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FIREFLY FLASHING IS CONTROLLED BY GATING OXYGEN TO LIGHT-EMITTING CELLS

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Summary

Although many aspects of firefly bioluminescence are understood, the mechanism by which adult fireflies produce light as discrete rapid flashes is not. Here we examine the most postulated theory, that flashing is controlled by gating oxygen access to the light-emitting cells (photocytes). According to this theory, the dark state represents repression of bioluminescence by limiting oxygen, which is required for bioluminescence; relief from this repression by transiently allowing oxygen access to the photocytes allows the flash. We show that normobaric hyperoxia releases the repression of light emission in the dark state of both spontaneously flashing and non-flashing fireflies, causing continual glowing, and we measure the kinetics of this process. Secondly, we determine the length of the barriers to oxygen diffusion to the photocytes in the aqueous and gas phases. Thirdly, we provide constraints upon the distance between any gas-phase gating structure(s) and the photocytes. We conclude from these data that the flash of the adult firefly is controlled by gating of oxygen to the photocytes, and demonstrate 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.

Movies available on-line.

Key words: bioluminescence, oxygen gating, light-emitting cell, photocyte, firefly, *Photinus pyralis.*

Introduction

The chemical reactions by which fireflies produce bioluminescence are well described (Reactions 1 and 2), the excited-state intermediates involved in light emission have been characterized, many of the genes for their luciferase have been sequenced, and the X-ray crystal structure of the *Photinus pyralis* luciferase enzyme has been solved (reviewed recently by Wilson and Hastings, 1998). However, the mechanism by which adults produce their bioluminescence as rapid flashes of light, which is vital for its biological courtship function, is not yet understood, despite a long history of scientific investigation. This has been comprehensively reviewed by Case and Strause (Case and Strause, 1978) and recently updated by Ghiradella (Ghiradella, 1998) and Wilson and Hastings (Wilson and Hastings, 1998), also see Movie 1 in the supplementary material for a demonstration.

$$\text{ATP} + \text{luciferase} + \text{luciferin} \xrightarrow{\text{Slow}} \text{Luciferase-luciferin-AMP} + \text{PP}_\text{i}\,, \quad (1)$$

$$\text{Luciferase-luciferin-AMP} + \text{O}_2 \xrightarrow{\text{Fast}} \text{Luciferase} + \text{oxyluciferin} + \text{CO}_2 + \text{AMP} + \text{light}\,, \quad (2)$$

Here we provide experimental evidence supporting the most commonly postulated theory of flash control, namely that the flash results from rapid gating of oxygen (O_2) to the light-emitting cells of the lantern (photocytes). There is a great deal of circumstantial evidence supporting such a control mechanism (Case and Strause, 1978; Wilson and Hastings, 1998), including (1) firefly bioluminescence absolutely requires oxygen (Reaction 2); (2) sequential hypoxia and reoxygenation causes a ‘pseudoflash’, simulating many features of a genuine flash (Alexander, 1943; Hastings and Buck, 1956); (3) the tracheolar system of the adult lantern (the structures supplying oxygen to insect cells) has a unique structure compared to the larval form (Ghiradella, 1977; Ghiradella, 1998); and (4) the nerves innervating the flash motor unit do not terminate upon the photocytes themselves but rather the specialised tracheal end

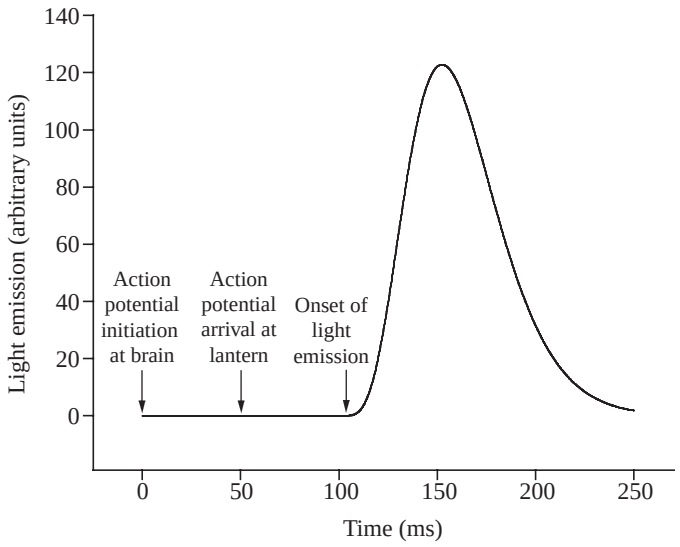

Fig. 1. Temporal sequence of events in a typical single firefly flash (adapted from data in Buck et al., 1963).

cells (Ghiradella, 1977; Ghiradella, 1998; Case and Strause, 1978).

