrograph was about 10 h for the whole night. Hence the spectrum recorded in the spectrograph could be expected to give more real representation. Just like the earlier reported[8] case for *L. praeusta* at 591 nm, for the present case also we speculate that the emission peak becomes narrower due to the preferential amplification at 579 nm within the FWHM. The ring cavity resonance determines the lasing

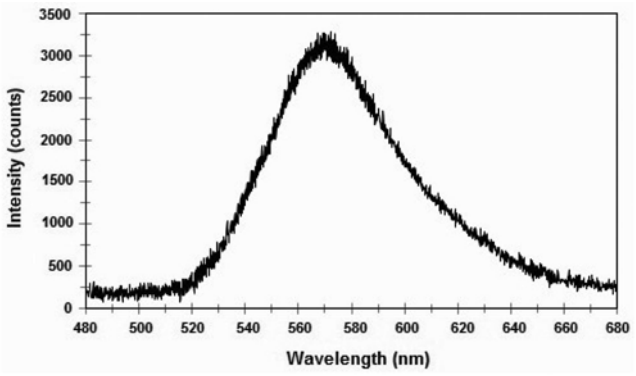

Figure 3. Emission spectrum of firefly *A. circumdata* (Motsch.) recorded using a high-resolution spectrometer. The peak wavelength appears at 570 nm and the full width at half maximum has a value of 57 nm.

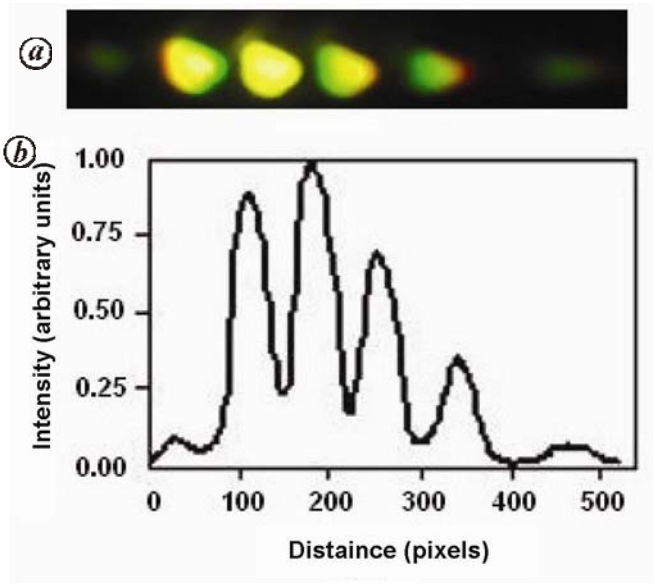

Figure 4. ***a***, Diffraction pattern produced by the transmission grating. The central principal diffraction maximum is evidently yellow, whereas in the other two orders, this sector gets suppressed by green and red ones. ***b***, Intensity profile of the diffraction pattern.

frequency, and we propose that the granules in the reflector region, acting like a diffusive material, keep the light inside the system long enough for the amplification to become effective. Another aspect of Figure 3 is that the red sector has higher intensity than the green sector – opposite to that in the case of *L. praeusta*.

The diffraction pattern produced by the transmission grating (Figure 4) is similar to the ones produced for the controlled light from the Indian species *L. praeusta*, and the Japanese species *Luciola cruciata* and *Luciola lateralis*[9]. The central principal diffraction maximum is predominantly yellow. It could be mentioned here that for a polychromatic source the central maximum is of the same colour as the source. Green and red-coloured bands appear from the first-order principal maximum onwards. With increasing orders, these bands become broader while the yellow one becomes narrower. This result suggests that the intense yellow region, as a matter of fact, is very narrow, and this species of firefly most probably also emits coherent yellow-coloured light.

Figure 5 presents the flashes of this species. It is clear that a typical flash is a combination of two pulses: a weaker pulse coming just before completion of the stronger or the main one. A close look at the lantern reveals that the lower segment starts flashing just before emission of light from the upper segment is stopped. That is, there are basically two groups of luminescent molecules which emit at slightly different times. This is the most probable reason for the appearance of the bimodal flash. At the normal flashing temperature range of roughly 27–33°C, the second pulse sometimes becomes indistinct as seen in Figure 5 *b* at 28°C – appearing to be an extension at the time of completion of the main one – which implies that the two segments blink almost simultaneously. At temperatures lower and higher than the normal range, the second 'combination' pulse becomes more prominent, almost at par with the first pulse (Figure 5 *a* and *c* at 22°C and 38°C respectively). Thus a change in temperature clearly affects the number of molecules in the two groups making the transition that results in the flash. It is worth mentioning here that flashes of the species *L. praeusta* are 'simple' ones, triangular in shape, and their start and end points are clear[25]. In the present case, depending on the timing of the second pulse in the combination, the flash duration varies greatly. This type

