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BROAD OXYGEN TOLERANCE IN THE NEMATODE *CAENORHABDITIS ELEGANS*

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Summary

This study examined the effects of oxygen tensions ranging from 0 to 90 kPa on the metabolic rate (rate of carbon dioxide production), movement and survivorship of the free-living soil nematode *Caenorhabditis elegans*. *C. elegans* requires oxygen to develop and survive. However, it can maintain a normal metabolic rate at oxygen levels of 3.6 kPa and has near-normal metabolic rates at oxygen levels as low as 2 kPa. The ability to withstand low ambient oxygen levels appears to be a consequence of the small body size of *C. elegans*, which allows diffusion to supply oxygen readily to the cells without requiring any specialized respiratory or metabolic adaptations. Thus, the small size of this organism pre-adapts *C. elegans* to living in soil environments that commonly become hypoxic. Movement in *C. elegans* appears to have a relatively minor metabolic cost. Several developmental stages of *C. elegans* were able to withstand up to 24 h of anoxia without major mortality. Longer periods of anoxia significantly increased mortality, particularly for eggs. Remarkably, long-term exposure to 100 % oxygen had no effect on the metabolic rate of *C. elegans*, and populations were able to survive for a least 50 generations in 100 % (90 kPa) oxygen. Such hyperoxic conditions are fatal to most organisms within a short period.

Key words: *Caenorhabditis elegans*, hyperoxia, hypoxia, oxygen toxicity, metabolic rate, nematode.

Introduction

All known multicelluar animals require oxygen to complete their life cycle (Fenchel and Finlay, 1995; Jensen, 1995), although many metazoans can tolerate varying degrees and periods of hypoxia. Under aerobic conditions, animals typically utilize oxygen in the mitochondrial electron transport chain for oxidative phosphorylation and the synthesis of adenosine triphosphate (ATP) (Saraste, 1999). In a typical aerobic organism, more than 90 % of the oxygen consumed is used in this manner (Lu et al., 1999). Aerobic metabolism is critical for most organisms and, to maintain normal metabolic function, metazoans have evolved a variety of mechanisms either to supply oxygen to cells or to compensate for reduced oxygen levels.

The oxygen tension required in mitochondria for aerobic function is relatively low. It has been estimated that mitochondria function at full aerobic capacity at less than 0.2 kPa of oxygen and can potentially continue aerobic function at oxygen tensions as low as 0.004 kPa (Atkinson, 1980; Gnaiger et al., 1998; Richmond et al., 1997). Because normal atmospheric air has an oxygen tension of 21 kPa, oxygen would not appear to limit aerobic respiration. However, oxygen must diffuse into cells, and the rate of diffusion declines rapidly with increased distance; this factor, combined with the high rate of oxygen consumption by the mitochondria, can cause cells to become oxygen-limited (Denny, 1993; Schmidt-Nielsen, 1997).

Organisms use a variety of mechanisms to supply oxygen to cells, including respiratory and circulatory systems to transport oxygen throughout the body and oxygen-binding compounds to increase the amount of oxygen that can be carried in the circulatory system. Animals can also use behavioral modifications to maintain cellular oxygen levels if ambient oxygen levels are reduced. For example, fish in hypoxic waters can gulp oxygen-enriched surface air, and some animals living in the oxygen-minimum regions of the oceans undergo daily migrations to more oxygen-rich waters (Childress and Seibel, 1998).

Many environmental factors, such as decreased oxygen partial pressures with increased altitude, can expose animals to hypoxic conditions. Oxygen levels in aquatic and terrestrial environments are often reduced because of chemical reactions that remove or displace oxygen, in addition to biological depletion of oxygen (Lee and Atkinson, 1976). Flooded soils commonly become hypoxic as a result of blocked soil pore spaces, which limits gas diffusion and convection. Soil oxygen levels can be reduced to near zero for many days, and organisms living in such environments must cope with periodic hypoxia (Baumgartl et al., 1994; Cooper and Van Gundy, 1971b; Drew, 1992; Lee and Atkinson, 1976; Rhode, 1971).

At the cellular level, organisms have developed many methods to cope with reduced oxygen levels (Hochachka,

1980). Adaptations include increasing rates of glycolytic flux (Pasteur effect), reducing metabolic rate (Baumgartl et al., 1994; Johansson et al., 1995) or increasing the efficiency of oxygen removal from the environment (Childress and Seibel, 1998). Even with such modifications, oxygen can still become limiting, forcing the organism to survive periods of hypoxia or anoxia (Hand, 1998). Exposure to hypoxia has a wide array of effects on an organism, including metabolic depression (Clegg, 1997) and the regulation of a variety of genes including those expressing heat shock proteins (Ko and Prives, 1996; Ratcliffe et al., 1998). Despite compensatory responses, hypoxia can have toxic effects, including the generation of reactive oxygen species (Drew, 1992) that are responsible for a wide variety of biological damage (Wyllie, 1997).

If unable to obtain sufficient oxygen to function aerobically (e.g. under anoxic conditions), organisms must rely on anaerobic metabolism (Hand and Hardewig, 1996). Anoxia can also induce a wide range of potentially detrimental physiological effects. In vertebrates levels of nicotinamide adenine dinucleotide hydride (NADH), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and inorganic phosphate can increase, while ATP levels and pH can decrease within 1 min of the onset of anoxia (Foe and Alberts, 1985). Anoxic conditions can also induce apoptosis (Wyllie, 1997). Despite such effects, many animals are able to withstand periods of anoxia, although the length of time varies widely between metazoan animals. At one extreme, copepod eggs have been found that are viable after burial in presumably anoxic lake sediments for 40–332 years (Hairston et al., 1995; Marcus et al., 1994). Other animals, including some vertebrates, can also survive long periods of anoxia (Hand, 1998; Lutz and Nilsson, 1997). For example, turtles (genera *Trachemys* and *Chrysemys*) survive up to 5 months of anoxia and brine shrimp embryos (*Artemia franciscana)* up to 4 years of anoxia (Clegg, 1997; Hand and Hardewig, 1996; Lutz and Nilsson, 1997). However, the great majority of free-living eukaryotic organisms cannot withstand more than 24 h of anoxia (Clegg, 1997).

