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Lifespan and oxidative stress show a non-linear response to atmospheric oxygen in *Drosophila*

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SUMMARY

Oxygen provides the substrate for most ATP production, but also serves as a source of reactive oxygen species (ROS), which can induce cumulative macromolecular oxidative damage and cause aging. Pure oxygen atmospheres (100 kPa) are known to strongly reduce invertebrate lifespan and induce aging-related physiological changes. However, the nature of the relationship between atmospheric oxygen, oxidative stress, and lifespan across a range of oxygen levels is poorly known. Developmental responses are likely to play a strong role, as prior research has shown strong effects of rearing oxygen level on growth, size and respiratory system morphology. In this study, we examined (1) the effect of oxygen on adult longevity and (2) the effect of the oxygen concentration experienced by larvae on adult lifespan by rearing *Drosophila melanogaster* in three oxygen atmospheres throughout larval development (10, 21 and 40 kPa), then measuring the lifespan of adults in five oxygen tensions (2, 10, 21, 40, 100 kPa). We also assessed the rate of protein carbonyl production for flies kept at 2, 10, 21, 40 and 100 kPa as adults (all larvae reared in normoxia). The rearing of juveniles in varying oxygen treatments affected lifespan in a complex manner, and the effect of different oxygen tensions on adult lifespan was non-linear, with reduced longevity and heightened oxidative stress at extreme high and low atmospheric oxygen levels. Moderate hypoxia (10 kPa) extended maximum, but not mean lifespan.

Key words: metabolism, oxygen, aging, lifespan, protein carbonylation, *Drosophila melanogaster*.

INTRODUCTION

Organisms and tissues can be exposed to varied atmospheric oxygen levels across space and evolutionary time. Hypoxia (atmospheric P_{O_2}<21 kPa) can occur at high altitudes, in soils, burrows and eutrophic waters, and tissues can experience P_{O_2} below normal when gas exchange fails to match tissue needs (e.g. hypoventilation, ischemia). Relative hyperoxic conditions (atmospheric P_{O_2}>21 kPa) can occur in environments where oxygen has been enriched by photosynthesis (e.g. sunlit algal pools), and tissues can experience higher than normal P_{O_2} when gas exchange exceeds tissue needs (e.g. hyperventilation, reperfusion injury). The atmospheric oxygen levels of Earth have varied quite dramatically over evolutionary time, from near zero to over 30 kPa, with hypothesized effects on many aspects of organic evolution (Berner et al., 2007). Aerobic life forms use oxygen for cellular ATP production. However, a consequence of this use is the formation of mitochondrial reactive oxygen species (ROS) (Turrens, 2003), which can damage biomolecules and influence the rate of senescence of organisms (Harman, 1956). Nonetheless, the complex dynamics between oxygen concentration, oxidative damage and senescence remain largely unclear.

Data to date suggest that both extreme hyperoxia and extreme hypoxia can induce increased oxidative stress. Rearing in pure oxygen (100% O_2) has been shown to increase the rate of ROS production (Beckman and Ames, 1998; Chance et al., 1979), levels of carbonylated (oxidized) proteins in houseflies (Sohal et al., 1993; Sohal and Dubey, 1994) and mitochondrial damage in *Drosophila* (Walker and Benzer, 2004). Moreover, pure oxygen is linked to the oxidative injury of mitochondrial enzymes such as cytochrome *c* oxidase (complex IV) (Walker and Benzer, 2004), aconitase and adenine nucleotide translocase (Das et al., 2001; Yan and Sohal, 1998), and induces brain damage (Kloek et al., 1978; Philpott et al., 1974), apotosis (Oh et al., 2008) and lung damage (Bin-Jaliah et al., 2009; Pace et al., 2009). Similarly, extreme hypoxia, and hypoxia followed by reoxygenation (reperfusion), can promote free radical production *in vivo*, as well as many types of oxidative injury (Chandel et al., 1998; Dirmeier et al., 2002; Duranteau et al., 1998; Yamamoto et al., 2006). Reported oxidative stress in both 100% oxygen atmospheres and extreme hypoxia suggests that the relationship between atmospheric oxygen level and oxidative damage is non-linear and perhaps parabolic in shape. However, because no studies have yet examined the effect of a wide range of atmospheric oxygen levels on oxidative stress in any single organism, the nature of the relationship between atmospheric oxygen level and organism-level oxidative stress is currently unclear.

Similarly, the shape of the relationship between atmospheric oxygen level and aging is unclear. Rearing in hyperoxia (Table 1) reduces the lifespan of invertebrates such as *C. elegans* and *Drosophil*a (Baret et al., 1994; Honda et al., 1993; Sohal et al., 1993) (Table 1). In vertebrates, to our knowledge, studies examining the effects of hyperoxia on lifespan are lacking, but cell culture studies have generally supported the concept that higher oxygen tensions increase the accumulation rate of age-related defects (von Zglinicki et al., 1995). But the relevance of cell culture lifespan to whole organism lifespan is questionable, which further highlights the need for organismal-level studies. Conversely, hypoxia effects on lifespan are not well studied and seem to differ between vertebrate and invertebrate models, and within cultured cells (Table 1). Low oxygen tension lengthened the lifespan of human diploid fibroblasts (Chen et al., 1995), but reduced the lifespan of human CD4 and T cell clones (Duggan et al., 2004). Mice showed a lifespan reduction in hypoxia and detrimental physiological changes such as severe

