Chapter 4
Seasonal variation of firefly flash
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4.1 Introduction

Firefly flashing has fascinated naturalist, biochemist and electro optic physicist for a millennium. We all know that firefly light is due to a chemical reaction called chemiluminescence reaction. The beginning and the end of firefly flash is controlled by the oxygen which initiates the light reaction in addition to the other chemicals through complex series of successive smaller tubes known as tracheoles. It is controlled according to a postulated theory which described that flashing is controlled by gating oxygen access to the light-emitting cells (photocytes). According to this theory, the dark state represents suppression of bioluminescence light by limiting oxygen, which is required for bioluminescence; relief from this suppression by transiently allowing oxygen access to the photocytes allows trigger the flash. So it was reported that the flash of the adult firefly is controlled by oxygen flow to the photocytes, and it was demonstrated that the tracheole fluid modulate the oxygen flux providing the extra amount of flashing. These tracheoles have uniquely strengthened structure so that the nerve which stimulates the flash motor unit terminates upon the tracheal end cells and not the photocytes. The foremost and lattermost observations led Ghiradella to conclude that some osmotic mechanism might be involved in controlling the flash. Maloeuf suggested that the modulation of the tracheolar fluid, which is due to the direct change in photocyte osmotic potential, controls the firefly flash. This mechanism was later concurred by Alexander in a way that modulation of tracheolar fluid levels occurred and was important in a ‘secondary
Nitric oxide (NO), which is a ubiquitous signalling molecule, has been found to play the 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 \( \text{O}_2 \) levels in the peroxisomes\(^6\). The firefly flashes are believed to be shaped by neural impulses generated in the brain that eventually impinge on the lantern tissue. By simply adjusting the frequency and duration of the stimulus activating the lantern nerves, as per reported hypothesis, it is possible to shape any kind of flash\(^7\). Another report suggests that NO may play the key role in producing continuous light by functioning as a neurotransmitter signal for bioluminescence\(^8\). It is widely believed that octopamine is the neurotransmitter responsible for the induction of luminescence in the light-producing organ of the firefly\(^9\). It is found in adult lanterns\(^10-11\) as well as in larval lanterns.

Measurements on a single flash have shown that the duration varies from about 70 ms\(^{12}\) to a few hundred milliseconds\(^{13-17}\) up to a couple of seconds. The flash pattern as well as flash duration is different for both males and females. Neither flash number nor flash length correlated with the flash rate is recorded for male population. It is found that female identify male flashes based on their rate but not their power which indicates the operation of a perceptual filter yielded by phasic neural response to flash onsets, followed shortly thereafter by adaptation and inhibition\(^18\). In recent times, time-resolved bioluminescence measurements have been performed for fireflies placed in pulsed and static magnetic fields\(^19\), which prompt speculations that the magnetically induced current inside the firefly in the pulsed magnetic field affected its nervous system or the photochemical processes in the light producing organ,\(^20\) while the diamagnetic torque and Lorentz forces induced by the field of strength 10 Tesla had inhibitory and stimulating
effects respectively, on the bioluminescence system\textsuperscript{19-21}. Regarding effects of temperature on bioluminescence of fireflies, Lloyd observed in four *Luciola* species of fireflies of Melanesia that flash periods decreased with an increase in temperature\textsuperscript{15}. Similarly, a significant negative correlation between ambient temperature and inter-flash separation was observed in an investigation on inter-flash intervals of *Luciola cruciata* which is recorded at five different sites in central Japan\textsuperscript{22}. In a very recent study of *in vitro* bioluminescence of the North American firefly *Photinus pyralis*, it is found that the intensity of the green component, the only temperature-sensitive quantity, decreases with an increase in temperature, while the life time is shorter at pH 7.0 than at pH 8.0, lengthening sharply above 30 °C at pH 8.0\textsuperscript{23}.

It is well-known that the rate of an enzyme-catalysed reaction increases as the temperature is raised. An understanding of the effects of temperatures on enzymes is important from the point of view of investigations of normal cellular functions as well as of the ability to manipulate these functions through enzyme and metabolic engineering. It is also important from the point of view of having available data on enzyme concentrations, activities and regulatory response in the cell, organism or system in question - for acquiring deep knowledge of system biology. Several models have been proposed to account for the effect of temperature on enzyme activity and stability. A comparison of those Equilibrium Models, proposed in the early part of this century, could be found in a recent review article by Daniel and Danson\textsuperscript{24}. The present chapter is an attempt to study the variation of firefly flash with temperature of different seasons for getting deep knowledge into system biology\textsuperscript{25}. 
4.2 Experimental detail

Seasonal variation of firefly flashes was observed by recording its flashes for the whole year at various seasons. The experiment was conducted mainly for the period from early March to late October or early November. In this experiment our room temperature varies from 21 °C to 34 °C. Prior to the experiment, we opened the doors and windows of the laboratory room to maintain the room temperature as that of the outside. For this particular experiment we have used a photomultiplier tube with a controlling voltage supply, a digital storage oscilloscope and digital thermometer.