Anoxic conditions may have been a powerful selective factor during the evolution of life. The oceans are thought to have been anoxic for most of earth's history, remaining anoxic until the Neoproterozoic, 5×10^8 to 10^9 years ago (Canfield, 1998). Although this hypothesis is controversial, it has been proposed that a world-wide anoxic event was responsible for the large extinction event at the end of the Permian period 250×10^6 years ago (Isozaki, 1997; Wignall and Twitchett, 1996; but see Rampino, 1996; Retallack and Holser, 1997). At this time, a presumed anoxic event occurred in the world's oceans that may have persisted for 20×10^6 years. It has been proposed that past anoxic events may have selected for the ability of organisms to reduce their metabolic rate during periods of hypoxia (Vermeij and Dorritie, 1996).

While anoxic conditions are detrimental to most organisms, increased oxygen levels can also have toxic effects. Hyperoxia toxicity is thought to be caused by an increased production of free radicals and the generation of hydrogen peroxide associated with higher oxygen levels (Joenje, 1989; Paget et al., 1987b). Among other effects, hyperoxia can disrupt metabolic pathways and reduce respiration rates (Gille and Joenje, 1992; Jamieson, 1989; Schoonen et al., 1990a).

The free-living soil nematode *C. elegans* has become a popular model organism for a wide array of biological studies. While there is a wealth of information on its genetics, development and molecular biology (Pennisi, 1998; Riddle et al., 1997; Wood, 1988), the basic physiology of *C. elegans* is less well understood. Nematodes lack both specialized respiratory systems and complex circulatory organs, and they must rely on diffusion from the surrounding environment into their tissues for gas exchange (Atkinson, 1980; Paget et al., 1987a). Although well-adapted to low-oxygen environments, all known nematodes require periods of aerobic respiration to complete their life cycle (Atkinson, 1973; Bolla, 1980). This study examined the effect of varying oxygen levels on metabolic rate, movement and survivorship in *C. elegans*.

Materials and methods

Metabolic measurements

Caenorhabditis elegans were maintained at 20 °C on nematode growth medium in 35 mm Petri dishes and fed live *Escherichia coli* (strain OP50) following standard *C. elegans* culture techniques (Brenner, 1974). Metabolic measurements were conducted using several hundred wild-type (N2 strain) *C. elegans* per experiment. Age-synchronized worm populations were obtained by allowing approximately 250 adult worms to lay eggs for an 8 h period and then removing the adult worms. These eggs were allowed to hatch and develop into young adult worms (3 days old), which were used for the metabolic measurements. Worms were washed from the dishes with 0.6× worm Ringer's solution into 15 ml centrifuge tubes. Antibiotics (Gentamicin, $1\,g\,l^{-1}$, and Kanamysin, $0.1\,g\,l^{-1}$) were added to the wash solution to prevent bacterial contamination from confounding the metabolic measurements. The tube was centrifuged at approximately $800\,revs\,min^{-1}$ for less than 1 min, and the supernatant was discarded. The worms in the bottom of the tube were collected with a 100 µl microcapillary tube and placed onto a thin 0.4 % (w/v) agarose spot (agarose was dissolved in 0.6× M9 *C. elegans* Ringer) and sealed in 5 ml glass metabolic chambers. To ensure that the worms were well-fed during the measurements, the agarose spot was coated with a layer of heat-killed *E. coli*. Metabolic chambers were kept at 20 °C during the measurements by placing them within a temperature-controlled chamber.

Each set of metabolic measurements consisted of an experimental group of worms exposed to variable oxygen tensions, a control group of worms maintained under normoxic conditions, an empty chamber and a chamber containing only an agarose spot and heat-killed bacteria. The four chambers were connected with Luer fittings that allowed air flow to be individually directed through each chamber. CO_2-free, water-

saturated air was passed through the sample chamber at 25 ml min^{-1}. Air flow was controlled with a Sierra mass flow meter (Sierra Instruments, Monterey, CA, USA) connected to a Sable System mass flow controller (Sable Systems International, Henderson, NV, USA). After passing through the sample chamber, water vapor was removed from the air flow using a magnesium perchlorate filter, and the airstream flowed into a Li-Cor 6251 (Li-Cor Inc., Lincoln, NE, USA) CO_2 gas analyzer and a Sable System PA-1 paramagnetic O_2 analyzer.

The CO_2 analyzer was zeroed daily against CO_2-free air and calibrated weekly against a 51 p.p.m. certified gas standard (Air Products, Long Beach, CA, USA). The CO_2 analyzer maintained very stable readings that were typically within 1 p.p.m. of the calibration standard. The oxygen analyzer was zeroed and calibrated daily using research-grade nitrogen (minimum purity of 99.995 %) and outside atmospheric air. After allowing the worms to stabilize in the chambers for approximately 1 h, a stream of research-grade nitrogen gas was mixed into a 1 l glass mixing flask leading into the metabolic chamber. Over the course of approximately 3 h, the oxygen level in the airstream leading to the experimental group was reduced from normoxic (21 %) to 0 % oxygen. Oxygen and carbon dioxide concentrations were analyzed with a 2 s sampling interval using Sable System Datacan data-acquisition hardware and software starting from when the worms were first sealed into the chambers under normoxic conditions. A typical group of worms produced an enrichment of approximately 15 p.p.m. CO_2 of the airstream under normoxic conditions. After exposing the worms to 1–20 min of anoxia, atmospheric air was introduced into the mixing flask. The metabolic rate (calculated as the amount of CO_2 produced) of the worms was then recorded for a 4 h period. Some of these groups were then exposed to hyperoxic conditions after this 4 h interval. Hyperoxia measurements were carried out under conditions identical to the hypoxic experiment, with the exception that 100 % gaseous oxygen rather then nitrogen was mixed into the 1 l flask. The CO_2 production of the control group of worms maintained continuously under normoxic conditions and of the sample chamber containing agarose was also measured several times during each experiment.

To determine whether the agarose spot in the chamber was a significant source or sink of oxygen or carbon dioxide, the approximate time required for a drop of fluid to come to equilibrium with the airstream was calculated (Nicholas and Jantunen, 1966). In addition, to ensure that the agarose was not an oxygen source under hypoxic conditions, one group of worms was placed on agarose spots that contained 200 mg l^{-1} sodium sulfite. This compound binds dissolved oxygen (Fernandes and Rantin, 1989; Gee and Gee, 1991), thus preventing the agarose from supplying any oxygen to the worms in the chamber.