Table 1. Summary of studies examining ambient oxygen effects on organismal lifespan

Organism	Common name	P_{O_2}	Lifespan	References
Caenorhabditis elegans	Roundworm	Hyperoxia	Reduced	Honda and Matsuo, 1992
		Hypoxia	Increased	Honda et al., 1993
Drosophila melanogaster	Fruit fly	Hyperoxia	Reduced	Philpott et al., 1974; Sohal et al., 1993; Baret et al., 1994; Mockett et al., 1999
		Hypoxia	Reduced	Vigne and Frelin, 2006
Musca domestica	House fly	Hyperoxia	Reduced	Yan et al., 2000
Mus musculus (C57BL/6 strain)	Mouse	Hypoxia	Reduced	Debonneuil et al., 2006

weight loss, trembling and polycythemia (Debonneuil et al., 2006). Conversely, low oxygen tensions have been shown to lengthen the lifespan of *C. elegans* (Honda and Honda, 2002). In *Drosophila*, the few studies regarding hypoxia and lifespan found contradictory results. Vigne and Frelin (Vigne and Frelin, 2006) showed that fruit flies reared in 5 kPa P_{O_2} had a shorter lifespan, whereas Strehler (Strehler, 1962) contended that *Drosophila* lifespan is extended in 1 kPa P_{O_2}. A collective examination of these results suggests that the effects of atmospheric oxygen on longevity, similar to the effects of oxygen on oxidative damage, can be non-linear and possibly parabolic in shape. However, this has never been empirically demonstrated in any organism.

To address these complex dynamics between oxygen, oxidative stress and lifespan, we examined the effect of varying oxygen levels (2, 10, 21, 40 and 100 kPa P_{O_2}) on longevity and oxidative damage accumulation rate of adult male *D. melanogaster*. Protein carbonylation was chosen as a marker of oxidative stress as injury to proteins may interfere with cellular homeostasis and with the antioxidant defense process itself (Dalle-Donne et al., 2003). Oxidative stress affects protein oxidation in a wide variety of species including *Drosophila* (Sohal, 2002; Stadtman and Levine, 2000), but no prior studies have examined the effect of a broad range of ambient P_{O_2} values on the production of carbonylated proteins in any species.

Organisms exhibit many physiological and developmental responses to changes in atmospheric oxygen level. Physiological responses include variation in ventilation and behavior, and changes at the molecular level. Many insects respond to variation in atmospheric oxygen level by varying the degree of spiracular opening, convective ventilation, and/or the level of fluid in the tracheoles (Harrison et al., 2006). It was recently proposed that discontinuous gas exchange in insects, a cyclical respiratory pattern characterized by three spiracular phases (closed, flutter, open), may function primarily to maintain low internal P_{O_2} levels to prevent oxidative damage (Hetz and Bradley, 2005). Although *Drosophila* do not breathe discontinuously, they can alter convective oxygen delivery (Lehmann et al., 2000). Further, hypoxic exposure during development causes the induction of Hypoxia Inducible Factor (HIF), a transcription factor that mediates a variety of responses to hypoxia including tracheal proliferation and cell growth (Centanin et al., 2008; Lavista-Llanos et al., 2002).

Such physiological and developmental responses complicate predictions about how hyperoxia and hypoxia will affect oxidative stress and aging. Rearing larval *Drosophila* in hypoxic conditions increases the diameter of tracheal tubes (oxygen delivery tubes) (Henry and Harrison, 2004) and augments the number of tracheoles (Jarecki et al., 1999), which could potentially lead to a higher tissue P_{O_2} at any given ambient P_{O_2}, heightened ROS production, and an ultimate reduction of lifespan. Conversely, larvae reared in hyperoxia have reduced tracheal diameters and fewer tracheoles (Jarecki et al., 1999; Henry and Harrison, 2004), which could possibly lower tissue P_{O_2} at any given ambient P_{O_2}, and hypothetically cause a lengthening of lifespan for flies exposed to hyperoxia as adults. Atmospheric oxygen level also alters adult body size in *D. melanogaster*; for oxygen tensions below 21 kPa P_{O_2}, body size linearly decreases in *Drosophila* (Peck and Maddrell, 2005) but above 21 kPa and up to 40 kPa P_{O_2}, body size slightly increases (Frazier et al., 2001), although this may be the result of selection during a single generation rather than a result of developmental plasticity (Klok et al., 2009). It seems possible that these body size differences could alter the rate of senescence due to accompanying changes in metabolic rate. The level of atmospheric oxygen is also likely to affect the concentrations of oxygen radical detoxification enzymes (Benedetti et al., 2004; Magalhaes et al., 2004), and could potentially affect genes and enzymes involved in repair of oxidative damage. Finally, it is possible that oxidative damage associated with higher atmospheric oxygen levels is cumulative, carrying over from juvenile to adult stages and reducing adult longevity. Although most adult tissues of holometabolous insects develop from imaginal discs, some adult tissues are directly derived from juvenile cells (Weaver and Krasnow, 2008), providing a mechanism for the transfer of oxidative damage from the juvenile to adult. To begin to address the complex question of how developmental responses to varying atmospheric oxygen levels can affect the senescence of adults at multiple atmospheric oxygen levels, we compared the longevity of *D. melanogaster* reared as juveniles (egg to eclosion) in 10, 21 and 40 kPa P_{O_2} and exposed as adults to 2, 10, 21, 40 or 100 kPa P_{O_2}. Furthermore, we measured the lifespan and rate of protein carbonyl production for adults reared in 2, 10, 21, 40 and 100 kPa P_{O_2}, when all larvae were reared in normoxia.

