9-13-1999

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The Journal of Experimental Biology 202, 2631–2638 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
JEB2120

MEASUREMENT OF OXYGEN PARTIAL PRESSURE, ITS CONTROL DURING HYPOXIA AND HYPEROXIA, AND ITS EFFECT UPON LIGHT EMISSION IN A BIOLUMINESCENT ELATERID LARVA

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Accepted 19 July; published on WWW 13 September 1999

Summary

This study investigates the respiratory physiology of bioluminescent larvae of *Pyrearinus termitilluminans* in relation to their tolerance to hypoxia and hyperoxia and to the supply of oxygen for bioluminescence. The partial pressure of oxygen (P_{O_2}) was measured within the bioluminescent prothorax by *in vivo* electron paramagnetic resonance (EPR) oximetry following acclimation of larvae to hypoxic, normoxic and hyperoxic (normobaric) atmospheres and during periods of bioluminescence (during normoxia). The P_{O_2} in the prothorax during exposure to an external P_{O_2} of 15.2, 160 and 760 mmHg was 10.3±2.6, 134±0.9 and 725±73 mmHg respectively (mean ± S.D., N=5; 1 mmHg=0.1333 kPa). Oxygen supply to the larvae *via* gas exchange through the spiracles, measured by determining the rate of water loss, was also studied in the above atmospheres and was found not to be dependent upon P_{O_2}. The data indicated that there is little to no active control of extracellular tissue P_{O_2} within the prothorax of these larvae. The reduction in prothorax P_{O_2} observed during either attack-response-provoked bioluminescence or sustained feeding-related bioluminescence in a normoxic atmosphere was variable, but fell within the range 10–25 mmHg. The effect of hypoxic atmospheres on bioluminescence was measured to estimate the intracellular P_{O_2} within the photocytes of the prothorax. Above a threshold value of 50–80 mmHg, bioluminescence was unaffected by P_{O_2}. Below this threshold, an approximately linear relationship between P_{O_2} and bioluminescence was observed. Taken together with the extracellular P_{O_2} measurements, this suggests that control of P_{O_2} within the photocyte may occur. This work establishes that EPR oximetry is a valuable technique for long-term measurement of tissue P_{O_2} in insects and can provide valuable insights into their respiratory physiology. It also raises questions regarding the hypothesis that bioluminescence can have a significant antioxidative effect by reduction of prothorax P_{O_2} through oxygen consumption.

Key words: oxygen consumption, EPR oximetry, *Pyrearinus termitilluminans*, hypoxia, hyperoxia, respiration, bioluminescence, elaterid.

Introduction

It has been suggested that oxygen-consuming bioluminescence might have evolved in some organisms from, as or into an important oxygen detoxification mechanism (McElroy and Seliger, 1962; Colepicolo et al., 1992), although there is an equally significant body of opinion that this is not the case (Seliger, 1975; Hastings, 1983). Such a mechanism would presumably operate by reducing the oxygen concentration within the organism, hence decreasing both free radical generation (e.g. the formation of superoxide through leakage of electrons from the electron transport chain to oxygen), by decreasing singlet oxygen formation and by decreasing the chain length of peroxidative reactions. Barros and Bechara (1998) recently hypothesized that such an oxygen detoxification pathway may be important in the bioluminescent prothorax of *Pyrearinus termitilluminans* larvae exposed to hyperoxia, because levels of products indicative of oxidative damage observed upon hyperoxic treatment could be substantially increased by co-administration of an inhibitor of luciferase, luciferin-6′-methylether (LME).

To provide direct evidence for this hypothesis, and also to provide information upon more general aspects of their respiratory physiology, we have measured the partial pressure of oxygen (P_{O_2}) within the prothorax of these larvae under a variety of normobaric atmospheres, using *in vivo* electron

paramagnetic resonance (EPR) oximetry with paramagnetic crystals of lithium phthalocyanine (LiPc) (Liu et al., 1993). The effects of normobaric hypoxia and hyperoxia on prothorax P_{O_2} were measured, as was the effect of bioluminescence induced both by provoking an acute 'attack' bioluminescent response and during the sustained feeding-related bioluminescence observed following dusk. In addition, the effect of varying degrees of normobaric hypoxia upon bioluminescent light emission (feeding-related) was studied. Finally, a parameter relating to larval respiratory physiology, namely oxygen supply through the spiracle (determined as water vapor porosity), was measured in hypoxic, normoxic and hyperoxic atmospheres.

Materials and methods

Larvae of *Pyrearinus termitilluminans* (Neto et al., 1986), 2–3 cm long, were collected from termite mounds and stored in individual plastic containers containing humidified tissue as described previously, except that they were fed with adult *Drosophila melanogaster* (Barros and Bechara, 1998). Between 10 and 15 LiPc crystals (dimensions approximately 20–40 μm×5 μm×5 μm) were loaded into a 26 gauge needle and injected 2–3 mm deep into the prothorax, thereby reporting on P_{O_2} in an area of approximately 0.25 mm^3. Initially, mortality of approximately 50 % was observed within 2–3 days after implantation, presumably as a result of trauma, but a later protocol in which the larvae were briefly cooled to 4 °C prior to and during implantation resulted in 0 % mortality (0/5), and the results reported here are from these five larvae. In either case, surviving larvae were long-lived and normal in appearance and behavior, and measurements began 1 week after implantation. Larvae were acclimated to normobaric hyperoxia and hypoxia for 1 day at room temperature (20–22 °C) in a humidified chamber before measurement, using nitrogen as the diluent gas. EPR oximetry was performed upon live unanesthetized larvae, gently restrained in a plastic holder and exposed to defined atmospheres at room temperature (20–22 °C), using a 1.2 GHz EPR system as previously described (Liu et al., 1993; James et al., 1997). Magnetic field modulation (at 27 kHz) at amplitudes of less than one-quarter peak-to-peak first-derivative linewidths and non-saturating microwave power levels were used. The first-derivative linewidth was calculated using a reiterative fitting program, and thence P_{O_2} was calculated from calibration curves and, in some cases, compared with oxygen electrode measurements made using a Clark electrode with a 10 μm tip (Diamond General Corp., Ann Arbor, MI, USA).