The theoretical relationship between the body size and metabolic rate of *C. elegans* and oxygen tension was calculated using a diffusional equation derived by Hill (1929). This equation can be used to predict the maximum metabolic rate that can be supported for a cylindrical organism of a given diameter and takes the following form:

$$P = mr^2/4k,$$

where P is the oxygen tension (atmospheres; 1 atm=101.3 kPa), m is the metabolic rate (ml O_2 ml^{-1} tissue h^{-1}), r is the radius of the animal (cm) and k is the permeability constant for oxygen diffusion through tissue.

Values of k vary with tissue type; two values of k were used to calculate the point at which metabolic rate is predicted to become diffusionally limited in *C. elegans*. A constant of 8.4×10^{-4} cm^2 atm^{-1} h^{-1} assumes diffusion through muscle, whereas a constant of 6.9×10^{-4} cm^2 atm^{-1} h^{-1} assumes diffusion through connective tissue (Atkinson, 1980; Krogh, 1918; Schmidt-Nielsen, 1997). The actual rate of diffusion in *C. elegans* is probably a combination of the diffusion rates for several different tissue types. The Hill equation was used to calculate the effects of different oxygen tensions on the metabolic rate of the worms using an average worm radius of 30 μm and a metabolic rate of 7 ml O_2 g^{-1} h^{-1}. The body radius of *C. elegans* was determined by measuring the body size of 19 adult worms of different ages using measurement methods described previously (Van Voorhies, 1996). The metabolic rate of individual *C. elegans* was based on previously determined measurements (Van Voorhies and Ward, 1999).

Movement in hypoxia

To determine the relationship between the metabolic rate of *C. elegans* and movement, worm movement was recorded during the induction of hypoxic conditions. Worm movement was recorded using a Panasonic time-lapse video cassette recorder connected to a Coho video camera mounted on an Olympus stereo dissecting microscope. This recording was replayed on a video monitor, and the movement distances were calculated for 10 worms at four different oxygen tensions. Movement was divided into two components: the total distance moved in 10 s (gross distance) and the distance of an individual from its initial starting point after this 10 s interval (net movement). Although all the measurements were performed on the same group of worms, the large number of worms in the chamber makes it unlikely that any individual worm was measured twice, and each measurement was considered to be independent.

Survivorship in anoxic/hyperoxic conditions

To determine the effect of acute anoxia on *C. elegans*, groups of worms at several stages of development (eggs, second-stage larvae, fourth-stage larvae and young adults) were placed on nematode growth medium in 35 mm Petri dishes. These were placed at 20 °C in 750 ml glass Mason jars sealed with rubber stoppers. These jars were continuously flushed with water-saturated, research-grade nitrogen at 25 ml min^{-1} through an 18 gauge needle inserted through the stoppers. Worms in each of the four developmental stages were exposed to 6, 12, 24, 48, 96 and 144 h of anoxia, and survivorship was assayed. A worm was scored as dead if it did

not undergo any further development and was not moving within 24 h of the return to normoxic conditions. Survivorship was also assayed in control groups exposed to identical conditions to the anoxic groups, with the exception that ambient atmospheric air flowed through the chambers.

To examine the long-term survivorship of *C. elegans* in hyperoxia, seven experimental populations and seven control populations of worms were placed on 35 mm Petri dishes filled with nematode growth medium and spotted with *E. coli*. These populations were sealed in 750 ml glass jars and flushed with either 100 % oxygen for the experimental group or room air for the control group. At weekly intervals, a small number (approximately 25 worms per population) of worms from each population were transferred to a new dishes.

Data analysis

Differences between groups were compared using analysis of variance (ANOVA). *Post-hoc* testing was performed using Fisher's least-significant-difference test.

Results

Metabolic rate was unaffected by oxygen tensions ranging from 3.6 to 90 kPa. Anoxic conditions arrested the movement and development of *C. elegans*, but worms in all stages of development were able to withstand 24 h of anoxia without any mortality. However, 144 h of anoxia was nearly 100 % lethal to *C. elegans* at all stages of development except for second-stage larvae.

Under hypoxia, *C. elegans* maintained a constant metabolic rate until oxygen tensions dropped to approximately 3.6 kPa (Fig. 1A). The metabolic rate of the worms then declined, but was still 50 % of normal when oxygen tensions were reduced to 1 kPa. Under anoxic conditions, the CO_2 output of *C. elegans* was reduced to approximately 5 % of that under normoxic conditions (Fig. 1B). Metabolic rate was significantly reduced at lower oxygen tensions. The first significant reduction (P<0.05) in metabolic rate occurred at approximately 3.6 kPa (F=5.6, P<0.001, $d.f._{14,57}$). The metabolic response of groups of worms placed on agarose containing sodium sulfite showed the same pattern. The straight lines in Fig. 1B represent the theoretical maximum metabolic rates for a given oxygen tension based on diffusional limitations calculated from the Hill equation. As oxygen tensions were reduced, the metabolic response of *C. elegans* was in close agreement with that predicted by the Hill equation.