MATERIALS AND METHODS

Maintenance of flies and oxygen rearing

Wild-type *Drosophila melanogaster* Meigen of Oregon-R strain were purchased from Carolina Biological Supply (Burlington, NC, USA). Upon receipt and prior to beginning our experiments, flies were randomly mated and reared in standard conditions [25±1°C, 12 h:12 h L:D cycle, 21 kPa P_{O_2} (normoxia)] for three generations on *ad libitum* medium containing water, dextrose, cornmeal, yeast and Tegosept. During this period, antibiotics (tetracycline, alternated with rifampicin, 0.02% and 0.002%, respectively) were used to rid cultures of possible *Wolbachia* infection.

For the juvenile and adult oxygen rearing study, fruit flies were reared from egg to adulthood in seven 237 ml bottles (Genesee Scientific, CA, USA). The founding population of each bottle consisted of approximately 35 mating pairs (obtained randomly) on 40 ml of medium. Flies were given 3.5 days to mate and oviposit. This procedure is well-suited to account for the low fecundity of this particular strain (Kaliss and Graubard, 1936) and the resultant population densities are in the range of those that are known not to have an effect on fitness components such as longevity and body size (Graves and Mueller, 1993; Miller and Thomas, 1958; Sorensen

and Loeschcke, 2001). Newly eclosed (<12-h-old) male offspring were then transferred to 25 mm5×95 mm food-containing glass vials (Genesee Scientific, San Diego, CA, USA) for the longevity assay, which was conducted in duplicate. A fruit fly cannot mate until its cuticle has hardened, and our experience indicates that when males are collected within 12 h, the vast majority are virgins. Male and female flies have different stress susceptibilities (Sorensen and Loeschcke, 2001), so it is important to note that the results of these experiments might differ if females or mated individuals had been used.

Fly bottles were placed inside Plexiglas chambers, each of which were regulated at a test P_{O_2} by a ROXY-8 oxygen regulation system (Sable Systems, Las Vegas, NV, USA). This system monitored chamber P_{O_2} approximately every 30 min and controlled the ratio of O_2 to N_2 within 0.5% of the setpoint. Relative humidity within the chambers was not regulated, but was monitored by Hobo data loggers (Onset, Bourne, MA, USA), and averaged near 90%. For handling purposes, fruit flies were cold anesthetized at 4°C. Cold rather than CO_2 anesthesia was used to avoid non-experimental exposure of flies to intermittent hypoxia and/or anoxia, as ambient oxygen was the primary experimental variable measured.

Experiment 1

In order to quantify the effect of varying oxygen environments on the mortality dynamics of adult flies, larvae were reared at three P_{O_2} levels (10, 21 or 40 P_{O_2}) and were then maintained in five P_{O_2} levels during the adult stage (2, 10, 21, 40 or 100 P_{O_2}; Fig. 1). Newly eclosed (<1-day-old) male offspring were transferred to 25 mm×95 mm food-containing glass vials within their respective adult P_{O_2} levels. To ensure that a random population of males was used for each of the adult test P_{O_2} levels, all male flies were pooled by juvenile P_{O_2} and were assigned to test vials. Each vial contained 30 male flies. Identically prepared duplicate test vials were included to control for inter-vial variation (Fig. 1). Every 5 days, flies were transferred to new vials with fresh food and dead flies were removed.

Lifespan measurements

Flies were monitored and times of death were recorded for all individuals (N=900, approximately N=60 for each juvenile P_{O_2} and adult P_{O_2} treatment combination) approximately every 12–24 h until the last fly was observed dead. Individuals that died as a result of an accident during routine transfers (<0.6% of total) were not included in the data analysis. Individual lifespans were calculated as the midpoint between the last two temporal observations for any fly.

Experiment 2: protein carbonylation

In *Drosophila*, protein carbonyl levels tend to increase during aging (Toroser et al., 2007). To obtain an index of oxidative stress across P_{O_2} groups in adults, we measured the rate of carbonylated protein production in median-aged flies maintained in 2, 10, 21 and 100 P_{O_2}; N=5 per P_{O_2}; see Table 4). For sample collection, juveniles were reared from egg to adulthood in 21 kPa P_{O_2}. As in Experiment 1, newly emerged males were transferred to 2, 10, 21 or 100 kPa and were maintained as previously described (see Fig. 1).

With the exception of the 100 kPa P_{O_2} group, flies were sampled at their median ages obtained from Experiment 1. Flies reared in 2 and 100 kPa P_{O_2} were measured at 7 days of age, whereas those reared at 10 and 21 kPa P_{O_2} were measured at 27–28 days of age. Flies were collected and flash frozen using liquid N_2.