However, perhaps most importantly, only a few tens of milliseconds (typically 40–60 ms) pass between the arrival of the nervous action potential at the lantern and the start of light emission *in vivo*, with maximal light emission occurring within 100–150 ms after action potential arrival (see Fig. 1; Buck et al., 1963). Only the reaction of pre-formed luciferase-luciferyl-AMP complex (LL-AMP) with oxygen (Reaction 2) occurs rapidly enough to be the point of control, with maximum light emission occurring within 60 ms of mixing LL-AMP and oxygen *in vitro* (De Luca and McElroy, 1974). If however, the reaction is instead initiated *in vitro* by mixing luciferin, luciferase, ATP and oxygen (i.e. proceeding *via* Reaction 1 followed by Reaction 2), a lag phase of 25 ms occurs before any light is emitted, and maximal light is not emitted until 300 ms after mixing (De Luca and McElroy, 1974). Hence these kinetics dictate that any control mechanisms acting at points prior to the reaction of LL-AMP with oxygen (e.g. *via* controlling ATP levels or luciferin release from photocyte vesicles) would simply occur too slowly and can be ruled out.

In vitro, the reaction of the LL-AMP complex with oxygen is rapid and spontaneous, and although one could argue that *in vivo* this might be subject to the repressive action of a cellular second messenger/binding protein (not present in the *in vitro* experiments), with this repression being rapidly and transiently lifted for the flash, no such consensus sequences/binding sites, other than those for substrate carboxyl group adenylation (Wood, 1995), were found after extensive database searching (Altschul et al., 1997) using all known coleopteran luciferase sequences (G. S. Timmins, unpublished results). [Note that although no 'control' sequences were found, a clear analogy between coleopteran luciferase and bacterial thiophene oxidation protein sequences was observed e.g. 45-AHIEVNITYAEY-56 for *Photinus pyralis* and 172-AHVEVNIDYPEY-183 for *Bacillus halodurans*. Given the similarities in molecular structure between the site of benzothiazole luciferin oxidation and thiophene, this may perhaps form a common oxidation and/or binding site, arrived at through convergent evolution.]

The flash is therefore almost certainly controlled by regulating the access of oxygen to the LL-AMP complex, most logically by control of oxygen entry to the photocytes, perhaps by a mechanism analogous to (albeit faster than) the spiracular control of discontinuous cyclic gas exchange (DCG) known to occur in many insects (Lighton, 1996), or perhaps through control of tracheolar fluid levels (Timmins et al., 2000). It has long been known that oxygen supply to tissues in insects is mediated by changes in tracheolar fluid levels, which result from changes in the balance of osmotic pressure of the tracheolar fluid and intracellular milieu (Wigglesworth, 1935). Indeed, Maloeuf (Maloeuf, 1938) proposed such a mechanism of control of oxygen supply to the photocyte by controlling tracheolar fluid levels some years ago, although Alexander (Alexander, 1943) later proposed that the tracheal end cell rather acts in the manner of a mechanical valve to control oxygen access. Irrespective of the actual mechanism, in such a model light emission during the dark phase of flashing would be repressed by restricting oxygen access to the photocytes; these are richly endowed with mitochondria around their internal periphery, adjacent to the photocyte plasma membrane that abuts the oxygen-supplying tracheoles (Ghiradella, 1977; Ghiradella, 1998; Case and Strause, 1978). The combination of limiting oxygen supply, together with the oxygen consumption by these mitochondria, could reasonably be expected to result in anoxia within the centre of the photocytes and thereby inhibit light emission. This is supported by the fact that the light-emitting subcellular structures, the photocyte vesicles that contain the LL-AMP complex, are found at the centre of the photocytes (Smalley et al., 1980). The flash of light emission would therefore be achieved by transiently increasing the supply of oxygen to the photocytes; their peripheral mitochondria would be unable to consume all of this increased oxygen supply, so some would reach the photocyte vesicles and result in light emission. The combination of oxygen consumption by mitochondria and bioluminescence itself would consume the 'pulse' of increased oxygen, resulting in anoxia around the photocyte vesicles and repression of light emission, ending the flash.