In their natural environment, the larvae live in an 'inquilinism' in oxic tunnels excavated in the surface of termite mounds, and their bioluminescence is related to their feeding activity at night upon their prey (termites), presumably as an attractant measure. In the laboratory, two different methods of eliciting bioluminescence were employed: manual prodding with a cotton-tipped applicator to 'provoke' the larva or the introduction of prey into their container just after dusk. In the former, the bioluminescence occurred after repeated prodding, usually immediately following attack of the probe by the larva, and took 2–10 s to reach a maximum that was maintained relatively constantly for 1–2 min before fading abruptly within 10–20 s. In the latter, once the larva was aware of the presence of the prey *Drosophila* (usually as a result of contact of the prey upon its abdomen), bioluminescence occurred within 1–5 s and was long-lived (sometimes for more than 1 h). If the prey was caught, bioluminescence usually ceased shortly afterwards over 0.5–2.5 min, and it was often not possible to induce its reoccurrence by addition of more prey. Prothorax P_{O_2} was measured during bioluminescence induced either by prodding the larvae repeatedly with a cotton-tipped applicator or during feeding after dusk, with the particular stimulus used being noted in the text or figure legend.

Bioluminescent light emission (during sustained feeding-related emission) was measured using a blue-green-corrected silicon photosensor connected to a picoammeter, and the larvae were gently restrained in a constricted transparent plastic holder in a gas stream that was analyzed using an online oxygen analyzer. At these light levels, the recorded current is proportional to light intensity. The experiment was conducted under highly subdued lighting at night with additional screening of the apparatus (black material). The P_{O_2} was decreased in a stepwise fashion, with 15–20 readings being taken at each P_{O_2} once a steady reading had been obtained, before it was again reduced further (approximately 2 min total at each P_{O_2}). If bioluminescence did not return to within 20 % of the initial reading when returned to the original (highest) P_{O_2}, as occasionally happened, the experiment was disregarded. Measurements of the 'pseudoflash' were conducted by holding the larvae at a lowered P_{O_2} until bioluminescence was stable and rapidly switching the gas stream to air (within 1 s). The photodiode current was calibrated using a blue-green standard light source with a Li Cor Li-189 light meter for absolute measurement of light output, and the bioluminescent prothorax was modeled as an isotropically emitting non-absorbing cylindrical emitter of the measured anatomical dimensions.

Larval desiccation studies were conducted by placing the larvae (N=5, no larva was used for more than one determination) in weighed glass test tubes and placing them in a desiccator containing anhydrous calcium sulfate and normobaric atmospheres as above. The larvae were periodically removed for weighing (<1 min), and the tubes and desiccator were reflushed with dry gas for 10 min. Data obtained before body mass loss exceeded 7.5 % were linear when plotted against time; beyond this, the rate of dehydration decreased substantially.

Results and discussion

Principles of the mechanism of P_{O_2} *measurement in relation to insect respiratory physiology*

The line-broadening of LiPc by oxygen is a spin-exchange phenomenon, with the crystal structure possessing a suitably

sized pore for oxygen diffusion throughout the crystal (oxygen diffusion constant 3.6×10^{-7} cm^2 s^{-1}, spin diffusion constant 6×10^{-2} cm^2 s^{-1}); it therefore effectively integrates and reports upon the average P_{O_2} around the surface of each individual crystal (Bensebaa and Andre, 1992). The actual measurement made is that of peak-to-peak first-derivative linewidth (Fig. 1A), and this linewidth increases with P_{O_2} (Fig. 1B). Since the first-derivative display is used here, as in most other oximetric studies, any LiPc crystals reporting lower P_{O_2} values will exhibit a greater signal intensity and so, in a heterogeneous environment, the P_{O_2} calculated from multiple probes may be 'skewed' to lower values. The quality of line fit was not improved in any case by including more than one linewidth component however, and this phenomenon is therefore unlikely to have made significant contributions to the calculated P_{O_2} values.

