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## DIRECT DETERMINATION OF THE KINETICS OF OXYGEN DIFFUSION TO THE PHOTOCYTES OF A BIOLUMINESCENT ELATERID LARVA, MEASUREMENT OF GAS- AND AQUEOUS-PHASE DIFFUSIONAL BARRIERS AND MODELLING OF OXYGEN SUPPLY

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### Summary

We describe the development and use of a direct kinetic technique to determine the time taken for oxygen to diffuse from the external environment into the light-producing cells (photocytes) in the prothorax of bioluminescent larvae of *Pyrearinus termitilluminans*. This was achieved by measuring the time course of the pseudoflash induced through sequential anoxia followed by normoxia. We have also determined the separate times taken for this oxygen diffusion in gaseous and tissue (predominantly aqueous) phases by using helium and nitrogen as the carrier gas. Of the total time taken for diffusion, that in the gas phase required  $613 \pm 136$  ms (mean  $\pm$  S.E.M.,  $N=5$ ) whilst that in the aqueous phase required  $1313 \pm 187$  ms. These values imply pathlengths

of diffusion in the gaseous and aqueous phases of  $4.80 \times 10^{-3} \pm 0.53 \times 10^{-3}$  and  $8.89 \times 10^{-5} \pm 0.61 \times 10^{-5}$  m, respectively. In addition, the pathlength of gas-phase diffusion was used to derive a parameter relating to the tortuosity of the tracheal system. These values, together with those obtained upon bioluminescent oxygen consumption, have been used to model oxygen supply to the photocyte. From these studies, it would also appear that the modulation of tracheolar fluid levels might be a significant mechanism of control of tissue oxygen levels in at least some insects.

Key words: bioluminescence, insect, oxygen, respiratory physiology, *Pyrearinus termitilluminans*.

### Introduction

The supply of oxygen for insect respiration is mediated through gas-phase transport in the tracheal network and, although it can be driven by active muscular pumping which causes tidal air volume changes in the tracheal network (mainly in flying insects and not elaborated upon further here, e.g. Greenlee and Harrison, 1998; Joos et al., 1997), it is often driven simply by diffusion, as is the case for most non-flying insects (e.g. Krogh, 1941; Buck, 1962; Snyder et al., 1995; Tenney, 1985). It is thought that the main site of resistance to gas-phase diffusional transport of oxygen is the spiracle (e.g. Buck, 1962; Tenney, 1985) and that control of spiracle opening is determined by balancing the requirements of supplying sufficient oxygen and off-loading carbon dioxide, whilst minimising loss of water. However, as recently and eloquently described by Lighton (1996, 1998), those insects displaying discontinuous, cyclic gas exchange do not do so primarily to retain water, but rather as an adaptation to hypercapnic and hypoxic environments.

Importantly, however, in addition to gas-phase diffusional

resistance, there will also be significant diffusional resistance to oxygen supply in the fluid-filled terminal branches of the tracheal network and in the cells themselves, because the diffusion coefficient of oxygen in the aqueous phase is approximately  $10^4$  times smaller than in the gas phase. Indeed, it was demonstrated by microscopic observation some time ago that the extent of fluid filling of the tracheolar endings is variable and correlates inversely with oxygen supply (for a review, see Wigglesworth, 1965). Although some study has been made of the relative contributions of these two diffusional barriers to oxygen supply, for example by determining the 'critical partial pressure of oxygen ( $P_{O_2}$ )' for movement in hypo- and normobaric hypoxia (Tenney, 1985) with some resistance to gas-phase diffusion being observed, no definitive conclusion could be drawn. Accurate quantification of the contributions of these two diffusional barriers to insect oxygen supply has not, to the authors' knowledge, been attempted, which is surprising considering the potential role of tracheolar fluid levels in the control of tissue oxygen supply (Wigglesworth, 1965).

We describe the development and use of a direct kinetic technique to determine the time taken for oxygen to diffuse from the external environment into the photocytes in the prothorax of bioluminescent larvae of *Pyrearinus termitilluminans*. We have also determined the relative contributions of gas- and tissue-phase (predominantly aqueous) diffusion time in this oxygen supply and used this data in modelling of oxygen supply. The ideas presented are also developed to provide further insight into a possible mechanism of control of insect tissue oxygenation.

