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**Environmental and Evolutionary Consequences of Altered Atmospheric Oxygen in *Drosophila*
*Melanogaster***

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**ENVIRONMENTAL AND EVOLUTIONARY CONSEQUENCES OF ALTERED
ATMOSPHERIC OXYGEN IN *DROSOPHILA MELANOGASTER***

By

Marc Charette

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ABSTRACT

Experimental evolution was used to independently evolve 12 replicate populations of *Drosophila melanogaster* for 34+ generations in one of three treatment environments of varying PO₂: hypoxia (5.0-10.1 kPa), normoxia (21.3 kPa), and hyperoxia (40.5 kPa). Several traits related to whole animal performance and metabolism were monitored during experimental evolution and several common garden assays were performed at various stages to directly compare evolved and acclimatory differences between treatments. Results clearly demonstrate the evolution of increased anoxia tolerance in hypoxia-evolved populations, suggesting adaptation to this environment. This was correlated with an increase in citrate synthase activity compared to normoxic (control) populations, suggesting an increase in mitochondrial density in these populations. In contrast, no direct evidence of increased performance of the hyperoxia-evolved populations was detected, although an evolutionary cost was observed as a substantial decline in anoxia tolerance. Changes in performance did not result in an increase in any of the fitness components measured, including productivity and longevity, suggesting that these assays failed to capture the components of fitness relevant to adaptation.

RÉSUMÉ

Douze populations, provenant d'une souche commune de *Drosophila melanogaster*, ont été divisé également entre trois environnements expérimentaux, soit : l'hypoxie (5.0 – 10.1 kPa), la normoxie (21.3 kPa) et l'hyperoxie (40.5 kPa). Les populations ont évolué dans ces environnements pour un minimum de trente-quatre générations d'évolution expérimentale. Des mesures ont été prises sur les traits liés à la performance de l'animal entier et de la taille corporelle pendant les premières générations de sélection pour observer les changements dû à l'acclimatation et au cours de l'évolution expérimentale, des aspects liés au métabolisme et à la déshydratation en addition aux traits mentionnés ultérieurement ont été mesurés dans un environnement contrôlé pour comparer les changements dû à l'évolution dans ces populations. Les résultats démontrent que la tolérance à l'anoxie chez les populations évoluées à l'hypoxie ont augmenté comparativement aux populations contrôles (c.-à-d. normoxie). Cette augmentation était corrélée avec une augmentation de l'activité de la citrate synthétase chez ces populations. Cependant, nos résultats ne démontrent pas que les populations évoluées à l'hyperoxie possèdent une performance plus élevée suite à un stress oxydatif. Par contre, ces dernières ont évolué une réduction dans leurs performances dans l'hypoxie, suggérant un coût évolutif quelconque associé à l'évolution dans l'hyperoxie. Ces changements en performance chez ces populations expérimentales n'ont pas été accompagnés par des augmentations de leur valeur adaptative associée à leurs productivités et longévité dans leurs environnements évolutifs. Ceci semble suggérer que ces mesures de leur valeur adaptative n'ont pas réussi à capter les composantes adaptatives pertinentes à l'adaptation.

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LIST OF ABBREVIATIONS

aPO₂, atmospheric oxygen partial pressure

ANCOVA, analysis of co-variance

ATP, adenosine triphosphate

BAH, beneficial acclimation hypothesis

BMR, basal metabolic rate

C-phase, closed-phase

CS, citrate synthase

DGE, discontinuous gas exchange

ETC, electron transport chain

F-phase, flutter-phase

G_{max}, maximal conductance

Gpx, glutathione peroxidase

GR, glutathione reductase

GST, glutathione-S-transferase

HIF, hypoxia inducible factor

HSP, heat shock proteins

O-phase, open-phase

O₂, oxygen

P_{crit}, critical partial pressure oxygen

RH, relative humidity

RMR, routine metabolic rate

ROS, reactive oxygen species

SOD, superoxide dismutase

INTRODUCTION

Aerobic organisms have the capacity to utilize aerobic metabolism to produce adenosine triphosphate (ATP; Lipmann, 1941) in great excess to what is produced during anaerobic metabolism. O_2 plays a critical role in aerobic ATP generation, as it is the final acceptor in the mitochondrial electron transport chain (ETC), and its availability in the environment strongly influences all aspects of aerobic metabolism. Concentration, or the partial pressure of oxygen (PO_2), has varied throughout geological time (Dudley, 1998; Berner et al., 2003) leading to periods of increased atmospheric PO_2 (a PO_2), which has influenced the evolution O_2 extraction and transport systems in aerobic animals (Berner et al., 2007; Bradley et al., 2009). The evolution of respiratory systems has been largely driven by environmental challenges related to O_2 extraction to meet metabolic demands (Dudley, 1998). Low a PO_2 (hypoxia) and high a PO_2 (hyperoxia) conditions pose different challenges to aerobic organisms. Hypoxia can potentially reduce the generation of aerobic energy (i.e. ATP), which limits the capacity of organisms for complex behaviours beyond organismal maintenance. Consequently, limiting the aerobic energy available to organisms for metabolic consumption can potentially hamper performance by limiting energetically expensive behaviours (e.g. resource gathering and mating) essential to assure adequate fitness. Conversely, in hyperoxia an overabundance of O_2 may lead to oxidative damage of cells and tissues that can potentially impact performance and ultimately may have fitness consequences.