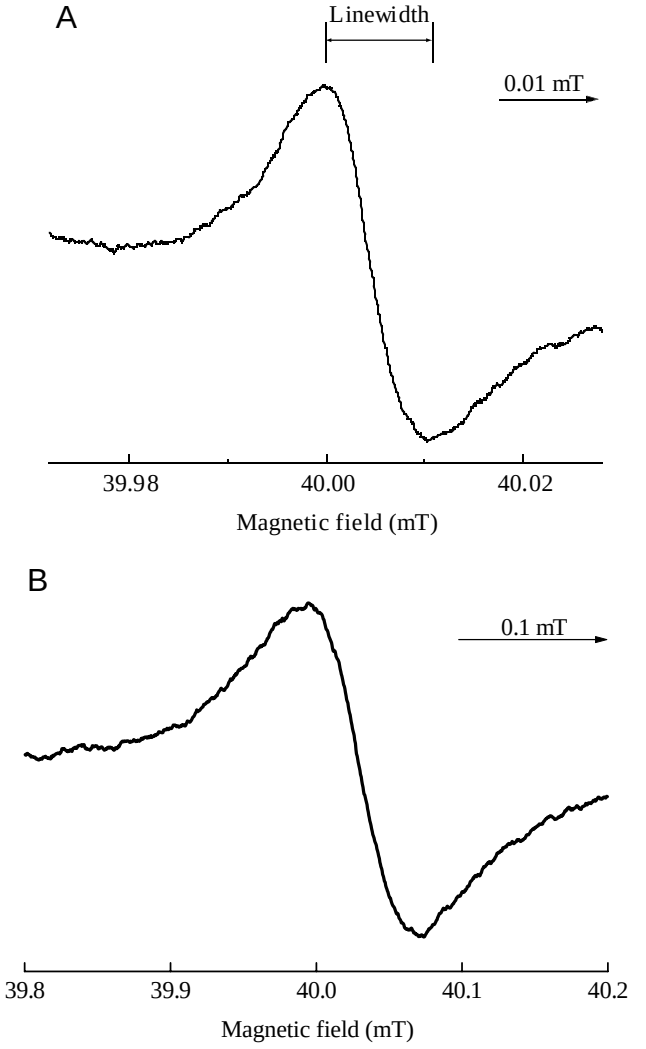

Fig. 1. Electron paramagnetic resonance (EPR) spectra of LiPc crystals implanted in larvae and recorded *in vivo* in atmospheres of (A) P_{O_2}=15.2 mmHg and (B) P_{O_2}=160 mmHg (1 mmHg=0.1333 kPa), showing peak-to-peak linewidth measurement and recorded using the apparatus and settings described in Materials and methods. Note the different magnetic field scan ranges.

Estimates of maximal inter-tracheolar distance in insect larvae are approximately 2 μm (Buck, 1962), and it is therefore likely that each LiPc crystal is in intimate contact on at least part of one of its faces with the tracheolar system, although the majority of the LiPc will be in contact with the extracellular space. It has been shown (Tenney, 1985) that, in another larval system, the critical P_{O_2} for movement was approximately 22 mmHg (1 mmHg=0.1333 kPa), and that all of the P_{O_2} gradient implicit from this measurement occurred through resistance to gaseous diffusion at the spiracles, the exterior airway openings. Similarly, the observation of the 'pseudoflash' caused by partial anoxia in the firefly (*Photinus pyralis*) requires an external P_{O_2} of approximately 1.5–4 mmHg, implying that this is the approximate magnitude of the total environment-to-intracellular oxygen gradient (Hastings et al., 1953). From this, one can conclude that, in insect systems measured at low external P_{O_2}, the average intracellular P_{O_2} is close to the average tracheolar and extracellular P_{O_2} (which is the value measured here), so that the values measured here relate directly to both the extracellular and intracellular spaces. At higher P_{O_2} levels, this relationship is less certain because no accurate data on intracellular P_{O_2} exist or can be inferred. Therefore, the data from LiPc oximetry can only be definitively interpreted as the P_{O_2} in the extracellular space. At no time during experimentation did any larvae undergo molting, so that tracheal reorganization due to any changes in external P_{O_2} will not have occurred (Loudon, 1989).

P_{O_2} *measurements in larvae in relation to respiratory physiology and tolerance of hypoxia and hyperoxia*

Typical EPR spectra recorded from LiPc in the prothorax of a larva exposed to a P_{O_2} of 15.2 and 160 mmHg are shown in Fig. 1A,B respectively. Spectra typically took less than 1 min to record, and the intrinsic response time of a free, non-implanted LiPc sample to changes in P_{O_2} was also complete within the 1 min taken to record the spectrum. The calibration curve of LiPc linewidth against P_{O_2} for the LiPc sample used in this study is shown in Fig. 2. Prothorax P_{O_2} could be measured between 0 and 760 mmHg, and the time resolution for changes in P_{O_2} of this experiment was 1 min. No long-term changes in the linewidth for individual larvae in ambient air were observed (over the course of 1 month), indicating that the LiPc was stable throughout the period examined.

The prothorax P_{O_2} in larvae acclimatized to, and measured at, normobaric atmospheres with P_{O_2} values of 15.2, 160 and 760 mmHg is shown in Table 1. Under conditions of normoxia, it can be seen that the prothorax P_{O_2}, at 134 mmHg, is close to the external P_{O_2}. This value is substantially higher than that of most mammalian tissues (other than the lung) and probably reflects the insect respiratory system of gas-phase transport into tissues through tracheae and smaller branching tracheolar airways and the high density of these structures within the insect (Buck, 1962; Tenney, 1985).