In this study we have confirmed this theory by first demonstrating that increasing oxygen supply by normobaric hyperoxia (rapidly changing the external gas stream to oxygen from air) can relieve this repression of light emission, and more importantly, measuring the kinetics of this hyperoxia-induced light emission. Secondly, we have measured pseudoflash kinetics in fireflies in gas mixtures where the gas-phase diffusion coefficient of oxygen varies to determine gas- and aqueous-phase barriers to oxygen diffusion (Timmins et al., 2000). Thirdly, we have used direct electrical stimulation of firefly flashing by insertion of electrodes into the lantern (Buck et al., 1963) and measured the delay between electrical stimulation and peak light emission in a range of normoxic gas

mixtures, in which the gas-phase diffusion coefficient of oxygen varies. From this, we have been able to provide constraints on the distance between any such gas-phase gating structure and the photocytes. Finally, we use these and previous data to provide the likely identity of the physical mechanism by which rapid oxygen gating is achieved, namely modulation of tracheolar fluid levels. The use of such an oxygen-gating control mechanism does not (to the author's knowledge) occur elsewhere in animal biology, and has most likely evolved *via* a series of sequential modifications of the pre-existing mechanism through which insects regulate tissue oxygenation, *via* changes in tracheolar fluid levels (Wigglesworth, 1935).

Materials and methods

Fireflies (*Photinus* sp., male) were collected locally from meadows near Hanover, NH, USA, and kept individually in plastic containers containing ventilation holes in ambient lighting conditions. Measurements of kinetics of bioluminescence induced by a rapid change from normobaric normoxia to hyperoxia (change in partial pressure of oxygen, P_{O_2}, from 21 kPa to 101 kPa) were determined as previously described at 294±1 K using a rapid gas-changing valve apparatus, photodiode and storage oscilloscope. The total time taken to change the external gas supply at the lantern was 25 ms, and the time from gas change to peak light emission was measured (Timmins et al., 1999; Timmins et al., 2000). Pseudoflash kinetics in fireflies (either after electrical stimulation or during periods of spontaneous flashing) were measured in low- and high-diffusivity carrier gases (N_2 and He, respectively; at least five measurements per sample in each gas) with the time-to-peak-pseudoflash intensity measured (Timmins et al., 2000). Root mean square (r.m.s.) pathlengths of diffusion in the gas and condensed phase were calculated as previously; the barrier in the condensed phase is produced almost entirely in the aqueous phase since the barrier to diffusion across the plasma cell membrane is exceedingly small (Subczynski et al., 1992). For electrical stimulation studies, fireflies were decapitated, mounted on a plastic carrier with adhesive tape, and electrically stimulated with electrodes inserted into the lantern (Buck et al., 1963) whilst held at 294±1 K (measured at the thorax) in a polystyrene case in a gas stream (both temperature-controlled). Flashing was stimulated by a pulse of nominal 50 V amplitude, 5 ms duration from a stimulator connected to the lantern-mounted electrodes, and the delay between stimulation and peak flash intensity was measured. At the voltage and pulse length used, only a single flash was stimulated. Fireflies were studied in either O_2/He and O_2/N_2, or in O_2/He and O_2/SF_6 gas mixtures, in two cycles of gas changes (except for sample 3). Spontaneously flashing fireflies were obtained by exposure to ambient light in a natural diurnal cycle, and measured in subdued light after dusk at 21.00–23.00 h. Non-flashing fireflies were studied as for flashing samples, but at 18.00–20.00 h (prior to dusk) in dimmed ambient lighting; delicate handling was required to avoid stimulation of flashing. Video recordings of hyperoxia and pseudoflash experiments (supplemental material) were made using a Canon MV series digital video camera and converted to MPEG1 format. All values are means ± 1 S.D.