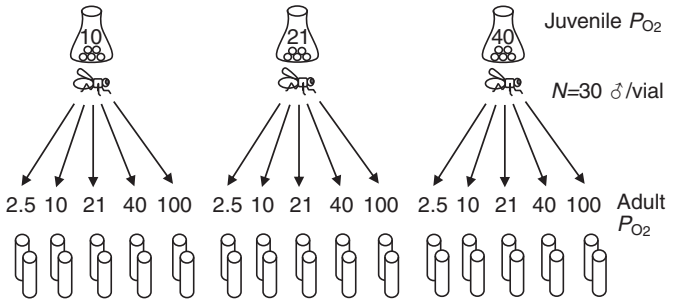

Fig. 1. Design for longevity experiment (Experiment 1). Flies were reared from egg to newly emerged adults at 10, 21 or 40 kPa P_{O_2} in replicate bottles for each juvenile P_{O_2}. Thereafter, all the available newly eclosed (<12 h) male offspring were transferred to 25 mm×95 mm food-containing glass vials and placed in 2, 10, 21, 40 or 100 kPa P_{O_2}, with two replicate vials of 30 flies each for each adult P_{O_2} and juvenile P_{O_2} treatment combination.

Protein carbonyl levels were measured using a dinitrophenylhydrazine (DNP)-based enzyme-linked immunosorbent assay (ELISA) kit (Biocell Corporation; Auckland, New Zealand). All chemicals were either included in the ELISA carbonylation kit or purchased via Sigma-Aldrich, unless otherwise specified. Each sample was composed of 15 fruit flies. Briefly, five samples (per P_{O_2} tested) were homogenized in 200 µl of cold 50 mmol l^{-1} sodium phosphate buffer (pH 7.4) containing 1% Triton X-100 and a cocktail of protease inhibitors (Roche, Indianapolis, IN, USA). Thereafter, samples were precipitated with ice-cold 28% (w/v) TCA as specified by the kit manufacturer's low protein procedure. Next, 5-µl samples were derivatized with DNP for 45 min and then proteins were adsorbed to the wells of the 96-well ELISA plate overnight in 37°C. Each well was then subsequently washed five times with wash buffer (included in kit) to remove unreacted DNP. Following the blocking of non-specific sites with the kit blocking agent for 30 min at room temperature, proteins were incubated with a primary anti-DNP-biotin antibody for 1 h at 37°C. As above, all cells were subsequently washed five times. Thereafter, the immunocomplex (DNP-conjugated proteins and anti-DNP) was incubated with streptavidin-linked horseradish peroxidase for 1 h at room temperature. After washing all plate wells five times, 200 µl of chromatin kit reagent was added to initiate the peroxidation of 3,3′,5,5′-tetramethylbenzidine (TMB). The oxidation of TMB with horseradish peroxidase produces a color change that allows for the detection and quantification of protein carbonyls. The 96-well sample ELISA plate was then read at 450 nm using an xMark microplate spectrophotometer (Bio-Rad, Hercules, CA, USA). Protein carbonyl content (nmol mg^{-1}) was determined from a standard curve based on values obtained from the oxidized albumin carbonyl controls included in the ELISA kit and the rate was calculated.

Statistical analyses

Individual lifespan data were analyzed using the survival and time failure analysis module in *STATISTICA* 6.0 (Statsoft). No significant differences were found among duplicate test vials for Experiment 1 within the same adult P_{O_2} group when the t-test was employed, so these were pooled within treatments. The multi-sample survival test (MS test) was used to compare survival in multiple oxygen groups and assess the overall significance of juvenile P_{O_2} and adult P_{O_2} on lifespan. Thereafter, a two-sample survival test, Cox's F-test, was used to test for significant differences between normoxic (21 kPa control) atmospheres and all other P_{O_2} treatment groups.