### Materials and methods

Larvae of *Pyrearinus termitilluminans* 2–3 cm long were collected from luminous termite mounds in the cerrado (open pastures) of Goiás, Brazil (Bechara, 1988), and stored as previously described, except that they were fed with adult *Drosophila melanogaster* (Barros and Bechara, 1998). The biology and behaviour of these larvae have been extensively documented in an account by Bechara (1988), to which the interested reader is directed. Briefly, during feeding just after dusk, when the larvae became aware of the presence of their prey (*Drosophila melanogaster* in these laboratory-based experiments), they underwent a prolonged period (usually between 0.5 and 1 h) of bioluminescence during which these experiments were performed. If the prey was captured, bioluminescence ended (within a few minutes). The bioluminescent light emission was measured *via* the voltage output from an unbiased blue-green-corrected silicon photodiode connected to a digital storage oscilloscope (Timmins et al., 1999). The experiment was conducted under highly subdued lighting at night in a laboratory maintained at 20–22 °C, with additional screening of the apparatus (black material). The larvae were gently restrained in a constricted transparent plastic holder in a gas stream that was analysed using an online oxygen analyser.

Measurements of the 'pseudoflash' were conducted by changing the oxic normobaric gas stream in which the larva was held to an anoxic one (either 100 % helium or 100 % nitrogen) for 1 min until bioluminescence was no longer visible. It was observed (although not quantified in this experiments) that extinction of bioluminescence was more rapid when helium was the carrier, indicating that diffusion of oxygen out of the larva can be important in causing anoxia, in addition to oxygen consumption. A rapid gas switching valve (Cole-Palmer model E-01367-72, dead time 15 ms) was then used to switch the gas stream rapidly to a normoxic gas mixture comprising 21 kPa oxygen in the appropriate carrier gas (i.e. 21 kPa O<sub>2</sub>, 80 kPa He for helium experiments). All gas mixtures were essentially free of CO<sub>2</sub>. From the known gas stream velocity, valve dead time and the dead space between the valve and the prothorax (minimised as far as experimentally possible), the total time taken to change the gas mixture supply to the larval prothorax was estimated to be 25 ms, which was subtracted from all experimental values. This technique was a substantial improvement in dead time upon

our previous observations of approximately 1 s (Timmins et al., 1999). The voltage used to activate the solenoidal gas valve was simultaneously used to trigger the time base of an oscilloscope, and the time of peak bioluminescence was directly measured from the oscilloscope (using 10× time base expansion for accuracy when useful).

The carrier gas identity was cycled every three pseudoflashes (i.e. three measurements in He, He/O<sub>2</sub> followed by three measurements in N<sub>2</sub>, N<sub>2</sub>/O<sub>2</sub>, etc.) Once baseline bioluminescence (i.e. during normoxia after each pseudoflash) did not return to within 25 % of the initial reading, indicating that the experimental procedure was overly affecting larval bioluminescence or respiratory physiology, the experiment was discontinued. Importantly, if bioluminescence was not long-lived enough to permit cycling through more than five changes of carrier gas (i.e. ≥15 individual measurements), the experiment was disregarded, as any observed differences between carrier gases could not be definitively assigned as being reproducible and, hence, might instead arise from a long-term 'drift' in the physiological state of the larva. These requirements limited the final number of larvae from which data are presented.

### Results and discussion

#### *Principles of measurement*

During the course of our studies of the oxygen supply to bioluminescent larvae of *Pyrearinus termitilluminans* (Barros and Bechara, 1998; Timmins et al., 1999), we observed that inducing a period of anoxia for a larva undergoing bioluminescence resulted in inhibition of light emission, and upon reoxygenation an intense 'pseudoflash' was observed (Fig. 1). In a similar manner to studies of *Photinus* sp. (Hastings et al., 1953; Hastings and Buck, 1956), we attributed the inhibition of bioluminescence as being due to anoxia within