Results and discussion

Kinetics of both hyperoxia-initiated light emission and the pseudoflash

When the gas stream in which spontaneously flashing specimens (at approximately 1–2 h after dusk, N=5) were held was rapidly changed from air to oxygen (a change of oxygen partial pressure, P_{O_2}, from 21 kPa to 101 kPa in approximately 25 ms), the entire lantern began to glow continuously, taking 5–15 s to reach peak light emission (5 measurements for each sample, mean 10.2±2.8 s). As the hyperoxia-induced continuous light emission increased, the apparent intensity of spontaneous flashing decreased and could not be observed at maximal hyperoxia-induced light emission; this continued for the longest periods of hyperoxia tested (30 s). Upon changing the gas stream back to air, the continuous hyperoxia-induced light emission steadily decreased to zero within 10–30 s, and spontaneous flashing again became apparent during this time (a typical experiment is shown in Movie 2 in the supplementary material). Analogous behaviour was also observed when the hyperoxic gas contained 96 kPa O_2 and 5 kPa CO_2, whereas changing the gas stream from air to 5 kPa CO_2 in air did not induce glowing, indicating that CO_2 levels play no role in this effect.

When samples that were not spontaneously flashing (prior to dusk, N=4) were studied, peak continuous hyperoxia-induced light emission due to changing the gas stream from air to oxygen was somewhat slower, taking 17–52 s (3 measurements on each sample, mean 31.6±10.7 s,) but still occurred, and upon changing the gas stream back to air, light emission similarly ended over a period of 10–30 s. A typical experiment is shown in Movie 3.

These observations, first reported by Kastle and McDermot (Kastle and McDermot, 1910) and studied extensively by Snell (Snell, 1932) and Alexander (Alexander, 1943), directly demonstrate that during the dark state, both between flashes and when not spontaneously flashing at all, the repression of light emission can be lifted simply by increasing the ambient oxygen concentration (by a factor of approximately five) and hence increasing the supply of oxygen to the photocyte by a similar factor. This in and of itself has been used to strongly support the theory of oxygen control of bioluminescence. Additionally, the overwhelming of spontaneous flashing at maximal hyperoxia further supports the hypothesis of oxygen control, because if normal spontaneous flashing were achieved by another mechanism that was independent of controlling oxygen levels, then the spontaneous flashing should rather have been superimposed upon the hyperoxia-induced continual bioluminescence. Analysis of the observed kinetics of light emission is discussed later.

Pseudoflash kinetics for five samples were obtained using helium and nitrogen as carrier gases (Timmins et al., 2000), with delays to peak pseudoflash intensity of 0.249±0.061 s and