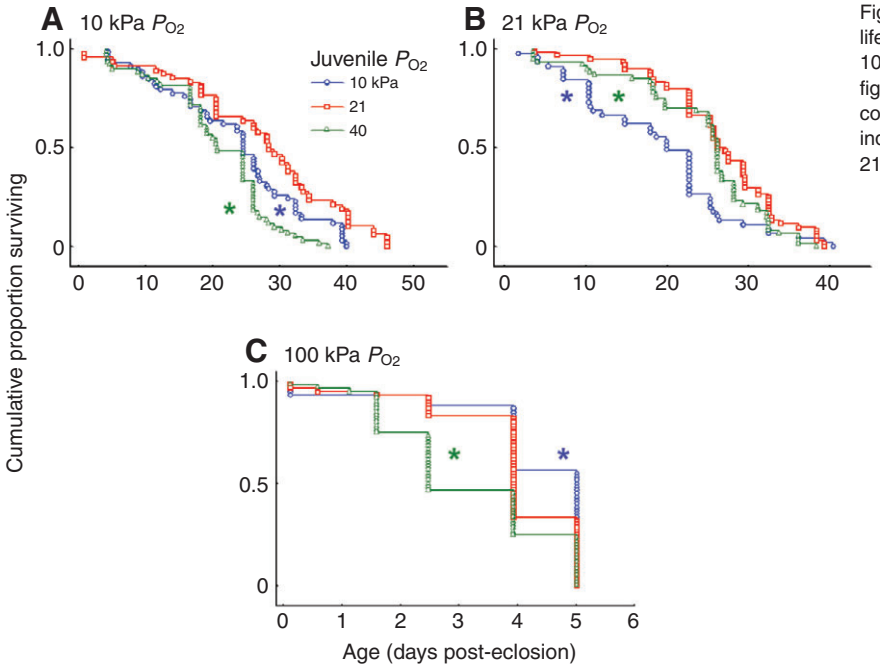

Fig. 2. Rearing juvenile P_{O_2} significantly affected adult lifespan when adult flies were kept in (A) 10, (B) 21 or (C) 100 kPa P_{O_2}. Adult maintenance P_{O_2} levels appear above figures. The different rearing P_{O_2} are shown in different colors (10 kPa, blue; 21 kPa, red; 40 kPa, green). Asterisks indicate significant differences in survival from that at 21 kPa P_{O_2} (Cox's *F*-test; Table 2).

The Kaplan–Meier estimator was used to approximate the survival function for fly populations in distinct oxygen groups (data shown as Kaplan–Meier estimator graphs). Protein carbonylation data were analyzed using the Kruskal–Wallis ANOVA test, and the Mann–Whitney *U*-test was used to test for significant differences between oxygen groups. Quantitative differences were considered significant if α-values were less than 0.05.

RESULTS

Effect of juvenile ambient oxygen on lifespan

Juvenile oxygen levels significantly affected longevity of adult flies tested at 10, 21, and 100 kPa P_{O_2} [Fig. 2A-C; multi-survival (MS) tests: 10 kPa P_{O_2} χ^2=12.28, *P*=0.002, *N*=165; 21 kPa P_{O_2} χ^2=19.04, *P*<0.001, *N*=165; 100 kPa P_{O_2} χ^2=22.27, *P*<0.001, *N*=180). For flies maintained as adults in 10 or 21 kPa P_{O_2}, lifespan was longer if they had been reared in 21 kPa P_{O_2} than if they had been reared in either 10 or 40 kPa P_{O_2} (Fig. 2; Table 2). When adult flies were tested in 100 kPa P_{O_2}, rearing in 10 kPa P_{O_2} (moderate hypoxia) extended lifespan, whereas rearing in 40 kPa P_{O_2} (moderate hyperoxia) reduced lifespan, relative to those raised in 21 kPa P_{O_2} (Fig. 2; Table 2). Rearing in 10 and 40 kPa P_{O_2} reduced adult lifespan in 10 kPa P_{O_2} relative to flies reared in 21 kPa P_{O_2} (Fig. 2; Table 2).

Table 2. Summary of two-sample *post-hoc* comparisons for Experiment 1 using Cox's *F*-test comparing survival for adults that were exposed to 10 or 40 kPa P_{O_2} as juveniles with those raised in 21 kPa P_{O_2}

		Juvenile P_{O_2}					
		10 *vs* 21			40 *vs* 21		
Adult P_{O_2}	Fig. 2	d.f.	*F*	*P*	d.f.	*F*	*P*
10	B	94, 116	1.670	0.004	94, 120	2.223	0.000
21	C	120, 90	1.749	0.003	120, 120	1.367	0.044
100	D	120, 120	1.407	0.031	120,120	1.379	0.040

P-values correspond to differences shown in Fig. 2.

Adult fruit flies in 2 kPa P_{O_2} died quickly regardless of their oxygen conditions when juveniles (MS test: χ^2=4.61, *P*=0.10, *N*=175; Fig. 3A). Similarly, adults placed in 40 kPa P_{O_2} died at approximately 15 days regardless of the P_{O_2} they experienced as juveniles (MS test: χ^2=0.92, *P*=0.63, *N*=178).

Effect of adult ambient oxygen on lifespan

The lifespan of flies reared in normoxia as juveniles was significantly affected by adult P_{O_2} (MS test: χ^2=196.03, *P*<0.001, *N*=284; Fig. 4). Lifespan was significantly reduced in 100, 40 and 2 kPa P_{O_2} and was extended in 10 kPa P_{O_2} compared to adult flies kept in 21 kPa P_{O_2} (Fig. 4; Table 3). The survival curves for flies in 10 kPa P_{O_2} and 21 kPa differed significantly (Cox's *F*-test; *P*-values in Table 3). Maximum lifespan was extended by 18% in 10 kPa P_{O_2} compared with normoxic controls, but mean lifespans were similar (Fig. 4; Table 4). In this study, mean and maximum lifespan of males (*N*=60) at 21 kPa P_{O_2} were 26 days and 39 days, respectively (Table 4). Although these lifespans were low relative to some other studies (Shen et al., 2009), they are similar to previously reported lifespan data on the Oregon-R strain (Lints et al., 1989). The oxygen level experienced by the larvae probably affected survival (Frazier et al., 2001) and density, which could affect lifespan. To partially test for this possibility, we tested the effect of population density on body size, a fitness component that is associated with density-dependent

Table 3. Summary of two-sample *post-hoc* comparisons for Experiment 1 using Cox's *F*-test for adults kept in 2, 10, 40 or 100 kPa P_{O_2} *vs* control adults kept in 21 kPa P_{O_2}

Adult P_{O_2}	d.f.	*F*	*P*
2	120, 114	5.129	0.000
10	120, 114	1.512	0.016
40	120, 114	2.398	0.000
100	120, 120	5.227	0.000

All juveniles (eggs, larvae, pupae) were reared in 21 kPa P_{O_2}.
P-values correspond to differences shown in Fig. 3.