4.2.1 Photomultiplier

For the detection of lower radiation power, photomultipliers are necessary. It is advantageous in the sense that it can limit noise by internal amplification of the photocurrent due to the secondary emission of electron from internal dynodes which multiplies the number of photoelectron. These photoelectrons emitted from the cathode will accelerate due to the potential difference of a few hundred volts. Then they will be focussed on to the metal surface of the first dynode. Each impinging electron will release on the average x secondary electron. These electrons will further accelerate to the second dynode. Again each secondary electron will produce about x tertiary electron on impinging to the second dynode, and so on. If m is the amplification factor that depends on the accelerating voltage V, on the incident angle α and on the dynode material, then each photoelectron in a photomultiplier with N dynodes produces at the anode a charge avalanche of

\[ Q = x^N e \]

with responding voltage pulse of

\[ V = \frac{Q}{C} = \frac{(x^N e)}{C} \]
C being the capacitance of the anode

For experiment demanding high time resolution, the rise time of the anode pulse should be as small as possible. Assuming a single photoelectron emitting from a photocathode accelerated to the first dynode, the initial velocity of the secondary electron emitted varies due to the depth in the dynode material. The time taken between two parallel electrodes with a distance d and a potential difference V for an electron of mass m and starting from rest is

\[ t = d \left( \frac{2m}{eV} \right)^{\frac{1}{2}} \]

The electron with initial energy \( E_{\text{kin}} \) reach the next electrode earlier by a time difference

\[ \Delta t_1 = \left( \frac{E_{\text{kin}}}{eV} \right)^{\frac{1}{2}} t \]

\[ = \left( \frac{d}{V} \right) \left( \frac{2mE_{\text{kin}}}{e} \right)^{\frac{1}{2}} \]

These electrons will travel slightly different path lengths through the tube. This causes an additional time spread

\[ \Delta t_2 = \Delta d \left( \frac{2m}{eV} \right)^{\frac{1}{2}} \]

Thus the rise time of an anode pulse starting from a single photoelectron, decreases with increasing voltage by an amount of \( V^{1/2} \). The rise time depends on the dynode structure.

The number of photoelectrons generated due to the short intense pulse in the time spread is further increased by two effects.

1. The initial velocity of the photoelectron emitted from the cathode.
2. The spot on the cathode where the photoelectron is emitted will determine the time of flight between the cathode and first dynode.
The main sources of noise in a photomultiplier tubes are

1. The dark current flowing in the photomultiplier tube.
3. Shot noise and Johnson noise which is caused by fluctuations of the amplification inside the tube and by noise of the load resistor\(^\text{26}\).

\[ \text{Figure 22. Schematic diagram of a photomultiplier Tube.} \]

4.2.2 Digital Storage Oscilloscope

The oscilloscope is basically a device that displays graphs. It basically draws a graph of an electrical signal. In most of the applications, the graph shows how signals change over time. Here the vertical (Y) axis represents voltage and the horizontal (X) axis represents time. The oscilloscope draws the behaviour of a voltage (V), but the absolute measurement has lower precision than a digital voltmeter (or multimeter). For example, to easily appreciate an exact 4.53 Volt battery voltage whereas the oscilloscope read out is just around 4.5 Volt. The screen is divided usually into 8 vertical and 10 horizontal divisions. Each square division has 5 further subdivisions per axis useful for better readings.
The oscilloscope is very useful not only in the world of electronics but also for response to physical stimuli, such as sound, mechanical stress, pressure, light, or heat. Thus an oscilloscope can measure all kinds of phenomena.

**Y axis – vertical -voltage**

At least one input channel is needed for the signal V to be shown. This signal is passed through a combination of amplifier and selected knob to set the amplitude value for each Y division. So 2 V/Div will give the maximum watching amplitude of each input signal which is 16V (i.e. 2 V × 8), while referring to centre, it is 8 V positive and 8 V negative.

**X axis-Horizontal-Time base**

X axis also has a selection knob to set the time base. For example the meaning of 10 ms/Div is that to trace the whole X axis it spends 0.1 seconds.