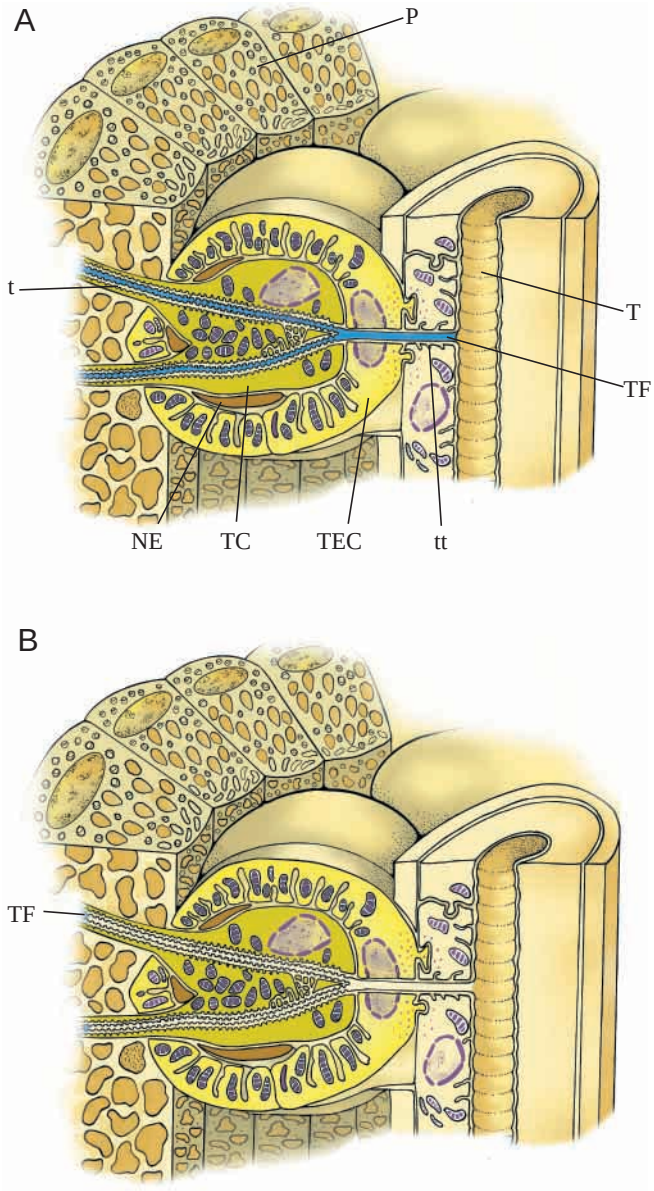

Fig. 2. Diagrams (adapted from Ghiradella, 1977; Ghiradella, 1998, with permission) depicting changes in tracheolar fluid length responsible for controlling oxygen access to the photocytes. (A) Increased fluid length during no light emission; (B) decreased fluid length during light emission. P, photocytes; NE, nerve ending; t, tracheole; TF, tracheolar fluid; T, trachea; TC, tracheolar cell; TEC, tracheal end cell. As explained more fully in the text and shown in Movie 4, neural stimulation leads to a transient increase in the osmotic potential of the tracheolar cell, resulting in decreased tracheolar fluid levels. The resultant decreased diffusional barrier allows greater oxygen supply to the photocytes, relieving intracellular anoxia and enabling light emission (molecular oxygen is required). As tracheolar cell osmotic potential returns to the resting state, tracheolar fluid levels increase, oxygen diffusion to the photocytes is decreased, intracellular anoxia occurs in the photocytes and light emission is inhibited.

0.154±0.05 s in nitrogen and helium carrier gases, respectively. The pseudoflash occurs due to accumulation of the LL-AMP complex within the photocytes (Reaction 1) caused by the inhibition of Reaction 2 by anoxia (sample held in gas stream of He or N_2); after rapid changing to a normoxic gas stream, the delay until peak pseudoflash intensity represents the time taken for diffusion of oxygen from the spiracle to the photocyte. These values are substantially lower than those obtained for larval *Pyrearinus termitilluminans* (1.95 s and 1.49 s, respectively), and indicate that the adult firefly has an enhanced system for oxygen supply compared to the larval light organ of *P. termitilluminans* (Timmins et al., 2000).

Table 1. *The delay between stimulation and peak flash intensity of fireflies in various gas mixtures*

Firefly identity	t_{SF_6/O_2}[a] or t_{N_2/O_2}[b] (ms)	t_{He/O_2} (ms)	$t_{N_2/O_2}/t_{He/O_2}$ or $t_{SF_6/O_2}/t_{He/O_2}$
A	171.1±4.9 (10)[a]	172.5±4.0 (13)	0.992
	176.9±2.2 (11)[a]	171.5±4.0 (10)	1.031
B	153.0±0.7 (5)[a]	150.9±1.1 (7)	1.014
	153.4±0.5 (7)[a]	152.8±0.8 (9)	1.004
C	146.4±0.5 (6)[a]	146.2±0.7 (8)	1.001
D	116.9±2.2 (8)[b]	119.2±1.8 (9)	0.981
	120±1.9 (8)[b]	120±2.8 (7)	1.000
E	121.5±1.4 (5)[b]	128.8±1.4 (6)	0.943
	120.5±1.2 (5)[b]	119.8±1.4 (5)	1.006
F	87.9±0.7 (11)[b]	88±0 (6)	0.999
	93.6±0.5 (8)[b]	94±0.6 (6)	0.996
Mean	ND	ND	0.997±0.022 (11)

Gas mixtures used were He/O_2, N_2/O_2 and SF_6/O_2. See text for details.

t, delay time taken for peak flash intensity after stimulation in the indicated gas mixture.

Values are means ± S.D. ($\pm\sigma_{N-1}$) (*N*=number of tests).

ND, not determined.

Effects of modulating gas-phase diffusion on kinetics of light emission in electrically stimulated fireflies

In order to provide constraints upon the gas-phase distance between the photocytes and any gating structure responsible for this control of oxygen supply (and hence to determine its possible nature), we studied the kinetics of light emission in electrically stimulated fireflies held in normobaric, normoxic gas mixtures of varying binary diffusion coefficients. These were 21 kPa O_2 in 80 kPa of either He, N_2 or SF_6, with binary diffusion coefficients of 7.91, 2.19 and 1.00×10^{-5} $m^2\,s^{-1}$, respectively, at 310 K (Chang, 1987). The delay time *t*, taken for peak flash intensity after stimulation, was measured (Fig. 2) and this and the ratio of delay times in either O_2/He and O_2/N_2, or in O_2/He and O_2/SF_6 gas mixtures, $t_{N_2/O_2}/t_{He/O_2}$ and $t_{SF6/O_2}/t_{He/O_2}$, respectively, were calculated and the data presented in Table 1. The greater the distance from the gating structure to the photocytes, the more this value will increase from unity. It can be seen from Table 1 that the mean ratio of delay time in low-diffusivity gas/high-diffusivity gas mixture in the same specimen was 0.997±0.022, implying either that a gas-phase gating structure must be close to the photocyte or that gas-phase gating is not important in flash control. The time taken for gas-