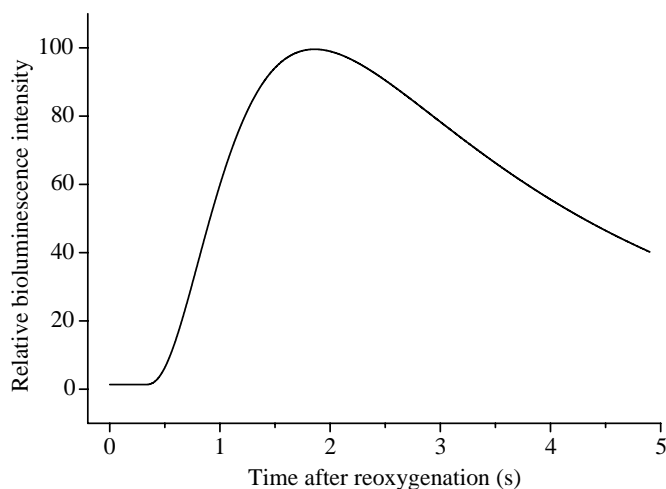
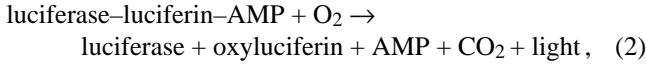
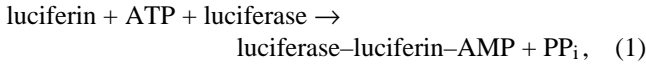


Fig. 1. Time course of a typical pseudoflash in *Pyrearinus termitilluminans* caused by sequential anoxia and reoxygenation with nitrogen as carrier gas.

the photocyte (molecular oxygen,  $O_2$ , is absolutely required for bioluminescence, see equations 1 and 2), resulting in an accumulation of the luciferase–luciferin–AMP intermediate in the photocyte:



where  $PP_i$  is inorganic phosphate.

Concurrently, there will be opening of the spiracle due either to normal respiratory control or to anoxia-induced narcosis of its controlling musculature. Upon external reoxygenation, oxygen diffuses through the tracheal network to the photocytes, resulting in a transitory phase of increased bioluminescence due to  $O_2$  reacting with accumulated luciferin–AMP (the pseudoflash), followed by a return to the normal state. The reaction of insect luciferase–luciferin–AMP with oxygen is rapid (maximal intensity *in vitro* occurs within 60 ms; De Luca and McElroy, 1974) and so light emission reports upon the kinetics of intracellular reoxygenation in the photocytes following anoxia. Since both this *in vitro* and the *in vivo* pseudoflash experiments involve the same reaction, we have assumed similar kinetics *in vivo* but have not corrected the data for this time delay of less than 60 ms because precise *in vivo* data are not available and its effect upon the data and interpretation is minor.

In the experiments described here, the effects of convection due to oxygen-consumption-mediated bulk flow of gas can be discounted because oxygen consumption cannot occur until oxygen has already diffused to its site of consumption (primarily bioluminescence in the photocytes). Thus, only once the pseudoflash has occurred can bulk flow contribute to oxygen transport. The delay between external reoxygenation and the peak of pseudoflash bioluminescence therefore directly measures the time taken for  $O_2$  to diffuse from the external to the intracellular environment, providing accurate kinetic data upon this phenomenon. Since the root mean square of the distance diffused by  $O_2$  in a given time is a function of its diffusion coefficient (equation 1), the time taken for oxygen to diffuse from the external environment into the photocyte will be given by simultaneous linear equations 4 and 5, assuming that there are two separate phases in which diffusion occurs (gaseous and tissue/aqueous), which have separate pathlengths and coefficients of diffusion, and that the peak of pseudoflash bioluminescence coincides with peak arrival of intracellular oxygen by diffusion following anoxia. In these larvae, as for larval fireflies, the control of bioluminescence is not thought to involve regulation of oxygen levels, although this has been hypothesised to occur in adult fireflies (Wilson and Hastings, 1998; Case and Strause, 1978).

$$\sqrt{\bar{x}^2} = \sqrt{2d_{O_2}t}, \quad (3)$$

$$\text{time lag}_{\text{nitrogen}} = \frac{\bar{x}_{\text{gas phase}}^2}{2d_{O_2\text{in nitrogen}}} + \frac{\bar{x}_{\text{aqueous phase}}^2}{2d_{O_2\text{in aqueous phase}}}, \quad (4)$$

$$\text{time lag}_{\text{helium}} = \frac{\bar{x}_{\text{gas phase}}^2}{2d_{O_2\text{in helium}}} + \frac{\bar{x}_{\text{aqueous phase}}^2}{2d_{O_2\text{in aqueous phase}}}, \quad (5)$$

$$2d_{O_2\text{in helium}} = 3.67 \times 2d_{O_2\text{in nitrogen}}. \quad (6)$$

where  $\bar{x}^2$  is the mean of length<sup>2</sup> of the diffusion path in a given phase ( $m^2$ ),  $d_{O_2}$  is the diffusion coefficient of oxygen in a given carrier ( $m^2 s^{-1}$ ),  $t$  is time and time lag is the delay between external reoxygenation and peak pseudoflash intensity.