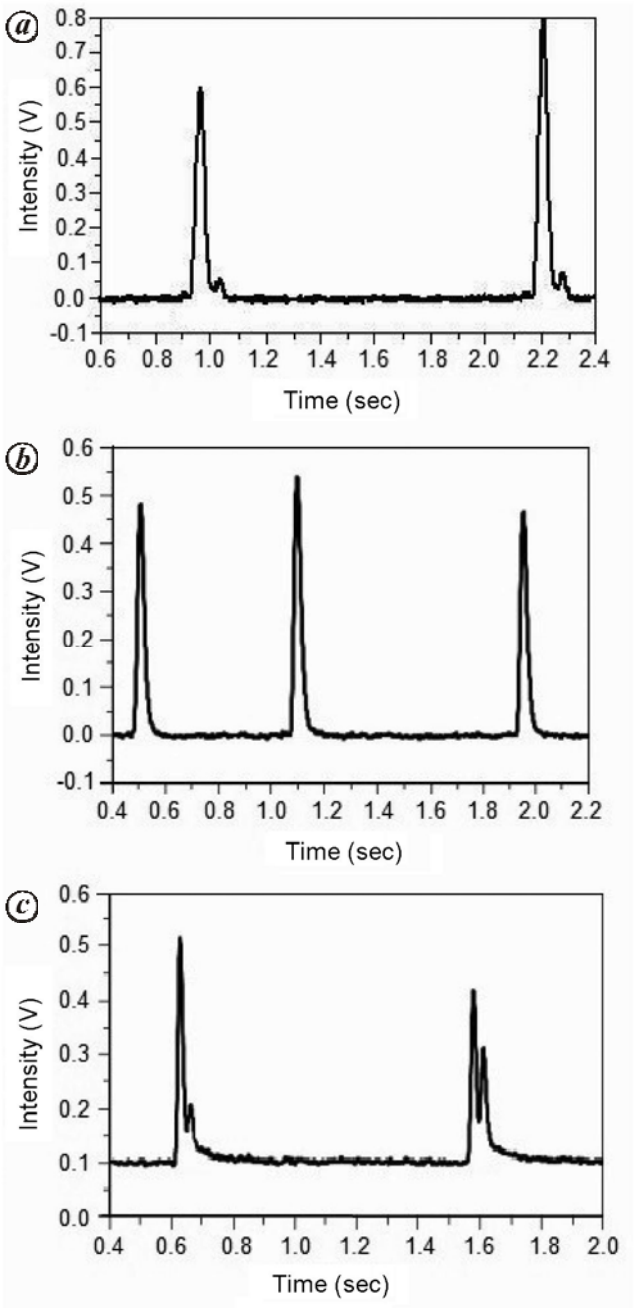

Figure 5. Typical flashes of the firefly at (***a***) at 22°C, (***b***) 28°C, (***c***) 38°C. The second or combination pulse is clearly prominent at both lower- and higher-than-normal flashing temperatures, especially at a high temperature of 38°C.

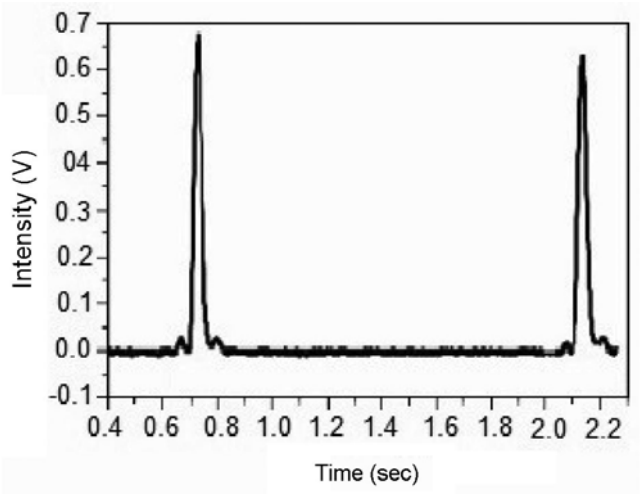

Figure 6. A rare occurrence: a weak pulse before the beginning as well as at the end of the main pulse.

Table 1. Different aspects of bioluminescence emissions of *Asymmetricata circumdata* and *Luciola praeusta*

Species	Spectrum using spectrometer: Peak wavelength (nm)	Spectrum using spectrometer: FWHM (nm)	Spectrum peak using spectrograph (nm)	Flash pattern
A. circumdata	570	57	579	Compound, bimodal flash
L. praeusta	562	55	591	Simple, triangular flash

of flash pattern has been observed in a couple of *Photinus* species of fireflies[13]. Non-simultaneous blinking of the segments, that is, time delay in flashing, definitely has scope for further research. A general observation is that the duration of the main as well as the second pulse increases with decrease in temperature. Another observation is that inter-flash intervals are generally longer than those of *L. praeusta*. Both these observations are difficult to quantify because of the reason given above and wide variation in the flashing rate respectively. Also, though rare, sometimes it has been noticed that the lower segment blinks both before and after that of the upper segment, producing weak combination pulses just before and after the main one (Figure 6).

The findings of this species are summarized in Table 1, and compared with those of *L. praeusta*.

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ACKNOWLEDGEMENTS. This work is supported by the Department of Biotechnology, New Delhi (project no. BT/PR4855/BRB/10/1030/2012). We thank Dr L. Ballantyne (School of Agricultural and Wine Sciences, Charles Sturt University, Australia), for identifying the firefly species of the specimens collected.

Received 22 May 2015; revised accepted 30 July 2015

doi: 10.18520/v109/i10/1838-1842