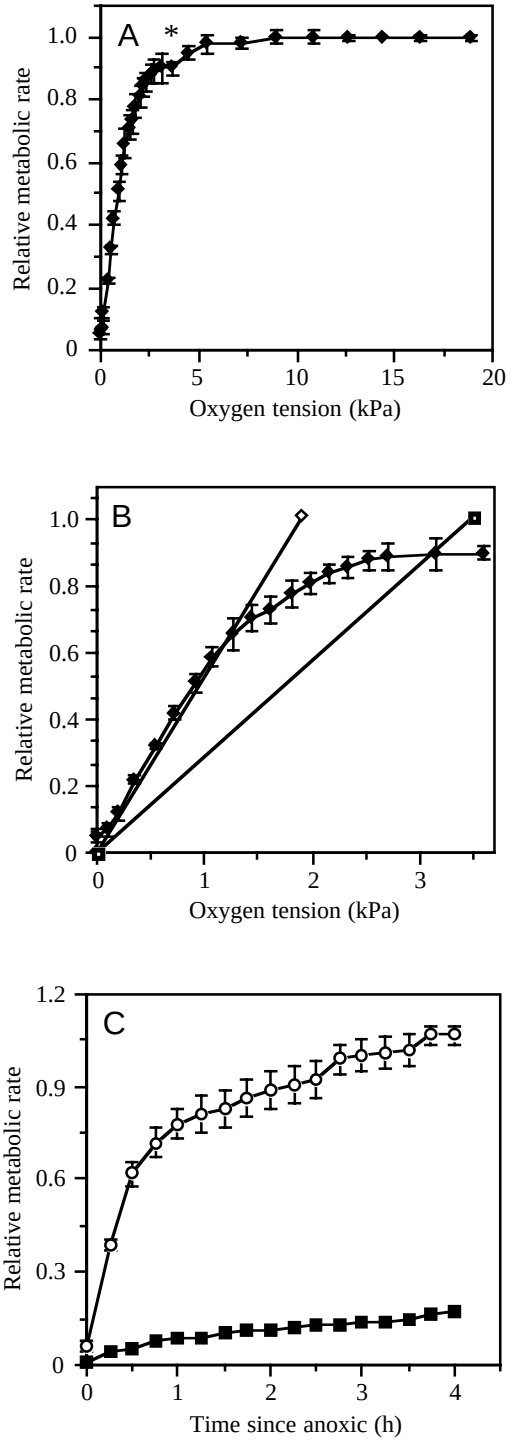

Fig. 1. Effects of reduced oxygen levels on the metabolic rate of *Caenorhabditis elegans*. (A) Relative metabolic rates at oxygen tensions ranging from normoxic to 0 kPa. The asterisk indicates when metabolic rates are first significantly reduced. (B) The lower range of the same data plotted at higher resolution. The two lines plot the theoretical effect of reduced oxygen tensions on *C. elegans* metabolic rate based on metabolic rate being dependent solely on oxygen diffusion rates. The left-hand line plots the predicted maximum metabolic rate for a worm with a body radius of 30 µm using the diffusion constant for muscle, while the right-hand line plots the predicted metabolic rate for a worm with a radius of 36 µm using the diffusion constant for connective tissue. (C) Recovery of worm metabolic rate after exposure to anoxia. The lower line (filled symbols) plots the metabolic rate of a group of worms exposed to 24 h of anoxia, while the upper line (open symbols) plots the metabolic recovery of worms exposed to between 1 and 20 min of anoxia. Data are plotted for metabolic rates at 23 different oxygen tensions measured in seven independent experiments. Results are presented as means ± S.E.M.

The left-hand line plots the predicted maximum metabolic rate for worms with a body radius of 30 μm using the diffusion constant for muscle, while the right-hand line plots the predicted metabolic rate for worms with a body radius of 36 μm and using the diffusion constant for connective tissue. This range of body radii encompassed the size range measured in 19 adult *C. elegans*.

Although *C. elegans* greatly reduced their metabolic rate and became immobilized under anoxic conditions, worms recovered quickly from anoxia when placed in normoxic conditions (Fig. 1C). Worms resumed moving within a few minutes after the resumption of normoxic air flow, and their metabolic rate returned to within 50 % of the control value within 30 min of normoxic conditions and was nearly normal after 3 h. *C. elegans* exposed to long periods of anoxia were much slower to resume normal metabolic function. A group of worms recovering from a 24 h exposure to anoxia had less than 20 % of their normal metabolic rate 4 h after a return to normoxic conditions (Fig. 1C).

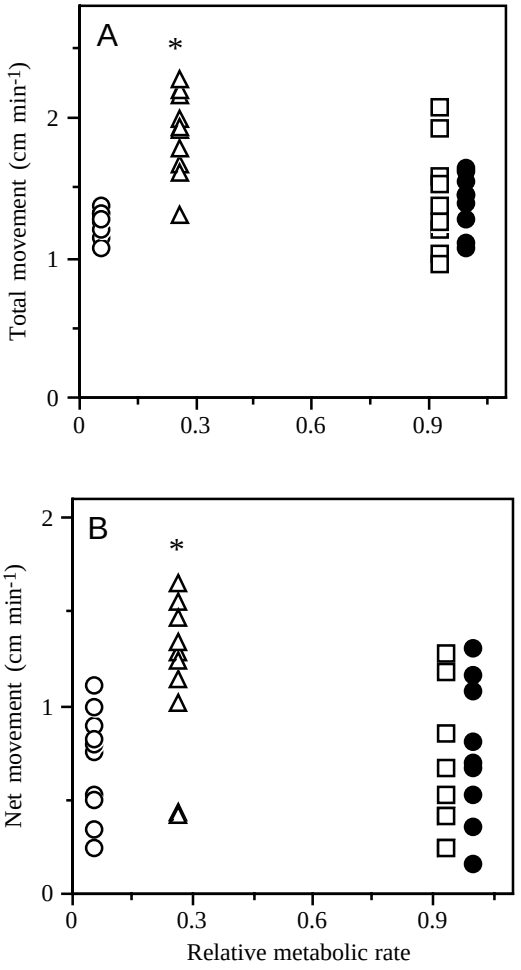

Fig. 2. The effect of reduced metabolic rate on the movement of *Caenorhabditis elegans*. (A) Total movement of worms at four different metabolic rates; (B) net movement of the same worms. Data are plotted from four groups of worms. The different metabolic rates of the worms was induced by exposing them to hypoxic conditions, which caused a reduction in metabolic rate. The total movement and net movement patterns of worms with a relative metabolic rate of 0.3 are significantly (*) greater than those worms under normoxic conditions with normal metabolic rates.

The hypoxia-induced reduction in the metabolic rate of *C. elegans* was not correlated with a reduction in worm movement (Fig. 2). The movement patterns of *C. elegans* whose metabolic rate was reduced by more than 90 % did not differ from that of worms in normoxic conditions with normal metabolic rates. The only effect of hypoxia on worm movement was a slight increase in the amount of movement at low oxygen levels (F=10.1, P<0.001, d.f.$_{3,34}$ for gross movement, F=3.7, P<0.05, d.f.$_{3,34}$ for net movement). This was consistent with the general movement pattern observed for worms in hypoxia. The worms moved continuously in small circles constantly feeding on the *E. coli* until oxygen levels dropped to approximately 2 kPa. At this point, there was a noticeable change in the movement pattern: the worms ceased feeding and moved off the *E. coli* and onto the edge of the agarose spot.