The binary gas-phase diffusion coefficient of oxygen in helium ( $6.98 \times 10^{-5} m^2 s^{-1}$  at 293 K) is 3.67 times greater than in air ( $1.90 \times 10^{-5} m^2 s^{-1}$ ), leading to root-mean-square velocities of oxygen that are 1.91 times higher in helium; however, diffusion of oxygen in the aqueous phase is unaffected by the carrier gas, with a diffusion coefficient of  $3.0 \times 10^{-9} m^2 s^{-1}$  (Witty and Minchin, 1994). Therefore, by performing the pseudoflash experiment in different carrier gases of helium and nitrogen, equations 4 and 5 can be solved through substitution with equation 6 to provide the time taken for oxygen to diffuse in the gaseous and tissue phases and, hence, the pathlengths of diffusion (as root mean square), assuming that that Fickian diffusion applies in the gas phase (Pickard, 1974) for both He and  $N_2$  as carrier gas.

A tracheal tortuosity parameter ( $P_t$ ), relating to the tortuosity of the gas-phase tracheal oxygen supply due to its repeated branching, can be defined from the ratio of the observed pathlength of gas-phase diffusion to the anatomical radius of the larva, with higher values indicating the extent of tortuosity of the tracheal network through which oxygen must diffuse. Although the value of  $P_t$  is dependent upon some assumptions, it does provide an intuitive estimate of tracheal tortuosity and limits to the modelling of tracheal structure and function.

#### Experimental results and modelling of oxygen supply

Fig. 2A,B shows representative histograms of time of pseudoflash peak intensity in helium and nitrogen carrier gases (respectively) for a typical larva. It can be seen that, although the means obtained with helium and nitrogen as carrier were significantly different, there were a range of times of pseudoflash peak intensity (similar ranges were observed for all larvae measured). Since this variation is greater than the error arising from the measurement procedure (maximum uncertainty approximately 50 ms), then it most probably represents short-term biological variations within the larva such as might arise from fluctuations in the degree of spiracular opening, changes in fluid levels in the tracheoles or body-movement-induced changes in tracheal volume or length (although no gross body movement was observed).

Table 1 shows the time of pseudoflash peak intensity with helium and nitrogen as carrier gas for the five larvae for which a full set of data could be obtained. Also shown are the calculated times and distances of diffusion in the gas (nitrogen) and aqueous phases, together with the calculated tracheal tortuosity parameter  $P_t$ . It is apparent that the calculated diffusion pathways and  $P_t$  values are similar for the larvae, as might be expected from their similar anatomical sizes.