An electronic beam lights up a dot on the screen. The position of the dot depends on the two deflection systems, horizontal and vertical. Without input signals the dot moves only due to the internal time base which gives a horizontal line. On giving the input signal the dot moves on the X-Y plane due to the combine effect of the two deflection system.

There are two types of Oscilloscopes, analog and digital types. In an analog oscilloscope the voltage is directly applied to electron beam moving across the oscilloscope screen. The voltage deflects the beam up and down proportionally, tracing the waveform on the screen. This gives the picture of the final waveform on the DSO screen. In contrast, a digital oscilloscope samples the waveform and uses an analog-to-digital converter (or ADC) to convert the voltage being measured into digital information. This digital information will then reconstruct the final waveform on the screen. People often prefer analog oscilloscopes when it is important to display rapidly varying signals
in "real time". Digital oscilloscopes are also advantageous in the sense that they allow capturing and viewing events that occur only once. It can process the digital waveform data or send the data to a computer for processing. It can also store the digital waveform data for later viewing and printing.

The digital oscilloscope is constructed of systems same as that of the analog oscilloscope; however, digital oscilloscope contains additional data processing systems. With these added systems, the digital oscilloscope collects data to form the entire waveform and then displays it.

When digital oscilloscope probe is attached to the circuit, the vertical system adjusts the amplitude of the signal, just as in the analog oscilloscope. The analog-to-digital converter (ADC) in the acquisition system then samples the signal at discrete points in time and converts the signal at these points to digital values called sample points. The horizontal system's sample clock determines how often the ADC takes a sample. The rate at which the clock "ticks" is measured in samples per second. The sample points from the ADC are stored in memory as waveform points. One waveform point is formed by taking more than one sample point. A number of waveform points together will make up one waveform record. The number of waveform points that forms waveform record is called the record length. This record is stored in the memory and then it goes to the display unit.

4.2.3 Procedure in detail

The *Luciola praeusta* found in Gauhati University was the firefly species chosen for the experiment. The experiment was performed from 19:00 hours to 21:00 hours local time. One of them was fixed in a thick piece of sponge with cello tape, and its lantern was positioned in front of a photomultiplier tube (Hamamatsu H10722 with power supply C10709). A digital storage oscilloscope (Tektronix TDS 2022C) recorded the pulses
generated by the photomultiplier tube. The room temperatures in the laboratory were recorded as 21 °C, 24 °C, 26 °C, 28 °C, 32 °C and 34 °C from winter to summer respectively. The experiment was performed immediately after catching the firefly and flashes were thus recorded. The fireflies were kept alive during the experiment. After recording at least 25 flashes the specimens were set free. A digital thermometer with a resolution of 0.5 °C was made by using IC LM 35, and placed adjacent to the fixed fireflies; the readings of temperature were noted from a multimeter (MASTECH MAS 830 L). The controlling voltage applied in the PMT was varied from 0.21 to 0.26 V for getting good intensity in the pulses. As the fireflies could not be kept in a close chamber for a number of days so we could not record the flash for all the temperature with the same specimen. Also as we were to analyse seasonal variation for the same species, specimen need not be the same. The sequences were recorded in the DSO, and a characteristic sequence, representing the average duration of pulses, was kept for the analysis. A USB device saved the values of waveforms in .CSV extension. The pulses were analysed in ORIGIN 6.1 after 15-point smoothening.
Figure 23. Experimental setup for recording of firefly flash.
4.3 Results and discussion