Although *C. elegans* can survive anoxic conditions, when placed in anoxic conditions, they ceased all movement or further development. They remained arrested in this state until returned to normoxic conditions. Control groups of worms placed in jars with a normoxic air flow developed normally without any mortality. Exposure to anoxia for more than 24 h significantly increased the mortality of *C. elegans* at several stages of development (Fig. 3A). Anoxia-tolerance varied significantly between the different developmental stages. Eggs were the most sensitive to long periods of anoxia, but the pattern in other developmental stages was more complex. Exposure to up to 24 h of anoxia did not affect progeny production. With the exception of the fourth stage of larval development, worms exposed to 24 h of anoxia were as fertile as worms that developed for their entire life in normoxic conditions (Fig. 3B). Eggs exposed to 24 h of anoxia took disproportionately longer to develop to adults and had a reduced rate of egg laying (Fig. 3C,D).

The metabolic rate of *C. elegans* appears to be insensitive to elevated oxygen tensions: it was not affected by increasing oxygen levels from normoxic to 100 % oxygen. The metabolic rate of a group of worms maintained in 100 % oxygen for 24 h was not significantly different from that of a control group kept in normoxic conditions (Fig. 4A,B). All seven of the *C. elegans* groups survived and appeared to be healthy after 50 generations (approximately 6 months) of continuous exposure to 100 % (90 kPa) oxygen.

Discussion

Caenorhabditis elegans requires oxygen to move, develop and survive. When placed in a reduced-oxygen environment, *C. elegans* maintains a constant metabolic rate until a lower critical oxygen tension (approximately 3.6 kPa) is reached and then reduces its metabolic rate. While this is a relatively low critical oxygen tension, the small body size of *C. elegans*

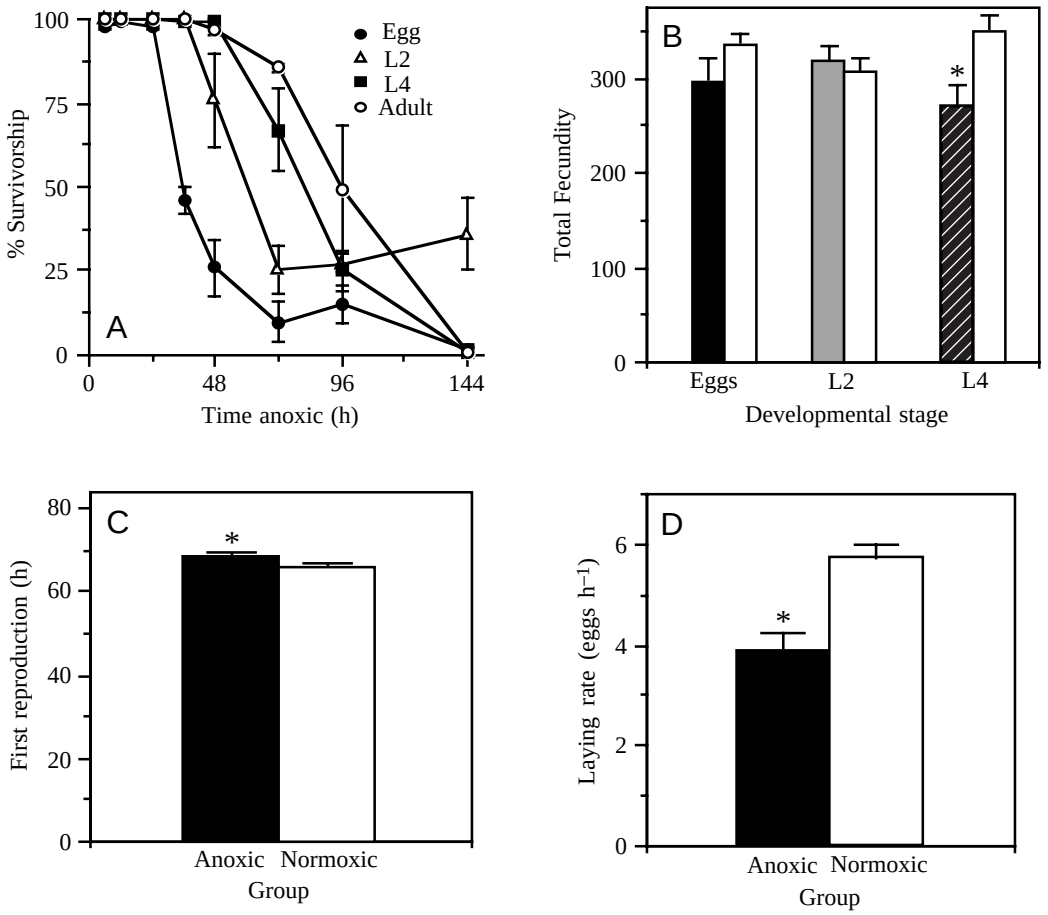

Fig. 3. The effects of anoxia on the survivorship and other life history traits of *Caenorhabditis elegans*. (A) Survivorship among *C. elegans* of different developmental stages (eggs, second-stage larvae, L2, fourth-stage larvae, L4, and young adults) exposed to anoxia for periods ranging from 6 to 144 h; *N*=3. (B) Lifetime fecundity of *C. elegans* of different developmental stages exposed to 24 h of anoxia (shaded columns) and of control worms (open columns). Fertility assays were measured on 10 worms per group. (C) Development time to adulthood (first reproduction) of eggs exposed to anoxia for 24 h. (D) Initial egg-laying rate of these same worms. Results are plotted as means ± S.E.M. An asterisk indicates a significant difference (P<0.05) from the respective normoxic value.

allows it to supply its tissues adequately with oxygen at low oxygen tensions without the apparent need for specialized respiratory or metabolic adaptations.