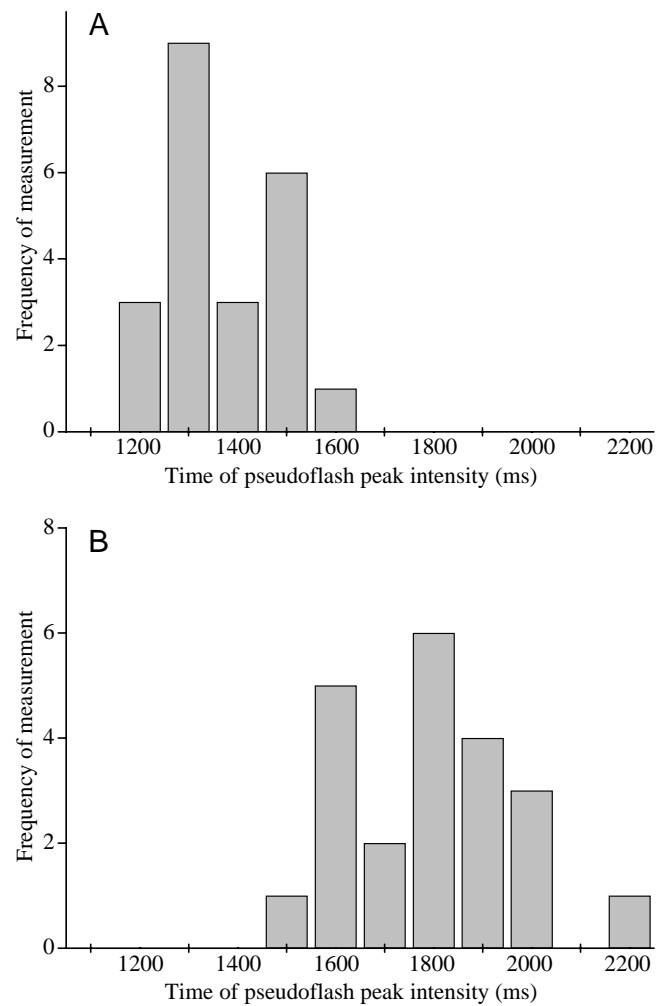


Fig. 2. Representative histograms of time of pseudoflash peak intensity in (A) helium and (B) nitrogen carrier gases (respectively) for a typical larva. Histogram bin size 100 ms.

Significantly, the time taken for aqueous diffusion was substantially greater than that for gas-phase diffusion in air by approximately twofold, with calculated mean pathlengths of diffusion of  $8.89\times10^{-5}$  m and  $4.80\times10^{-3}$  m respectively.

*Gas-phase diffusion in tracheae and tracheoles*

The mean pathlength of gas-phase diffusion of  $4.80\times10^{-3}$  m gives a mean tracheal  $P_t$  of 2.19, implying that the nature of the gas supply system, requiring as it does diffusion through the spiracle and the branching tracheal network, only increases this pathlength of gas-phase diffusion by a factor of approximately 3.1 when compared with a hollow gas-filled cylinder into which gas can diffuse through the entire surface. Such a figure bears witness to the effective branching nature of the tracheal system. One may model this tortuosity as effectively producing a reduction in the gas-phase diffusion coefficient of  $1/3.1^2$  in such a cylindrical model, implying that, were gas-phase diffusion the sole resistance to oxygen supply, the decrease in tracheal and tracheolar gas-phase  $P_{O_2}$  from the exterior of the larva to its centre should be of the order of 0.5 kPa, assuming a rate of oxygen consumption of  $200\times10^{-9}$  mol s<sup>-1</sup> cm<sup>-3</sup> (Timmins et al., 1999) and, hence, that bioluminescence should only be limited by hypoxia approaching this value. However, it was previously experimentally observed that bioluminescence began to become limited at hypoxic  $P_{O_2}$  values between 6.7 and 10.7 kPa, and so the resistance to oxygen diffusion to the photocytes in the gas phase must be relatively minor compared with other factors.

*Aqueous-phase diffusion in tracheoles and tissue*

The magnitude of the aqueous-phase diffusional pathlength of  $8.89\times10^{-5}\pm0.61\times10^{-5}$  m argues strongly against it occurring within the cellular tissue of the light organ itself because (i) the average distance between tracheoles in insect tissue is

Table 1. Measured and calculated respiratory parameters in larvae of *Pyrearinus termitilluminans*

Larva	Pseudoflash peak		Gas phase		Aqueous phase		Tracheal tortuosity, $P_t$
	Time in N <sub>2</sub> /O <sub>2</sub> (ms)	Time in He/O <sub>2</sub> (ms)	Calculated diffusion pathlength (m)	Time in gas phase (ms)	Calculated diffusion pathlength (m)	Time in aqueous phase (ms)	
1	2401±388 N=19	1803±198 N=17	$5.60\times10^{-3}$	825	$9.72\times10^{-5}$	1576	2.24
2	1779±170 N=22	1351±114 N=22	$4.73\times10^{-3}$	589	$8.45\times10^{-5}$	1190	1.72
3	2010±352 N=20	1581±252 N=20	$4.74\times10^{-3}$	591	$9.37\times10^{-5}$	1419	1.87
4	1654±110 N=25	1288±115 N=21	$4.11\times10^{-3}$	445	$8.52\times10^{-5}$	1209	2.35
5	1897±148 N=18	1440±129 N=18	$4.84\times10^{-3}$	616	$8.38\times10^{-5}$	1170	2.77
Mean			$4.80\times10^{-3}\pm0.53\times10^{-3}$	613±136	$8.89\times10^{-5}\pm0.61\times10^{-5}$	1313±178	2.19±0.42