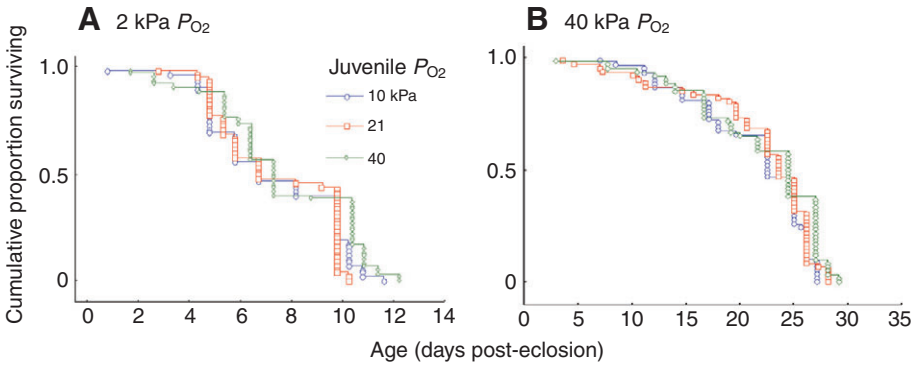

Fig. 3. Rearing P_{O2} had no significant effect on adult lifespan when adult flies were kept in (A) 2 or (B) 40 kPa P_{O2}. The different rearing P_{O2} are shown in different colors (10 kPa, blue; 21 kPa, red; 40 kPa, green).

changes in longevity (Miller and Thomas, 1958). Body sizes were unaffected by a 10× variation in the larval population densities centered around the larval densities utilized in this study (regression of male body mass on density, $F_{1,142}$=1.56, r^2=0.01, P=0.21).

Protein carbonylation in adults

Adult P_{O2} affected the rate of protein carbonyl accumulation for flies reared in 21 kPa P_{O2} [normoxia; Kruskal–Wallis test; H (3, N=20)=14.8, P=0.002; Fig. 5]. Fruit flies maintained at 2 or 100 kPa P_{O2} as adults accumulated carbonylated proteins at a higher rate than those kept at intermediate P_{O2} values (Fig. 4). Rates of protein carbonylation did not differ statistically for flies reared at 2 or 100 kPa P_{O2} (Mann–Whitney U-test; P=0.55). Individuals kept in 21 kPa P_{O2} accumulated carbonylated proteins at a lower rate than those kept in 10 kPa P_{O2} (Mann–Whitney U-test; P=0.008).

DISCUSSION

Effects of adult P_{O2} on longevity and oxidative stress

Rearing ambient oxygen levels had non-linear effects on fruit fly lifespan for larvae reared in normoxia, with the optimal P_{O2} concentration for longevity being moderately hypoxic (10 kPa, Fig. 4). Our whole-body protein carbonylation results also showed a non-linear, parabolic response to varying oxygen, with heightened rates of carbonyl accumulation at extreme levels of hypoxia (2 kPa) and hyperoxia (100 kPa) relative to adults reared in moderate hypoxia (10 kPa) and normoxia (Fig. 5). The fact that lifespan and protein carbonylation rates show similar parabola-like patterns with ambient oxygen provides support for the hypothesis that accumulated ROS-induced damage accelerates the process of aging. Here we also show that the rearing of *Drosophila* in moderate hypoxia can alter survival characteristics of adults by lengthening maximum, but not mean lifespan. Oxygen that is moderately low, but still permits ATP synthesis, may reduce ROS production in the mitochondria and enhance longevity by increasing mitochondrial efficiency and decreasing leak rates (Gnaiger et al., 2000). However, the accumulation rate of protein carbonyls was slightly higher in 10 kPa P_{O2} in comparison to carbonylation levels for normoxic controls, so factors other than a general reduction of oxidative damage must be responsible for the lifespan extension in moderate hypoxia. In larvae, 10 kPa P_{O2} can reduce feeding rates (Frazier, 2007); if a similar effect occurs in adults, longevity extension might be related to reduced caloric intake of these flies.

Negative effects of hypoxia

Moderate hypoxia may have some beneficial organismal consequences, but extreme hypoxia can have detrimental outcomes on growth and lifespan. In addition to increased rates of oxidative damage and reduced lifespan, flies exposed to 2 kPa P_{O2} probably experienced hypoxic suppression of developmental and growth processes, and the reduction of ATP synthesis. Both larvae and adults demonstrate suppression of metabolic rates with critical P_{O2} values in the range of 1–3 kPa (Klok et al., 2010; Van Voorhies, 2009). Thus, at 2 kPa P_{O2}, it is likely that ATP production is limited, with subsequent effects on processes such as protein synthesis, turnover and repair.