Under hypoxic conditions, *C. elegans* maintains metabolic rates near or slightly higher than the levels predicted if metabolism were limited by oxygen diffusion, as calculated from an equation developed by Hill (1929). The difference between the predicted and actual values depends on the body radius and the value of the diffusion constant used to calculate the equation. That *C. elegans* may have a slightly higher metabolic rate than predicted is not surprising. Hill's equation calculates the rate of gas diffusion into a cylinder without accounting for adjustments that an organism might make to increase its oxygen supply under hypoxic conditions. Accordingly, it is an over-simplification to use this equation to predict categorically the maximum metabolic rate that could be supported at a given oxygen level. This equation is most useful as a theoretical predictor of the metabolic level an animal could maintain solely on the basis of the simple diffusion of oxygen. Other studies have shown that the Hill equation generally provides a reasonable prediction of critical oxygen tensions in nematodes, although some nematodes have higher critical oxygen tensions than predicted (Atkinson, 1973, 1980; Rogers, 1962). However, the cases in which the measured critical oxygen tension is higher than predicted may be due to factors other than reductions in ambient oxygen levels reducing metabolic rate.

There are several explanations for the potentially higher than predicted metabolic rate of *C. elegans* in hypoxia. One possibility is that the agarose spot used in the metabolic chambers supplied oxygen to the worms at low oxygen tensions. The rapid flush rate of the metabolic chambers and the small volume of the agarose spot make this explanation unlikely. The metabolic chamber volume was flushed approximately four times per minute. This flush rate, combined with the small volume of the agarose spot (>0.1 ml), should allow gas levels in the agarose to equilibrate to within 1 % of the airstream values in approximately 30 s (Nicholas and

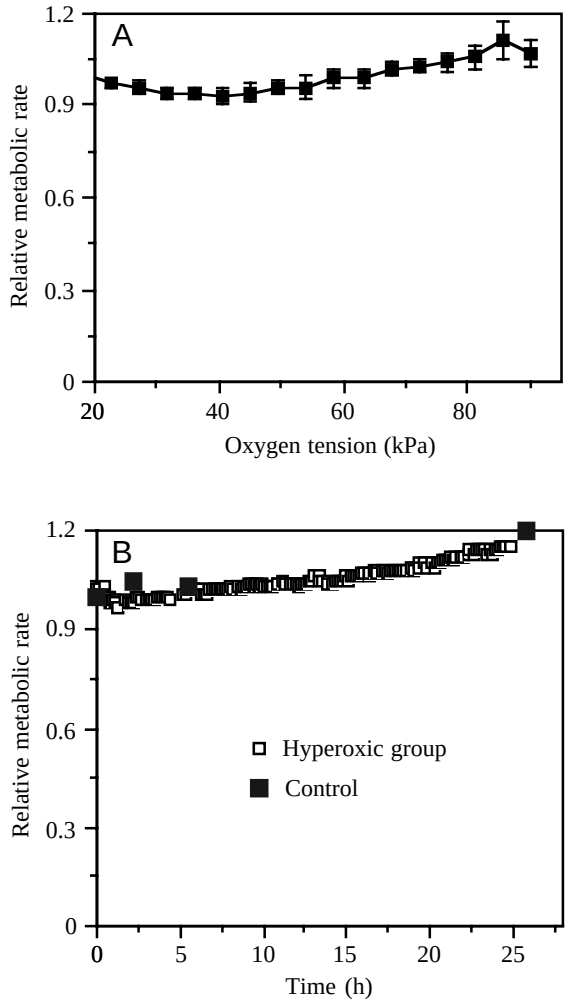

Fig. 4. The response of *Caenorhabditis elegans* to hyperoxia. (A) Relative metabolic rates from normoxia to 100 % oxygen; N=4. (B) Relative metabolic rates of a group of *C. elegans* exposed to 100 % oxygen (91 kPa) for 24 h; N=1. Results are plotted as means ± S.E.M.

Jantunen, 1966). Further evidence that the agarose was not a significant source of oxygen is that the metabolic response pattern of *C. elegans* to hypoxia was the same for worms placed on oxygen-free agarose and for worms placed on normal agarose.

It is more likely that *C. elegans* use a host of simple mechanisms to maintain a relatively high metabolic rate in hypoxia, such as locating metabolically active tissue closer to its surface, moving air through the digestive tract or using locomotory movements to increase mixing rates in the pseudocoelomic fluid (Lee and Atkinson, 1976). In addition, the relatively high gas permeability of lipids combined with the high lipid content of *C. elegans* would facilitate the rapid diffusion of oxygen throughout the worm (Childress and Seibel, 1998; Sidell, 1998).

Another potential method of increasing oxygen levels under hypoxic conditions is the use of oxygen-transport pigments. Such pigments have been found in many nematodes (Paget et al., 1987a); however, it has been proposed that, in some nematodes at least, these pigments may have evolved to prevent oxygen from reaching the cells rather than as a mechanism to supply oxygen to the cells (Minning et al., 1999). While *C. elegans* does have a gene coding for a hemoglobin-like protein (Moens et al., 1996), it is not clear whether the function of this protein is to transport oxygen. Hemoproteins can serve other biological functions such as sensing oxygen or in detoxification (Bunn et al., 1998; Hand, 1998; Hand and Hardewig, 1996; Lebioda et al., 1999); functions such as this, rather than oxygen transport, may be the role of this protein in *C. elegans*.

A previous determination of the critical oxygen tension for *C. elegans* reported a much higher value than those in this study. Bair (1955) determined a critical oxygen tension of over 17 kPa for a nematode species tentatively identified as *C. elegans* (the research facility was destroyed by fire before the nematode used in that study could be positively identified). This high value may be an artifact of the measurement method used. In Bair's study (1955), the metabolic rate of *C. elegans* was measured with the worms placed in liquid. Because oxygen diffuses much more slowly through liquid (Denny, 1993) than air, the actual oxygen levels of the microenvironment surrounding the worms may have been much lower than the oxygen tension recorded by the oxygen electrode. A later study by Anderson and Dusenbery (1977) measured the metabolic response of *C. elegans* to hypoxia in a well-stirred chamber and reported a critical oxygen tension of approximately 4 kPa, similar to values determined in the present study.

De Cuyper and Vanfleteren (1982) used a variant of the Hill equation to calculate the predicted critical oxygen tension for an individual *C. elegans* of a given metabolic rate. On the basis of their calculations, a *C. elegans* with a metabolic rate similar to those that we and others have measured (Ferris et al., 1995; Van Voorhies and Ward, 1999) would have a critical oxygen tension near 20 kPa, which is much higher than the values observed in the present study. This discrepancy appears to be due to an error in the diffusion constant used in the calculations of De Cuyper and Vanfleteren (1982), which is an order of magnitude lower than that listed by Atkinson (1980). The equation of De Cuyper and Vanfleteren (1982) predicts a critical oxygen tension of approximately 2 kPa when the correct diffusion constant is used (Atkinson, 1980), similar to that observed in the present study.