Values are means ± S.E.M.



much less than this value (e.g. an average of  $2 \times 10^{-6}$  m quoted by Buck, 1962; between  $0.7 \times 10^{-6}$  and  $7 \times 10^{-6}$  m based on a volume fraction of 10–0.1 %, Tenney, 1985), (ii) the maximal distance of radial diffusion of oxygen from the tracheole through the oxygen-consuming photocytic tissue is approximately  $3.8 \times 10^{-5}$  to  $1.7 \times 10^{-5}$  m (modelled using a Krogh–Erlang cylindrical model with values of oxygen consumption of  $200 \times 10^{-9}$  and  $1000 \times 10^{-9}$  mol cm $^{-3}$  s $^{-1}$  from Timmins et al., 1999), and (iii) for oxygen to diffuse a distance of  $8.89 \times 10^{-5}$  m into the photocytic tissue would require an oxygen consumption rate of only approximately  $7 \times 10^{-9}$  mol cm $^{-3}$  s $^{-1}$ , a value much lower than that observed. In the light of these constraints, one must attempt to explain the nature of the observed aqueous-phase diffusion pathlength.

The most likely explanation for the size of the aqueous diffusion barrier is that it primarily represents the amount of fluid partly filling the terminal extent of the tracheoles. This was first described in a qualitative fashion by Wigglesworth (1965), with the fluid level decreasing during hypoxia. As described above, the variation in the time of pseudoflash peak intensity implies some variation in this parameter during the course of the experiment, but it is likely that the ‘time constant’ for change in tracheolar fluid length exceeds the hypoxia/normoxia cycling time used in this experiment (approximately 1 min), resulting in some averaging of any minimal (hypoxic) and maximal (normoxic) values. In accordance with this explanation, Wigglesworth (1965) noted that rapid changes in tracheolar fluid levels only occurred at elevated temperature during extreme struggling during asphyxiation.

This was confirmed by direct observation of larvae using the transmission light microscopy approach of Wigglesworth, with no changes in the extent of tracheolar fluid filling observed in the bioluminescent prothorax being observed during 60 s of hypoxia at either 40× or 100× magnification, although it must be stated that it is possible that changes in tracheal/tracheolar fluid length in this organism might occur in tracheoles of insufficient diameter to resolve optically.

#### *Quantitative assessment of a mechanism of control of tissue $P_{O_2}$ through modulation of tracheolar fluid levels*

It would appear that, under these experimental conditions, the most significant source of the gradient in  $P_{O_2}$  between the external environment and the intracellular space of the photocyte (i.e. barrier to diffusion) is not in the gas phase of the tracheal/tracheolar system, but rather in the condensed phase, most likely the fluid filling the ends of the tracheoles. Thus, modulation of tracheolar fluid length might be a significant control mechanism by which tissue oxygen levels could be controlled in insects, operating in addition to controls acting upon the gas phase in the larger tracheae (spiracle opening/closing, active ventilation, etc.).

Since the extent of tracheolar fluid might reasonably vary between the full length of the tracheole (say  $300 \times 10^{-6}$  m) and zero, the overall time for diffusion from the external environment to the photocytes might vary between

approximately 16 and 0.7 s respectively, thus modulating the overall diffusion coefficient for oxygen transport (and hence flux for the same gradient in  $P_{O_2}$ ) from the external environment to the photocyte by a factor of approximately 20-fold. Although we currently lack quantitative values in other insects, this large factor would appear (in addition to other mechanisms such as spiracle opening/closing, active ventilation of larger airways) to account in great part for the plasticity of oxygen supply in insects (e.g. Greenlee and Harrison, 1998). In addition, if one assumes that the cross-sectional area of the tracheoles in which this mechanism operates is constant along their length and that the ends of the tracheoles are never completely free of fluid *in vivo*, then there is no respiratory water loss penalty for such a mechanism because the same area of fluid will always be exposed to the tracheal gas phase.

#### *Concluding remarks*

It has been demonstrated that the kinetics of intracellular reoxygenation can be measured in a bioluminescent larva following hypoxia. Through the use of different gas mixtures, it can be also demonstrated that the major barrier to oxygen diffusion is in the fluid, and not in the gas phase, this barrier most likely occurring in the fluid-filled termini of the tracheoles.

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