phase diffusion from such a structure to the photocyte must therefore be less than the observed experimental error, approximately 2 ms, corresponding to an r.m.s. distance in the gas phase from the gating structure to the photocyte of about 300 μm. This is substantially less than the average gas-phase distance from the spiracles to the photocyte (see later) and so these cannot be sites of gas-phase oxygen gating. However, the distance from the tracheal end cell to the photocyte is within this value (Ghiradella, 1977; Ghiradella, 1998; Case and Strause, 1978) and so these measurements do not discount its prior assignment as the potential oxygen gating structure (Alexander, 1943), although this is now thought not to be the case.

Calculation of gas- and aqueous-phase diffusional barriers for oxygen supply to photocytes

By analysing the delay required for peak pseudoflash intensity in different gas mixtures, it is possible to calculate the r.m.s. pathlengths of diffusion in the gaseous and aqueous phases (Timmins et al., 2000), and this leads to values of 2.21±0.24 mm and 27.4±5 μm, respectively. These values are in agreement with overall morphology of the firefly lantern (Ghiradella, 1977; Ghiradella, 1998; Case and Strause, 1978). During the period of anoxia prior to initiation of the pseudoflash, the levels of fluid in the tracheoles will be minimised, decreasing the barrier against oxygen diffusion to the photocytes they supply (Wigglesworth et al., 1935). This presumably occurs through the osmotic mechanism proposed by Wigglesworth, in which intracellular accumulation of metabolites from anaerobic metabolism during hypoxia increases the intracellular osmotic potential, with diffusion of fluid from the tracheoles into the cells being driven by this change in osmotic pressure. Thus the pathlength of aqueous-phase diffusion measured in these experiments approaches the minimum possible dictated by lantern morphology and physiology, although that occurring during flashing may be smaller.

Analysis of the delay to maximum light emission in hyperoxia-induced glowing is more complicated than the transient pseudoflash, as the former represents attainment of a new steady state. However, the long times indicate the barrier to diffusion occurs primarily in the aqueous phase, with the time to maximal glowing in spontaneously flashing and non-flashing samples (10.2 and 31.6 s) implying r.m.s. distances of diffusion in the aqueous phase of approximately 120 and 220 μm, respectively (Boag, 1969). [From type 'B' boundary conditions for planar geometry, with 99% maximal diffusion into photocyte at dt/l^2=2.0, where l is diffusion pathlength; pp. 170–171.] These must represent the extent of fluid filling the terminal portions of the tracheoles during the period between flashes and during non-flashing periods, respectively, as these are much greater than the r.m.s. aqueous-phase diffusion distance measured in pseudoflash experiments (such values are within the typical length of tracheoles in a variety of insect tissues, i.e. 200–300 μm, although some filling of the smaller trachea supplying the tracheoles may also occur). The large differences in the length of the aqueous-phase diffusional barrier dependant upon oxygen supply also provide a hint as to how oxygen supply to the photocyte might be gated, and hence provide the mechanism of flash control.

From Krogh's equation of diffusion in a planar model (applicable to supply by tracheoles where length >> area), one can define the value of any particular barrier to oxygen diffusion in terms of the gradient in P_{O_2} required to maintain a given oxygen flux through that barrier

$$\Delta P_{O_2} \times \dot{M}_{O_2}{}^{-1} = \alpha^{-1} \times d_{O_2}{}^{-1} \times l \,, \quad (1)$$