The elevation in oxidative damage observed in flies exposed to extreme hypoxia may be partly due to increased nitric oxide production in hypoxic flies (Wingrove and O'Farrell, 1999). Nitric oxide can contribute to mitochondrial superoxide formation (Yamamoto et al., 2006), and bind to and inhibit cytochrome oxidase, thereby causing the reduction of upstream electron carriers and favoring additional superoxide formation (Cooper and Davies, 2000).

Hypoxia survivorship heterogeneity

An interesting feature of the adult survivorship pattern occurred at approximately day 27 when a crossover point between the survival of the 21 kPa and the 10 kPa P_{O2} group was observed

Table 4. Survivorship of adults kept in 2, 10, 21, 40 or 100 kPa P_{O2} in Experiment 1

Adult P_{O2} (kPa)	N	Mean	Median	Minimum	Maximum	Variance	s.d.	s.e.m.
2	57	7.396	6.650	2.764	10.201	5.277	2.297	0.304
10	47	27.456	28.257	0.844	46.035	140.736	11.863	1.730
21	60	26.439	26.701	3.802	39.264	59.513	7.714	0.996
40	60	21.759	23.597	3.593	28.257	38.164	6.178	0.798
100	60	3.932	3.946	0.116	5.012	1.383	1.176	0.152

Values are days.
All juveniles (eggs and larvae) were reared at 21 kPa P_{O2}.

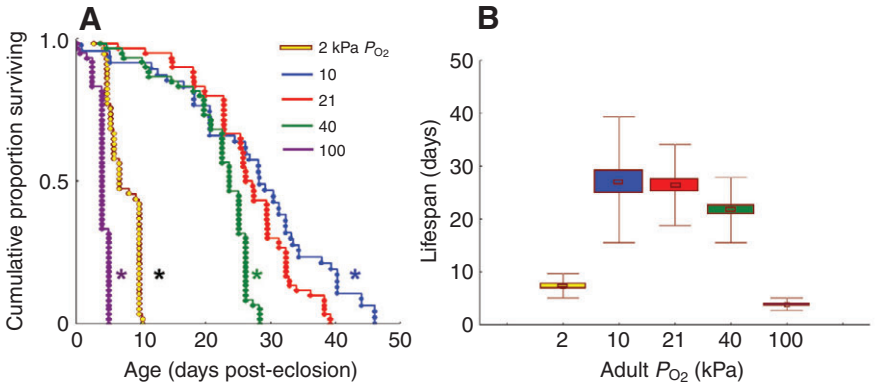

Fig. 4. (A) The effect of adult P_{O_2} on survival for flies reared in 21 kPa P_{O_2} (normoxia) as juveniles. Lifespan was extended in 10 kPa P_{O_2}, was moderately reduced in 40 kPa P_{O_2}, and severely reduced in 2 and 100 kPa P_{O_2}. Asterisks indicate significant differences in survival from 21 kPa P_{O_2} controls (Cox's *F*-test; *P*-values in Table 3). (B) Parabolic relationship between mean adult lifespan and P_{O_2} for flies reared in 21 kPa P_{O_2}. Values are means ± s.e.m. and whiskers denote standard deviation.

(Fig. 4). This crossover point may demarcate two separate subcohorts within the 10 kPa P_{O_2} population, one with higher death rates in response to hypoxia than the other. Once individuals with higher sensitivity to hypoxia perish, the demography of the population appears to become a more selected one consisting of individuals with an increased capacity to cope with hypoxia (Carey, 2003). One possible explanation for the crossover phenomenon observed may involve hormesis, a phenomenon in which low potency stressors produce beneficial biological responses in some individuals (Rattan, 2008). In this particular case, 10 kPa P_{O_2} may have acted as a moderate stressor for the adults that remained alive after the crossover point. This moderate stressor may have set a cascade of compensatory actions (e.g. increased tracheolar proliferation) into motion that could have subsequently conferred increased survival rates to this subpopulation. In support of this concept, Schulz et al. (Schulz et al., 2007) found that glucose restriction in *C. elegans* heightened mitochondrial metabolism, oxidative stress, ROS formation, antioxidant level and survival rates presumably as a result of mitochondrial hormesis. In support of this hypothesis, the 10 kPa P_{O_2} population exhibited greater variation in lifespan than normoxic controls (Table 4; *F*-test; F=2.074, ν=41, ν=59, P=0.005).