There appears to be little relative metabolic cost of movement in *C. elegans*. Neither movement rates nor movement patterns in worms whose metabolic rate was reduced by over 90 % differed significantly from those of worms with normal metabolic rates. This result is consistent with other studies on the metabolic costs of movement in both *C. elegans* and other nematodes. A relatively small proportion of oxygen consumption in the nematode *Nippostrongylus brasiliensis* appears to be involved in motility (Roberts and Fairbairn, 1965). Previous studies have also found that there were essentially no difference between the metabolic rates of

moving and stationary *C. elegans* (Dusenbery et al., 1978). It also appears unlikely that *C. elegans* uses anaerobic energy production to supply the energetic requirements of movement. Föll et al. (1999) found no evidence for the presence of any anaerobic metabolites until oxygen tensions were reduced to 0.4 kPa. Although this oxygen tension reduces the metabolic rate of *C. elegans* by approximately 80 % relative to worms in normoxia, their rate of movement is unchanged relative to normoxic worms.

The movement pattern of *C. elegans* under hypoxic conditions is consistent with an adaptive behavioral response of the worms to hypoxia. In their natural soil environment, *C. elegans* commonly encounter hypoxic conditions. Oxygen levels would be especially reduced near sources of biological activity such as local concentrations of bacteria. While *C. elegans* requires bacteria for food, the worms also require oxygen to function. In hypoxic conditions, *C. elegans* may have to leave a potential food source to meet its oxygen demands. Such a response was seen in this study: *C. elegans* remained feeding on a food source until oxygen levels reached a lower critical level and then ceased feeding and attempted to move to a new location.

C. elegans recover quickly from short-term (<1 h) anoxia. *Drosophila melanogaster* embryos show a similar pattern when exposed to short-term anoxia. These embryos arrested development within 5–10 min of the onset of anoxia but resumed normal development within 10 min after the return of normoxic conditions (Foe and Alberts, 1985). Periods of anoxia shorter than 24 h generally had relatively minor effects on the subsequent fertility or rate of development of *C. elegans*, indicating that *C. elegans* can quickly recover normal function even after greatly reducing its metabolic rate.

Periods of anoxia longer than 24 h did cause significant mortality. The ability of animals to tolerate anoxia varies widely. While some animals can survive months to years of anoxia, anoxic conditions can be lethal within a few minutes to some animals (e.g. most mammals). The tolerance of nematodes to anoxia also varies widely. At one extreme, the parasitic nematode *Ascaris suum* was once thought to be able to complete its life cycle in the absence of oxygen. Oxygen is now known to be required by *Ascaris suum* for both embryonic development and collagen synthesis (Fairbairn, 1969), and earlier reports of *Ascaris suum* completing its life cycle in anoxia were probably flawed by the presence of small amounts of oxygen in the sample chambers (Fenchel and Finlay, 1995; Laser, 1944). The larval stages of the nematode *Apheleninus avenaeve* can survive up to 60 days of anoxia with recovery greater than 85 % (Cooper and Van Gundy, 1971b). At the other extreme, mortality in adult *Nippostrongylus brasiliensis*, a parasitic gut-dwelling nematode, occurs after 2–3 h of anoxia (Paget et al., 1987a; Roberts and Fairbairn, 1965). The mortality values reported here for *C. elegans* are similar to those reported in previous studies of nematodes in the genus *Caenorhabditis*. Föll et al. (1999) reported that the mortality rates of *C. elegans* increased significantly after a 24 h exposure to anoxia. Anderson (1978) found that adult *C. elegans* can survive 2 days of anoxia, and Nicholas and Jantunen (1964) reported that *C. briggsae* can survive up to 24 h of anoxia.

The inability of *C. elegans* to withstand long periods of anoxia may be due to its high energetic requirements and the fact that toxic metabolic end-products can accumulate during anaerobic metabolism (Cooper and Van Gundy, 1971a). In addition, as a free-living nematode, the method of energy storage used by *C. elegans* is not conducive to surviving long-term anoxia. Nematodes are generally grouped into two classes, parasitic and free-living. Many parasitic nematodes, especially those living in animal intestines, must tolerate low-oxygen environments caused by gut micro-organisms that consume oxygen (Lee and Atkinson, 1976; Minning et al., 1999). The potentially limited availability of oxygen to parasitic nematodes is reflected in their method of energy storage. Parasitic nematodes often store energy as glycogen, whereas free-living nematodes tend to store energy as lipids. Although inefficient in terms of the storage mass required, glycogen can be metabolized under anaerobic conditions, while the metabolism of lipids requires oxygen (Bolla, 1980; Cooper and Van Gundy, 1971a). The biochemical composition of *C. elegans* is consistent with its classification as a free-living nematode: 35 % of its dry body mass is lipid, and only approximately 3.3 % of its dry mass is glycogen (Atkinson, 1980; Cooper and Van Gundy, 1971a; Lee and Atkinson, 1976). In contrast, the lipid content of parasitic nematodes can be less than 1 % of dry body mass, while glycogen levels can be as high as 20 % of dry body mass (Bolla, 1980; Cooper and Van Gundy, 1971a). The relatively low levels of glycogen in *C. elegans* may limit the amount of energy available for surviving long periods of anoxia (Föll et al., 1999), as was the case in an unidentified species of *Caenorhabditis* that used up available glycogen reserves after less than 72 h of anoxia (Bolla, 1980).

Of the different developmental stages placed in anoxia, the eggs were the most affected by a lack of oxygen. This is consistent with results from *Drosophila melanogaster* in which flies in early embryonic development were the most sensitive to anoxia (Foe and Alberts, 1985). *Drosophila melanogaster* embryos exposed to anoxia at the fifteenth stage of development survived 36 h of anoxia, while only 50 % of stage 9–13 embryos developed normally after 5 h of anoxia. This difference in survivorship may be due to the inability of early embryos to produce heat shock proteins that protect their cells from anoxia (Velazquez and Lindquist, 1984); heat shock proteins are only inducible after the thirteenth cell cycle in *Drosophila melanogaster* embryonic development.