where $\dot{M}_{O_2}$ is oxygen flux (moles s^{-1} m^{-2}), α is oxygen solubility (at 0.01 moles m^{-3} kPa^{-1}), d_{O_2} is the diffusion coefficient of oxygen (3.0×10^{-9} m^2 s^{-1}), ΔP_{O_2} is the difference in oxygen partial pressure (kPa) and l is diffusion pathlength (m). Using this we can calculate the overall barrier to diffusion from the spiracle to the photocyte in the aqueous phases for two cases of tracheolar fluid length observed, namely the value of 120 μm during the dark phase between spontaneous flashes, and the near-minimum possible value of 27.4 μm from pseudoflash measurements, with a constant air pathlength of 2.21 mm. This leads to values of 4.0×10^6 and 9.13×10^5 kPa mol^{-1} s m^2, respectively, during the dark phase between flashing and during anoxia, respectively (the value for the gaseous phase is much smaller, at 10.5 kPa mol^{-1} s m^2), indicating that the difference in P_{O_2} must be 4.3-fold greater in the former case to ensure oxygenation in the photocyte. This correlates with the observation that in the dark phase in a normoxic environment, bioluminescence is achieved by maintaining anoxia within the photocyte, and that by increasing the gradient in P_{O_2} from the external environment to the photocyte by fivefold (21–101 kPa) this repression of bioluminescence is lifted.

The even greater tracheolar fluid length in non-spontaneously flashing specimens (approximately 220 μm) implies that during periods when flashing does not occur, an even greater barrier to oxygen diffusion to the photocytes is maintained. Anoxia within the central portions of the photocytes (to maintain repression of light emission from the photocyte vesicles) is a function both of controlling oxygen diffusion by the length of the tracheolar fluid and of the oxygen consumption by mitochondria in the periphery of the photocytes (adjacent to the tracheoles). Hence, increasing the length of the tracheolar fluid when spontaneous flashing does not occur (i.e. most of the time) will allow a decrease in mitochondrial respiration during this time, and this may be important in minimising the overall energetic cost of this mechanism. The requirement for a short period of time between physical stimulation and competency for light emission observed by Case and Buck (Case and Buck, 1963), during which atypical light emission (dim, localised glowing, blushing and a final flash) can occur, may in part result from the time taken to induce a reduction in tracheolar fluid length from approximately 220 to 120 μm, i.e. changing from a 'resting' to a 'primed' state. However, and perhaps more importantly, during this time one might reasonably expect temporary mismatches between increasing oxygen supply (by decreasing tracheolar fluid levels) and increasing mitochondrial consumption (to maintain anoxia within the

photocyte centre between flashes), which may result in this atypical light emission.

Thus, a system of controlling oxygen access to the photocytes by modulating tracheolar fluid levels is in agreement with the calculated diffusional barriers from fireflies *in vivo*. Since it occurs by changes in the length of the aqueous-phase diffusional pathlength, it is also consistent with the observation that alteration of gas phase diffusion coefficients had no measurable effect upon flash kinetics in electrically stimulated fireflies.

A possible mechanism for modulating tracheolar fluid levels to control flashing

It has been argued that the repression of firefly bioluminescence is achieved by regulating oxygen access to the photocytes, and shown that the gating of oxygen access to photocytes by modulation of tracheolar fluid levels between values that have been directly measured *in vivo* provides a mechanism for controlling oxygen access to the photocytes. We also know that adult firefly lantern tracheoles have a uniquely strengthened structure (Ghiradella, 1977; Ghiradella, 1998), that the nerves stimulating the flash motor unit terminate upon the tracheal end cells and not the photocytes, and that these cells show morphological features characteristic of cells adapted for active transport of ions and fluid; indeed the foremost and lattermost observations led Ghiradella (Ghiradella, 1977; Ghiradella, 1998) to conclude that some sort of osmotic mechanism might be involved in controlling the flash. Maloeuf (Maloeuf, 1938) observed that injection of a hypertonic solution into fireflies resulted in continual and prolonged glowing, and interpreted this as showing that a decrease in tracheolar fluid levels (due to the resultant increased osmotic pressure in the insect abdomen) resulted in an increased transport of oxygen to the light-emitting cells. Maloeuf suggested that a direct change in photocyte osmotic potential would modulate tracheolar fluid levels and therefore control the flash (although later studies showing a lack of direct photocyte innervation make this less likely; Ghiradella, 1977; Ghiradella, 1998; Case and Strause, 1978). This mechanism was later argued against by Alexander (Alexander, 1943) who, although concurring that modulation of tracheolar fluid levels occurred, and was important in a '*secondary regulatory nature rather than being involved in the flash itself*', rather suggested that the primary mechanism of controlling the flash through oxygen supply to the photocytes was the tracheal end cell acting as a mechanical gas-phase valve; however, this has been discounted (Case and Strause, 1978).