The firefly flashes were recorded almost every day during the entire season. In winter there was not an abundance of firefly compared to summer (25 °C - 38 °C), therefore we could record a few firefly flashes in winter (20 °C – 24 °C). We record flashes of total 24 specimens. The recorded room temperature on the multi meter were 21 °C, 24 °C, 26 °C, 28 °C, 32 °C and 34 °C. Average flash durations for 24 specimens of the same species (*Luciola praeusta*) at temperatures 21°, 24°, 26°, 28°, 32° and 34 °C were measured as 128.15, 117.69, 108.1, 104.07, 9.53 and 86 ms, respectively. These values along with their standard deviations and standard errors are given in Table 1. It is evident from the figures that, with the increase of temperature, flash duration decreases almost linearly. It is found that there is a 3 ms decrease of flash duration with each °C rise in temperature on average. Typically ordered triangular-shaped flashes from a specimen of the firefly at the above-mentioned temperatures are shown in Figures 24, 25, 26, 27, 28 and 29, respectively, and variation in the pulse width with temperature is shown in Figure 30. A regression line is also presented along with the experimental one in this figure. An analysis of the regression line reveals that the regression equation is Y=191.73-3.129X along with the p-value of the slope which is 5.97521x10^{-4}. The coefficient of determination is $R^2 = 0.983$ which definitely shows a good correlation of theoretical line with experimental one. It is worth mentioning here that this particular species of fireflies usually flashes in the yellow region at temperature ranges 27 °C – 32 °C, approximately. In this experiment when the temperature fell below 23 °C, it was noticed that the ordered nature of the flashes coming out from many specimen was lost. At such low temperatures, those specimens were observed to emit flashes in a random fashion. Those flashes were found to be compound or the start-and end points were not clear. Hence those could not be considered for analyses. Again a close look at the lanterns of the fireflies emitting such type of flashes revealed that the upper light-emitting segment blinked slowly while the lower one showed a continuous glow only: no blinking at all. In a couple of cases, the specimens appeared to lie still with continuous glows from their lanterns, which resembled the condition of fireflies
affected by vapours of ethyl acetate\textsuperscript{22}. Only those specimens which could withstand the low temperature of 21 °C, flashing normally in an ordered manner, were used. It is also to be mentioned that specimens emitting pulses of reasonably good intensity were used in this study. As the experiment was not performed in a controlled environment i.e. the temperature of room was not controlled, the recorded room temperatures were found to be of irregular interval. The availability of firefly only at the evening time is also another reason for this. The temperature of the laboratory room was maintained as that of the outside by opening all doors and windows of the room. As we know most animal enzymes rapidly become denatured at high temperature above 40 °C, and also not to show cruelty to the insect by allowing it to withstand various temperature changes, the temperature of the room was not controlled. We propose that a change in temperature affects the functioning of the catalytic activity of enzyme luciferase, which results in a change in the reaction rate - variation in the flash duration being a manifestation of this change. According to the equilibrium model depending on Michaelis-Menten kinetics on enzyme, reported earlier is that the reaction rate increases exponentially with the increase in temperature due to the simultaneous decrease in the active enzyme through thermal irreversible inactivation\textsuperscript{23-25}. The result obtained in the present experiment is not in total agreement with this view, as the reaction rate, whose increase is indicated by a decrease in the flash duration, varies linearly with temperature. Values of pulse widths were found to be independent of time because of the fact that the total (potentially) active enzyme remains constant in the living insect. As the specimens were kept fixed, the flashes could be lightly termed as SOS signals, not carrying the usual codes or meanings. As such, inter-pulse spacing was not so important in this study. In the present study, in any case, inter-pulse separation showed so much variation, from almost zero to several seconds, that determining average values could be considered as pointless. It was generally observed that interpulse separations were longer at lower temperatures.
Figure 24. Time resolved flashes at temperature 21 °C, average flash duration is 128.15 ms.
Figure 25. Time resolved flashes at temperature 24°C, average flash duration is 117.69 ms.
Figure 26. Time resolved flashes at temperature 26 °C, average flash duration is 108.1 ms.
Figure 27. Time resolved flashes at temperature 28 °C, average flash duration is 104.07 ms.
Figure 28. Time resolved flashes at temperature 32 °C, average flash duration is 93.5 ms.
Figure 29. Time resolved flashes at temperature 34\(^0\)C, average flash duration is 86 ms.
Figure 30. Graph showing variation of average flash duration with temperature, along with $R^2$ value.

Table 1: Flash duration of firefly at different temperatures along with standard deviation and standard errors.

<table>
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<th>Temperature ($^\circ$C)</th>
<th>Number of specimen</th>
<th>Number of flashes</th>
<th>Average flash width (ms)</th>
<th>Standard deviation</th>
<th>Standard error</th>
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4.4 Conclusion

In this work, a study has been done on the firefly flashes at different temperature of different seasons. Variation of temperature of various seasons is found to have effect on the duration of the flash of the firefly, consequently on the rate of reaction. As durations of the flashes indicate durations of the chemiluminescence reaction that produces the light of the firefly we could make a conclusion here that the rate of this enzyme catalysed reaction decreases with the increase of temperature, change of flash width with temperature is the manifestation of this. Here we have found that there was a decrease of approximately 3 ms per degree rise of temperature. Hence we conclude that the reaction rate is almost linearly dependant on temperature. Thus the temperature affects the catalytic activity of enzyme luciferase thereby effecting the chemilluminescence reaction. In this chapter the study was done only with one species available in Gauhati University campus. Therefore the work could easily be extended to other species of fireflies for investigating changes in pulse durations with changes in temperature, and consequently to arrive at a general conclusion.
References