In larvae undergoing hypoxia (external P_{O_2} 15.2 mmHg), the prothorax P_{O_2} was 10.3 mmHg, a value that is roughly in

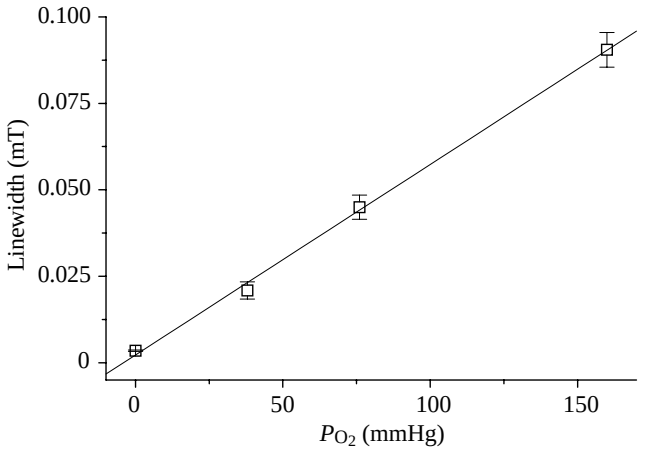

Fig. 2. The calibration curve of LiPc linewidth against *in vitro* P_{O_2} used to calculate P_{O_2} *in vivo* (1 mmHg=0.1333 kPa). Values are means + S.D. (N=5).

accord with the measurements of Tenney (1985) and that validates the use of his assumptions here: no larva undergoing this level of hypoxia stopped moving, indicating that this was above the 'critical' P_{O_2} for movement (Tenney, 1985). The fact that the external:prothorax P_{O_2} gradient is smaller under hypoxia than during normoxia (4.9 *versus* 26 mmHg) suggests that there is either some increase in gas diffusion into the prothorax (e.g. via spiracle opening) and/or a decrease in the rate of oxygen consumption. Under conditions of hyperoxia, the prothorax P_{O_2} was 725 mmHg, with the gradient to the external environment not being statistically significant.

In three larvae during normoxia, the prothorax P_{O_2} was measured using a fine-tipped, micromanipulator-mounted Clark electrode, following gentle puncture of the cuticle with a 30 gauge needle to provide access. In each case, the P_{O_2} in the prothorax was measured as below 5 mmHg (the limit of confident sensitivity of the electrode). The P_{O_2} of hemolymph that could be exuded from the needle hole by gentle pressure was also below 5 mmHg. These values are considerably different from those measured by EPR oximetry and could indicate either that the acute mechanical damage by the electrode (releasing intracellular cytoplasm and hemolymph) stimulates oxygen consumption in the fluid around the electrode or that this fluid was already of low P_{O_2}. Since EPR oximetry allows time for this acute damage after probe placement to heal, and is non-invasive after probe implantation, it was not susceptible to these artifacts.

Oxygen supply in relation to respiratory physiology

As noted above, the difference between prothorax and external P_{O_2} was slightly dependent upon the external P_{O_2}, possibly as a result of control of the spiracular aperture. The rate of loss of water from the larvae, a measure of gaseous exchange (Buck, 1962; Tenney, 1985), was therefore measured as a function of P_{O_2}, using the normobaric atmospheres as above. In this case, the rate used was the initial rate before the larva had lost more than approximately 7.5 % of its body mass (up to approximately 5 h), as it is felt that this more closely measures purely respiratory control; beyond this point, the larva will undergo additional stresses from dehydration that will complicate the measurement (as witnessed by marked slowing in the rate of water loss beyond this figure; data not shown). Table 2 shows that there was no significant effect of hyperoxia or hypoxia upon the rate of water loss and, hence, upon gaseous exchange.

The data on tissue P_{O_2} and oxygen supply suggest that there is little control of prothorax tissue extracellular P_{O_2} with regard to the external oxygen supply; at most a slight decrease in the P_{O_2} gradient between the prothorax and environment was observed. Thus, the prothorax P_{O_2} simply passively follows that of the external environment, and during hyperoxia this can result in tissue P_{O_2} values that are exceedingly high and would result in severe consequences in mammals. However, *P. termitilluminans* larvae showed no overt signs of toxicity after 3 days of exposure to a P_{O_2} of 760 mmHg, and the levels of tissue oxidation products were not greatly raised (Barros and Bechara, 1998); hence, it was suggested that bioluminescence might play a role in protection against hyperoxia.

The effect of bioluminescence upon prothorax P_{O_2}

When the larvae were observed undergoing hyperoxia, at most a slight bioluminescence was intermittently observed. However, a much greater degree of bioluminescence could be provoked in normoxic conditions by repeated gentle prodding of the larvae in the midsection with a wooden applicator; this induced them to bite at the applicator and they then shone brightly for approximately 1 min (termed the 'attacking response'). The prothorax P_{O_2} was therefore measured at time points after such provocation to determine whether such strong

Table 1. *External and prothorax* P_{O_2} *in larvae equilibrated in varying normobaric atmospheres*

External P_{O_2} (mmHg)	Prothorax P_{O_2} (mmHg)[a]	External/prothorax P_{O_2} gradient (mmHg)
15.2	10.3±2.6	4.9
160	134±0.9	26
760	725±73	35[b]

[a]Data from five individual larvae, presented as means ± 1 S.D.
[b]Not significantly different from the normoxic gradient.
1 mmHg=0.133 kPa.

Table 2. *Rate of dehydration by larvae as a function of external* P_{O_2}

External P_{O_2} (mmHg)	Rate of dehydration (% loss in body mass h^{-1})[a,b]
15.2	1.087±0.260
160	0.958±0.155
760	0.832±0.438

[a]Data from five individual larvae, presented as means ± 1 S.D.
[b]No larva was used twice or with an implanted LiPc probe; body mass range within 0.1–0.15 g.

bioluminescence could substantially alter the prothorax P_{O_2} through oxygen consumption.