All of these disparate observations are most convincingly integrated into one mechanism by which the access of oxygen to the photocyte is gated by modulation of tracheolar fluid levels, as hypothesised by Malouef (Malouef, 1938), but with changes in fluid levels being actively brought about by nervous stimulation of the cells that are innervated, the tracheal end cells. This nervous stimulation is postulated to result in rapid and transient fluid uptake into the tracheal end cell and the tracheolar cell that ensheathes the tracheoles, probably by movement of an ion across a membrane (analogous is the case for Ca^{2+} activation of muscle contraction). The morphology of both the tracheolar and tracheal end cells appears to be classically adapted to sodium pumping (Ghiradella, 1977; Ghiradella, 1998), and so this may well be the ion utilised. This increases tracheolar cell osmotic pressure and results in the absorption of fluid from the tracheoles into the tracheolar cell (and perhaps, hence, the tracheal end cell), with the uniquely strengthened structure of these tracheoles (Ghiradella, 1977) being required to withstand the lateral forces due to the large and rapid changes in pressure. The decreased barrier against oxygen diffusion to the photocytes is now sufficiently low to allow transient oxygenation of the photocyte interior, and light emission. Fig. 2A,B shows diagrams of the postulated mechanism, and Movie 4 provides a time course of this mechanism. Rapid ending of increased tracheal end cell osmotic pressure reverses the process, and results in an increase in tracheolar fluid level, raising the barrier against oxygen diffusion to the photocytes, resulting in internal anoxia and repression of light emission.

A key question is whether such a mechanism could operate with the speed required in order to be compatible with the observed kinetics of light emission, i.e. is there sufficient time for the movement of a length of tracheolar fluid of approximately 100 μm within the time period between arrival of the nervous action potential at the lantern and light emission (typically 40–60 ms)? It would appear that firefly tracheoles are highly permeable to water (Ghiradella, 1998), and this may result from the presence of specific water-channel proteins, aquaporins, known to occur in other insect tracheoles (Pietrantonio et al., 2000). Such high permeability is a fundamental requirement for the operation of such a mechanism. Since the transport of tracheolar fluid into the tracheolar cell will presumably occur axially along the strengthened wall of the tracheole, the diffusional path length of water from inside the tracheole into the tracheolar cell (resulting from the change in osmotic potential) will only be of the order of approximately 10 μm, requiring approximately 25×10^{-6} s, with bulk flow along the tracheole (which is not restricted by diffusion) being responsible for most of the required length of movement of fluid. Thus, such a mechanism could indeed operate sufficiently rapidly to be consistent with the observed kinetics of light emission *in vivo*.

The evolution of such a mechanism for the rapid control of light emission (as opposed to the slower acting mechanism in the larval form, presumably acting *via* control of the reaction of luciferin with ATP; Case and Strause, 1978) can therefore be envisaged as having merely required progressive modifications to one of the preexisting mechanisms by which insects control tissue oxygen supply, rather than the less likely concept of development of an entirely novel mechanism for gating oxygen access. Since present-day selection by females for more rapidly flashing males (in *Photinus consimilis*) is known to occur (Branham and Greenfield, 1996), a similar selection process could feasibly have provided the evolutionary pressure required for its initial development and

refinement. It is also interesting to speculate that the active control of tracheolar fluid length in modulating oxygen supply to tissues, as opposed to that passively caused by intracellular accumulation of metabolites from anaerobic metabolism during hypoxia as proposed by Wigglesworth (Wigglesworth, 1935), might be more widespread in insects.

Finally, it is also worth noting that an analogous system of oxygen control by using an aqueous-phase diffusion barrier of variable length at the ends of a predominantly gas-phase oxygen supply system, although more slowly acting, has evolved to control the oxygen supply to the symbiotic bacteroids in legume nodules. These must meet the requirement for oxygen for aerobic energy metabolism (nitrogen fixation is highly endergonic) whilst avoiding oxygen-induced destruction of the bacterial enzyme nitrogenase, in the face of an oxygen supply that can vary widely, as soil oxygen concentrations greatly vary due to flooding (Witty and Minchin, 1998). Thus, similar mechanisms to achieve control of cellular oxygen concentrations would appear to have evolved in both animal and plant kingdoms, which is an interesting demonstration of convergent evolution.

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