The survivorship pattern seen in the other developmental stages of *C. elegans* is more complicated. The higher anoxia-tolerance of the second-stage larval worms may be related to a well-known stress response of *C. elegans* and other nematodes (Wharton, 1986). During development under stressful conditions, *C. elegans* can enter a stress-resistant, morphologically distinct, stage known as the dauer larva (Wood, 1988), which exhibits increased resistance to anoxia relative to other developmental stages (Anderson, 1978). *C.*

elegans can only develop into this dauer form during the second stage of larval development. Differential survivorship of different developmental stages has also been observed in other nematodes. In the marine nematode *Theristus anoxybioticus*, 100 % of adults died within a 12 h exposure to anoxia, while the juvenile stage survived more than 15 days of anoxia (Jensen, 1995).

It is difficult to explain why long-term exposure to hyperoxia did not reduce the metabolic rate of *C. elegans* or how *C. elegans* is able to survive for many generations in 100 % oxygen. In what has been termed the oxygen paradox (Davies, 1995), all metazoans require oxygen, yet oxygen, particularly at high concentrations, has many toxic effects. Oxygen concentrations greater than 40 % are lethal to cultured cells (Joenje, 1989), and mice die after 3–4 days of exposure to 100 % oxygen (Barazzone et al., 1998). In mammals, high oxygen levels cause central nervous system dysfunction, including induction of potentially fatal convulsions (Hampson et al., 1996; Lawrence, 1996; Wang et al., 1998). In humans, the lungs, eyes and central nervous system are quickly affected by hyperoxia, and changes in human lung function are seen within 6 h of continuous exposure to pure oxygen (Stogner and Payne, 1992). The toxic effects of oxygen are not limited to vertebrates. Continuous hyperoxia is 100 % lethal, usually in less than 24 h, to the lepidopterans *Heliothis zea* and *Trichoplusia ni* (Brown and Hines, 1976).

Hyperoxia toxicity is thought to be caused by an increased production of free radicals and hydrogen peroxide generation associated with higher oxygen levels (Joenje, 1989; Paget et al., 1987b). Among other effects, hyperoxia disrupts metabolic pathways by the rapid oxidation of the pyridine nucleotides (Jamieson, 1989) and by selective inactivation of critical mitochondrial metabolic enzymes, including NADH dehydrogenase, succinate dehydrogenase and α-ketoglutarate dehydrogenase (Gille and Joenje, 1992). The rate of oxygen consumption of Chinese hamster cell cultures is reduced by 80 % in cells exposed to 100 % oxygen for 3 days (Schoonen et al., 1990b). Hyperoxia is also associated with a generalized shutdown of macromolecular synthesis, a depletion of ATP and compromised mitochondrial energy production (Gille and Joenje, 1992; Joenje, 1989). In addition, hyperoxia can cause extensive cellular damage, including cellular apoptosis and necrosis (Barazzone et al., 1998; Kazzaz et al., 1996) and damage to mitochondria (Gille and Joenje, 1992).

Because hyperoxia has detrimental effects on many important metabolic enzymes, it is surprising that high oxygen tensions did not reduce the metabolic rate of *C. elegans*. It is also unclear how *C. elegans* can survive for many generations in 100 % oxygen. Other investigators have also reported a similar ability of *C. elegans* to withstand multiple generations of hyperoxia (Goldstein and Modric, 1994). The hyperoxic tolerance of *C. elegans* also runs counter to the prediction that organisms with higher metabolic rates are more susceptible to oxygen toxicity (Barthelemy et al., 1981). On a mass-specific basis, the metabolic rate of *C. elegans* is close to maximal human metabolic rates (Van Voorhies and Ward, 1999) as predicted by an allometric relationship between metabolic rate and body mass (Atkinson, 1980).

Tissues in organisms that either carry out photosynthesis or contain photosynthetic symbionts can potentially be exposed to high oxygen tensions. It is not obvious, however, when a soil-dwelling nematode would be exposed to hyperoxic conditions either in its normal life cycle or in evolutionary history. In addition, a high tolerance to hyperoxia does not appear to be intrinsic to all nematodes (Rhode, 1971). Respiration rate in the parasitic gut-dwelling nematode *Nippostrongylus brasiliensis* declines above 60 μmol l^{-1} O_2 (6 kPa), and metabolic rates are reduced by 66 % in normoxic conditions (Paget et al., 1987b). In a marine nematode, *Theristus anoxybioticus*, 100 % of juveniles worms died after a 30 min exposure to normoxic air (21 kPa) (Jensen, 1995), and the parasitic nematode *Ascaris suum* is killed after several hours of exposure to 100 % oxygen (Laser, 1944). In addition, a 24 h exposure to an oxygen tension of 35–50 kPa is lethal to the gut parasite *Heterakis gallinae* (Roberts and Fairbairn, 1965).

There are no obvious attributes of the respiration of *C. elegans* that should allow it to withstand high oxygen tensions. Aerobic metabolism in *C. elegans* is very similar to mammalian respiratory metabolism (Blum and Fridovich, 1983; Murfitt et al., 1976; O'Riordan and Burnell, 1990), and there are no apparent differences in its respiration pathway that would allow it to withstand hyperoxia. One potential method for *C. elegans* to cope with hyperoxic conditions would be the presence of elevated levels of antioxidant enzymes to help minimize oxidative damage. However, levels of antioxidant enzymes such as superoxide dismutase and catalase in *C. elegans* are comparable with those found in mammals (Larsen, 1993; Sohal et al., 1990; Stogner and Payne, 1992; Vanfleteren, 1993). It is difficult to provide a simple explanation for how *C. elegans* can tolerate long periods of hyperoxia.

C. elegans is currently one of the most studied of all organisms. Additional studies of the physiology of *C. elegans* can potentially provide further insights into basic biological function. These studies could address questions such as the bioenergetic costs of movement, the factors responsible for the differential ability among different developmental stages to withstand anoxia and how *C. elegans* is able to survive extended exposure to hyperoxia. Of particular value will be the combination of physiological studies with molecular studies to explain fully the mechanisms by which organisms survive in different environments.

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