Fig. 3 shows the prothorax P_{O_2} at time points after a typical series of such experiments conducted under normoxia, where the insect was provoked into bright bioluminescence. Five such experiments were usually performed per session, and the data were averaged. It can be seen that a small decrease in prothorax P_{O_2} (usually approximately 10–20 mmHg, never more than 25 mmHg) was induced, which then returned to normal within 10–12 min (10 min point not shown). There was no cumulative effect from multiple provocation within the same session provided that the P_{O_2} was allowed to recover to normal levels before repetition. This indicates that, under normoxia, the bioluminescence of this organism is not limited by oxygen supply when bioluminescence is of an acute, intense nature. The control of this bioluminescence is therefore unlikely to involve the local control of oxygen levels, as has been hypothesized to occur in photogenic cells of *Photinus pyralis* (McElroy and Hastings, 1957).

It proved possible to measure prothorax P_{O_2} in several larvae during the sustained period of bioluminescence (0.5–1.5 h) that was manifest upon feeding larvae just after dusk (a condition that approximates their natural behavior). The reduction in prothorax P_{O_2} (larvae in normoxic conditions) was variable between larvae (but consistent throughout the period measured) and was within the range 10–25 mmHg (data not shown). Thus, a sustained period of bioluminescence did not induce a significantly greater reduction in prothorax P_{O_2} than did the brief period induced by provocation, so that the potential rate of oxygen supply is significantly greater than the rate of consumption. This finding is in accord with the known reserve capacities of oxygen supply relative to demand in other insects (Joos et al., 1997).

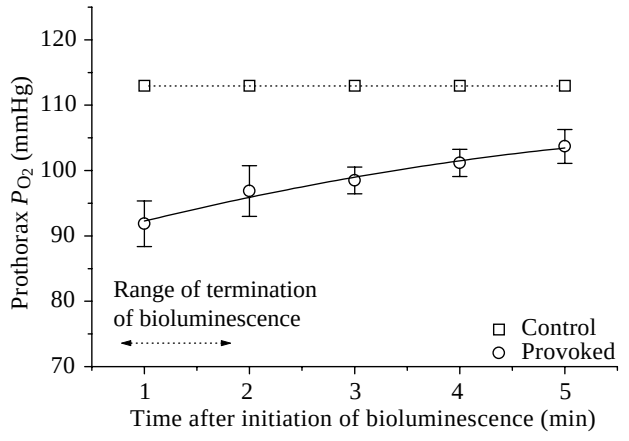

Fig. 3. Prothorax P_{O_2} recorded during a typical series of attack-response-provoking experiments conducted under normoxia. The larva was provoked into bright bioluminescence, and P_{O_2} was measured over time; bioluminescence continued for approximately 1 min after the start of the recording (range 0.5–2.5 min). Results of five such experiments performed in one session were averaged; error bars represent ±1 S.D. 1 mmHg=0.1333 kPa.

The effect of external P_{O_2} *upon bioluminescence intensity*

To determine the effects of external P_{O_2} upon bioluminescence, larvae undergoing sustained feeding-related bioluminescence were placed in a normobaric gas stream with a P_{O_2} of 160 mmHg, which was then switched to P_{O_2} values of 38, 15.2, 7.6 and 0 mmHg, and bioluminescence was observed visually. In all cases (8/8, separate non-implanted larvae), exposure to a gas stream of P_{O_2}=0 mmHg (larvae initially equilibrated at P_{O_2}=160 mmHg) caused bioluminescence to stop within approximately 60 s. If the gas stream was rapidly switched back to a P_{O_2} of 160 mmHg, the bioluminescence reached a transient maximum within 2–3 s that decreased to normal levels (see below). In P_{O_2} environments of 7.6, 15.2 and 38 mmHg (prior to hypoxia larvae were equilibrated at P_{O_2}=160 mmHg), bioluminescence was partially and reversibly inhibited (cf. 160 mmHg) in 8/8 cases, with a lowered level of bioluminescence at lower P_{O_2}.

The level of light emission from a typical prothorax at maximal bioluminescence was estimated (as described in Materials and methods) at 6×10^{-9} mol photons s^{-1} assuming that the prothorax acts as a spherical isotropic source (due to scattering of photons, with no absorption). Since this bioluminescence occurs in a volume of approximately 30 µl, and assuming a quantum yield of 1 photon per molecule of oxygen consumed, this would indicate that the bioluminescence-specific rate of oxygen consumption within the prothorax is 200×10^{-9} mol s^{-1} cm^{-3}. The likely combined errors in this value are estimated at ± two- to fivefold, and the value is most probably underestimated (e.g. some photons will be absorbed by the prothorax, quantum yield <1). This is a moderately high rate of oxygen consumption compared with whole-body values reported in larvae by others, e.g. rates for *Carpocapsa salitans* were measured within the range 8×10^{-9} to 16×10^{-9} mol s^{-1} cm^{-3} (Tenney, 1985), although much lower than values reported in active insect flight muscle that can range from 1600×10^{-9} to 6000×10^{-9} mol s^{-1} cm^{-3} (Weis-Fogh, 1964).

More quantitative experiments measuring the effect of hypoxia also proved possible in the case of several larvae in which feeding-related bioluminescence was prolonged (more than 10 min): in others (data reported above), the duration of bioluminescence was only long enough to assess visually, although the data were included as showing the generality of the response. Typical results from a series of these quantitative experiments are shown in Fig. 4. It can be seen that bioluminescence was not oxygen-limited above a P_{O_2} of approximately 80 mmHg (range for three larvae 50–80 mmHg). Once the P_{O_2} had decreased below this level, there was an approximately linear decrease of bioluminescence with P_{O_2}, an intercept of zero bioluminescence at between 0 and 10 mmHg, and half-maximal bioluminescence occurred in the P_{O_2} range of approximately 30–50 mmHg.