Effects of juvenile P_{O_2} on adult longevity

Juvenile P_{O_2} had significant, but complex and non-linear effects on adult longevity (Figs 2 and 3). For adults maintained at 10 or 21 kPa P_{O_2}, those that had been reared in 10 or 40 kPa, had shorter lifespans than flies reared in 21 kPa P_{O_2} (Fig. 2; Table 2). Adults maintained at 100 kPa P_{O_2} had shorter lifespans the higher the rearing P_{O_2} (Fig. 2; Table 2). Adults maintained at 2 or 40 kPa P_{O_2} showed no effect of rearing P_{O_2} on adult lifespan (Fig. 3). The effects of rearing P_{O_2} probably involves changes in tracheal structure, protein turnover and repair, endocrine regulation, body size, and metabolic rate, all of which may affect lifespan. Without the careful study of the multiple mechanisms potentially involved, it is impossible to determine the interactions for these non-linear responses. The shorter lifespan of flies reared in 10 or 40 kPa P_{O_2} and then maintained as adults in 10 or 21 kPa P_{O_2} compared with control flies that were continuously reared in normoxia, could be due to higher rates of oxidative damage since the least oxidative damage occurred in flies reared in 21 kPa P_{O_2} (Fig. 4). Alternatively or additionally, this pattern could be due to costs associated with compensatory changes initiated by rearing in a novel P_{O_2} (e.g. alterations in gene expression and protein allocation). Careful physiological measures of the relevant systems will be necessary to resolve these questions. One major question is: to what extent do physiological responses observed in larvae carry over to adults? For example, does larval P_{O_2} affect adult tracheal system structure, mitochondrial characteristics, and levels of tissue antioxidants? Secondly, the degree of plasticity of such systems to ambient P_{O_2} within adults is unknown.

It is possible that accumulated developmental oxidative damage could be transferred to adults *via* an epigenetic process that would alter gene expression, as was suggested in an early version of the free radical theory of development (Hitchler and Domann, 2007). In general, mutations are kept at a minimum during development to ensure organismal viability, but some developmental studies focused on glutathione modifications in *Drosophila* and in a slime mold, indicate that an oxidizing or reducing environment could potentially affect differentiation (Sohal and Allen, 1985; Sohal et al., 1986). Thus, although the effects of oxygen on gene expression during early development are not well known, it is possible that adult lifespan patterns could stem from oxygen and ROS influences on gene activity during development, especially in holometabolous insects like *D. melanogaster*.

Different oxygen environments (10, 21 or 40 kPa) experienced by larvae may have led to altered population densities since egg-to-adult survival is reduced in 10 kPa P_{O_2} (Frazier et al., 2001), and atmospheric oxygen level may affect egg-laying rate. Since high larval density can reduce longevity (Graves and Mueller, 1993), this could account for some of the effects of juvenile P_{O_2} on adult longevity. For example, such an effect could explain the enhanced longevity of flies reared in 10 kPa P_{O_2} compared with other treatment

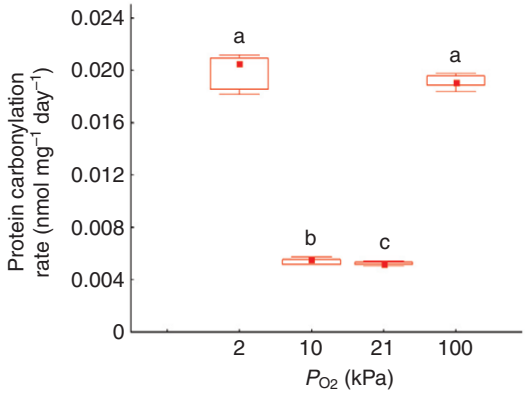

Fig. 5. Protein carbonylation rate differed in adults maintained at 2, 10, 21 or 100 kPa P_{O_2} (Kruskal–Wallis ANOVA; P=0.002; all juveniles reared in 21 kPa P_{O_2}). Data are presented in medians and quartiles. Different letters above box plots denote significant differences (*post-hoc* Mann–Whitney *U*-tests).

groups when adults were kept in a P_{O_2} of 10 or 100 kPa (Fig. 2). However, variation in larval population densities cannot explain the general non-linear pattern we observed for effects of oxygen on lifespan (Fig. 4) since this pattern was observed when the egg-laying mother and all larvae experienced identical rearing environments of 21 kPa (normoxia) prior to exposure to oxygen test levels (2, 10, 21, 40, 100 kPa). Furthermore, high larval densities are known to decrease body mass in *D. melanogaster* (Barker and Podger, 1970; Miller and Thomas, 1958). Miller and Thomas noted effects of larval density on longevity and found this to be true when densities were high enough to reduce the size of adult flies (Miller and Thomas, 1958). However, the fact that a 10× variation in larval population density (based on standard measures used here) did not affect adult body mass, suggests that the range of larval densities that occurred in this study were not stressful.

Many factors affect longevity, including potential trade-offs between investment in somatic maintenance and reproduction, mediated by hormonal pathways (Hwangbo et al., 2004; Tatar et al., 2003). At present, it is unknown how ambient P_{O_2} affects reproduction or insulin-dependent signaling, both of which are known to strongly influence lifespan. However, ambient P_{O_2} is known to affect body size, growth rates, developmental rates and cell cycle duration in *Drosophila* (reviewed by Harrison et al., 2006). It seems possible that the aforementioned developmental responses to ambient P_{O_2} could be mediated by insulin and/or TOR-dependent signaling (Corradetti and Guan, 2006), which may explain some or all of the lifespan effects shown in the present study. The demonstration of a parabolic effect of P_{O_2} on oxidative damage and lifespan in flies reared in normoxia as juveniles, as well as the strong but complex effects of juvenile oxygen levels on adult lifespan, suggests that the manipulation of atmospheric oxygen levels may provide a powerful tool for the experimental evaluation of the aging mechanisms and developmental plasticity of organisms.

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