Since it is known that the light emission from the isolated beetle luciferin/luciferase system is unaffected by hypoxia until a P_{O_2} lower than 3.0 mmHg is achieved, with 50 % of emission at 0.1 mmHg (Hastings et al., 1953), the observed dependence of bioluminescence upon external P_{O_2} can be

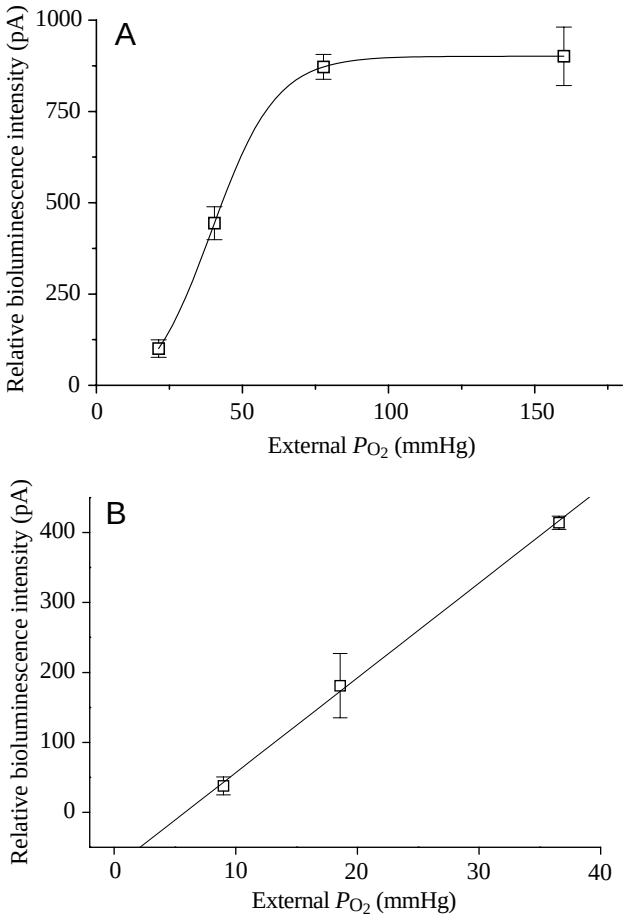

Fig. 4. Bioluminescence of a typical larva exposed to stepwise decreases in P_{O_2}. (A) An experiment conducted over a range of P_{O_2} from 160 to 20 mmHg; (B) another experiment conducted from 40 to 9 mmHg. Each point represents an average of 15 data points with error bars of ±1 S.D. 1 mmHg=0.1333 kPa.

taken to imply one of two limit conditions. In either case, the decrease in bioluminescence is not due to a decrease in substrate provision because the observation of a 'pseudoflash' upon reoxygenation (see below) implies that the supply of ATP and/or luciferin is not limiting.

Case 1

There are no large gradients in extracellular P_{O_2} throughout the prothorax, the intracellular P_{O_2} within the photocytes is quite homogeneous and is much lower (by approximately the 'threshold' value) than in the extracellular prothorax tissue and intracellular P_{O_2} decreases with external P_{O_2}, as does light intensity.

Case 2

There are large gradients in extracellular P_{O_2} through the prothorax (approximate in value to the 'threshold value'), because oxygen delivery is heterogeneous (perhaps photocytes deep in the prothorax are less well ventilated than those near its surface), and as the external P_{O_2} is decreased, the intracellular P_{O_2} of increasing numbers of photocytes drops to very low levels (less than 0.1 mmHg), thus directly inhibiting bioluminescence.

It is thought that case 2 is unlikely, because the main limit to oxygen transport into the larvae is thought to be at the level of the spiracle, not the tracheolar network (see Tenney, 1985). In addition, the variation in our measurements of prothorax P_{O_2} between larvae is smaller than would have been expected due to the randomly differing probe placement sites within the prothorax, and the inherent 'skewing' of the first-derivative spectra to low P_{O_2} would ensure that any areas of lowered P_{O_2} due to any significant gradients would have been readily observed.

Case 1 would require that a mechanism exists for the maintenance of intracellular P_{O_2} at levels greatly below extracellular levels, a contentious issue (Subczynski and Hyde, 1992; Subczynski et al., 1998). There is, however, observational support for the occurrence of such lowered intracellular P_{O_2} values (Erdmann et al., 1987; Glockner et al., 1989; James et al., 1995; Grinberg et al., 1998). This would certainly help explain the extreme resistance of these larvae to the measured extracellular tissue P_{O_2} values of over 700 mmHg during hyperoxia, and it is tempting to hypothesize that it is also linked to the known extreme radioresistance of both insects and insect cells (Koval, 1983). In addition, the low P_{O_2} values measured by needle electrodes in the prothorax could be interpreted as showing a low intracellular P_{O_2}. A definitive conclusion as to this point will, however, require direct assessment of the intracellular P_{O_2} in larvae *in vivo*.

Upon switching from a P_{O_2} of 0 mmHg to 160 mmHg, there was a transient increase in bioluminescence that ranged between 1.5 and five times steady-state levels in 160 mmHg and which lasted for a several seconds before returning to the normoxic steady state. A typical trace of bioluminescence intensity is shown in Fig. 5, and it can be seen that the

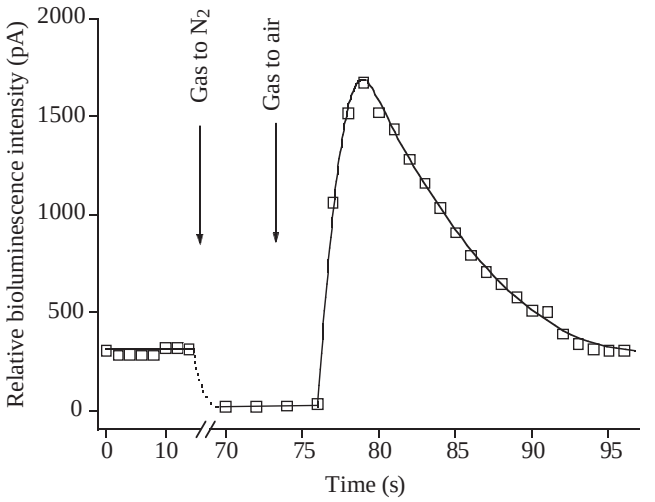

Fig. 5. Bioluminescence of a larva in a typical 'pseudoflash' experiment. The larva (undergoing sustained feeding-related bioluminescence) was first equilibrated at an external P_{O_2} of 160 mmHg, held at P_{O_2}=0 mmHg for 60 s, and then re-exposed to an external P_{O_2} of 160 mmHg. 1 mmHg=0.1333 kPa.

pseudoflash consisted of two approximately exponential processes, a rise in bioluminescence with a half-life ($t_{1/2}$) of approximately 1 s, and a decay with a $t_{1/2}$ of approximately 8–9 s. This behavior is analogous to the 'hypoxic pseudoflash' observed in *Photinus* sp. fireflies (Hastings and Buck, 1956), and is similarly assigned in the larvae studied here to an accumulation of the luciferyl-AMP intermediate within the photocyte due to a low intracellular P_{O_2} resulting from external anoxia, followed by a 'burst' of oxidation of the intermediate (and hence bioluminescence) upon reoxygenation of the photocyte. When the larva was equilibrated at low P_{O_2} in the range 15–80 mmHg, and the gas was then switched to air, a similar pseudoflash was observed, although at 80 mmHg the transient increase in light emission was only modest (approximately 25 %). This is in accord with data obtained above upon the dependence of light emission upon external P_{O_2} showing that, even at an external P_{O_2} of 80 mmHg, some oxygen-limitation of bioluminescence occurs.

Bioluminescence in relation to oxygen toxicity

From the data presented here one can conclude that oxygen consumption by bioluminescence is unlikely to be a factor causing a significant decrease in prothorax P_{O_2} in the case of hyperoxia for the following reasons: (a) in the observed absence of changes in rates of gaseous diffusion (such as spiracle opening or closing), the maximal value of the external:prothorax P_{O_2} gradient generated by maximal bioluminescence (10–25 mmHg) will also be that occurring under conditions of hyperoxia; this 25 mmHg reduction is not significant compared with the prothorax P_{O_2} during hyperoxia (734 mmHg); (b) importantly, bioluminescence (and hence bioluminescent consumption of oxygen) was only reproducibly observed during attack or feeding responses, not during hyperoxia; (c) the rate of bioluminescent oxygen consumption is well within the limits of oxygen supply 'plasticity' of insects; and (d) bioluminescence itself was not limited by oxygen level above an external P_{O_2} of approximately 80 mmHg.

In the light of these results, one must therefore attempt to explain the observation that inhibition of luciferase with LME caused a substantial increase in levels of markers of oxidative stress upon hyperoxia (Barros and Bechara, 1998). This is most probably an indirect effect, independent of any effect of bioluminescence on prothorax P_{O_2} for the following reasons: (a) bioluminescence, as described above, is unlikely significantly to reduce oxidative stress through oxygen consumption, so there is little or no effect expected by actually blocking luciferase specifically with an inhibitor; (b) the observed decrease in reduced to total glutathione ratio was much greater for luciferase inhibition during normoxia (54 %) than for hyperoxia alone (20 %; Barros and Bechara, 1998). However, even assuming all oxygen consumption can be inhibited by LME, the increase in prothorax P_{O_2} during normoxia can only be from 134 to 160 mmHg, whereas prothorax P_{O_2} increases from 134 to 725 mmHg upon hyperoxia. Clearly, the effect of LME cannot be mediated *via* changes in prothorax P_{O_2}.

The indirect effect of LME probably occurs as a result of LME metabolism in the larvae, perhaps as a result of glutathione consumption *via* glutathione-*s*-transferase-mediated conjugation of LME or a cytochrome P450-derived metabolite(s). Certainly, the amount of LME administered was much greater than the steady-state control concentration of glutathione (approximately 4 nmol mg^{-1} *versus* 0.3 nmol mg^{-1}, respectively). A substantial, long-term depletion of reduced glutathione level, induced by LME, could then lead to the observed increase in oxidative damage upon concurrent LME treatment and hyperoxia, simply as a result of the resultant reduction in antioxidant defences caused by the former.

This work was supported by NIH grant RO1 GM34250 (H.M.S.) using the facilities provided by P41 RR11602 (H.M.S.), and by FAPESP, CNPq and PRONEX (E.J.H.B.).

References

Barros, M. P. and Bechara, E. J. H. (1998). Bioluminescence as a possible auxiliary oxygen detoxifying mechanism in elaterid larvae. *Free Radical Biol. Med.* **24**, 767–777.

Bechara, E. J. H. (1988). Luminescent elaterid beetles; biochemical, biological and ecological aspects. In *Advances in Oxygenated Processes* (ed. A. L. Baumstark), pp. 123–178. London: JAI Press Inc.

Bensebaa, F. and Andre, J. J. (1992). Effect of oxygen on phthalocyanine radicals. I. ESR study of lithium phthalocyanine spin species at different oxygen concentrations. *J. Phys. Chem.* **96**, 5739–5745.

Buck, J. (1962). Some physical aspects of insect respiration. *Annu. Rev. Ent.* **7**, 27–56.

Colepicolo Neto, P., Camarero, V. C. C. P., Eickstein, J. and Hastings, J. W. (1992). Induction of bacterial luciferase by pure oxygen. *J. Gen. Microbiol.* **138**, 1–6.

Erdmann, W., Chen, C. F., Armbruster, S. and Lachmann, B. (1987). Autoregulation of intracellular P_{O_2} in *Aplysia* giant neurons and response of neuronal activity following changes in extracellular P_{O_2}. *Adv. Exp. Biol. Med.* **222**, 397–402.

Glockner, J. F., Swartz, H. M. and Pals, M. A. (1989). Oxygen gradients in CHO cells: measurement and characterization by electron spin resonance. *J. Cell. Physiol.* **140**, 505–511.

Grinberg, O. Y., James, P. E. and Swartz, H. M. (1998). Are there significant gradients of P_{O_2} in cells? *Adv. Exp. Biol. Med.* **454**, 415–423.

Hastings, J. W. (1983). Biological diversity, chemical mechanisms and evolutionary origins of bioluminescent systems. *J. Mol. Evol.* **19**, 309–321.

Hastings, J. W. and Buck, J. (1956). The firefly pseudoflash in relation to photogenic control. *Biol. Bull.* **111**, 101–113.

Hastings, J. W., McElroy, W. D. and Coulombre, J. (1953). The effect of oxygen upon the immobilization reaction in firefly luminescence. *J. Cell. Comp. Physiol.* **42**, 137–150.

James, P. E., Goda, F., Grinberg, O. Y., Szybinski, K. G. and Swartz, H. M. (1997). Intrarenal P_{O_2} measured by EPR oximetry and the effects of bacterial endotoxin. *Adv. Exp. Biol. Med.* **411**, 557–568.

James, P. E., Grinberg, O. Y., Michaels, G. and Swartz, H. M.

(1995). Intraphagosomal oxygen in stimulated macrophages. *J. Cell Physiol*. **163**, 241–247.

Joos, B., Lighton, J. R. B., Harrison, J. F., Suarez, R. K. and Roberts, S. P. (1997). Effects of ambient oxygen tension on flight performance, metabolism and water loss of the honey bee. *Physiol. Zool.* **70**, 167–174.

Koval, T. M. (1983). Intrinsic resistance to the lethal effects of X-irradiation in insect and arachnid cells. *Proc. Natl. Acad. Sci. USA* **80**, 4752–4755.

Liu, K. J., Gast, P., Moussavi, M., Norby, S. W., Vahidi, N., Walczak, T., Wu, M. and Swartz, H. M. (1993). Lithium phthalocyanine – a probe for electron paramagnetic resonance oximetry in viable biological systems. *Proc. Natl. Acad. Sci. USA* **90**, 5438–5442.

Loudon, C. (1989). Tracheal hypertrophy in mealworms: design and plasticity in oxygen supply systems. *J. Exp. Biol.* **147**, 217–235.

McElroy, W. D. and Hastings, J. W. (1957). Initiation and control of firefly luminescence. In *Physiological Triggers* (ed. T. H. Bullock), pp. 80–84. Washington: American Physiological Society.

McElroy, W. D. and Seliger, H. H. (1962). Origin and evolution of bioluminescence. In *Horizons in Biochemistry* (ed. M. Kasha and B. Pullman), pp. 91–101. New York, London: Academic Press.

Nealson, K. H. and Hastings, J. W. (1977). Low oxygen is optimal for luciferase synthesis in some bacteria – ecological implications. *Arch. Microbiol.* **112**, 9–16.

Neto, P. C., Bechara, E. J. H. and Costa, C. (1986). Oxygen toxicity aspects in luminescent and non-luminescent elaterid larvae. *Insect Biochem.* **16**, 381–385.

Seliger, H. H. (1975). The origin of bioluminescence. *Photochem. Photobiol.* **21**, 355–361.

Subczynski, W. K. and Hyde, J. S. (1992). Is the mammalian cell plasma membrane a barrier to oxygen transport? *J. Gen. Physiol.* **100**, 69–87.

Subczynski, W. K., Hopwood, L. E. and Hyde, J. S. (1998). Membranes: barriers or pathways for oxygen transport. *Adv. Exp. Biol. Med.* **454**, 399–408.

Tenney, S. M. (1985). Oxygen supply and limiting oxygen pressures in an insect larva. *Respir. Physiol.* **60**, 121–134.

Weis-Fogh, T. (1964). Diffusion in insect wing muscle, the most active tissue known. *J. Exp. Biol.* **41**, 229–256.