Insects (Class: *Insecta*) are of particular interest as many insect species have shown themselves to be hypoxia as well as hyperoxia tolerant (Chown, 2001), while possessing the highest known mass specific metabolic rates, which are associated with flight (Sacktor, 1976). In order to minimize adverse effects related to variations in a PO_2 , while still ensuring high mass specific metabolic rates, insects have shown acclimations (i.e. developmental and environmental plasticity) and adaptations that presumably increase performance and fitness in these environments (Frazier et al., 2001; Klok et al., 2009; Klok et al., 2010; Rascón & Harrison, 2010). However, direct measurement of increased

performance and fitness in populations acclimated/evolved to hypoxia and hyperoxia have never been explicitly examined in insects. This thesis will serve to examine the performance of evolved populations under altered aPO_2 and examine certain physiological and metabolic traits potentially underlying these altered performances. Additionally, performance will be examined to determine if they impact fitness traits in hypoxic and hyperoxic environments. However, before examining these topics, it is essential to review mechanistic aspects of insect respiration utilized to extract O_2 and purge CO_2 necessary for and generated during aerobic metabolism, respectively. Additionally, an overview will be presented about what is the current state of knowledge regarding the affects of hypoxia and hyperoxia and how insects respond to hypoxia and hyperoxia, after which a brief outline of hypotheses, experimental design and predictions will be given.

Insect respiratory systems

To ensure optimal aerobic metabolism, sufficient O_2 must be delivered to cells to meet aerobic demand, while allowing CO_2 to be discarded to the environment. While insects lack a circulatory system containing vascular fluid dedicated to O_2 transport (Chown & Nicolson, 2004; Sacktor, 1976), they have evolved a tracheated respiratory system to deliver O_2 to the cells/tissues and excrete CO_2 into the environment (Hedges et al., 2004). The tracheal network is composed of tracheae that are epidermally derived hollow branching airways that ramify into ever smaller tubes which end as blind-ended tracheoles. Spiracles are located at the junction where tracheae meet the exoskeleton and act as a barrier that can strongly modulate gas exchange by increasing or decreasing the spiracle opening which is controlled by two spiracle lips attached to a closer muscle (Chown & Nicolson, 2004). This respiratory system utilizes air, rather than fluid, as the conductive medium for gas exchange through the respiratory system (Chown & Nicholson, 2004). However, liquid is not absent from tracheae/tracheoles as they are lined with a variable water layer, which can be utilized to modulate gas

exchange to the surrounding tissues by increasing or decreasing its thickness (Chown & Nicholson, 2004). The principal site of gas exchange between cells and the gaseous environment inside the tracheal system occurs at the tracheoles because of their close proximity to metabolizing tissues (Chown & Nicholson, 2004). Gas transfer in insects (e.g. O₂ uptake, CO₂ release) at the tracheole/cell interface is principally mediated by diffusion, although several species use convective mechanisms (e.g. abdominal pumping) to complement diffusive gas exchange (Miller, 1966; Lehmann & Heymann 2005). Additionally, because of the diffusive nature of the tracheal system, water is lost as it diffuses from the saturated tracheae through the spiracle, which can modulate water loss via spiracle opening as alluded to previously. In insects, all diffusion of gases to and from tissues is principally governed by Fick's law:

$$\text{Diffusion Rate} \propto D \cdot P_{\text{gas}} \cdot S_{\text{gas}} \cdot A \cdot L^{-1} \cdot MW^{-1/2},$$

whereby for the diffusion rate any one gas in a medium is proportional to D , the gas specific effective diffusion coefficient, P , the differential partial pressure of the environment and tissues, S , the capacitance coefficient of the water located in the trachea, A , the cross-sectional area of the tracheae and spiracles, L , the length of the tracheae and MW , the molecular weight of the gas in question. Contemporarily, gas diffusion in insects has been studied in species exhibiting discontinuous gas exchange (DGE) while at rest (Bot, 2002; Chown & Holter, 2000; Lighton, 1996; Quinlan & Gibbs, 2006; Terblanche et al., 2008). This behaviour is thought to be adaptive and enables insects performing it to maximize and simultaneously limit exchanges of specific gases (Lighton, 1996). DGE is characterized by periodic bursts of CO₂ and short periods of O₂ uptake, which is modulated by the strict control of spiracle opening patterns (Lighton, 1996). The spiracle opening patterns of DGE serve to optimize gas exchange while at rest by maximizing the partial pressure difference of O₂ and CO₂ in addition to enabling CO₂ to dissolve in the tracheal fluid, while minimizing water loss. As this behaviour is present in many insect species, several hypotheses have been proposed to explain the

evolution of DGE. Levy and Schniederman (1966) proposed the hygric hypothesis, which postulates that the open-phase (O-phase) is minimized and closed-phase (C-phase) maximized in order to minimize water loss. Lighton (1998) presented the chthonic hypothesis which argued that the O-phase, flutter-phase (F-phase) and C-phase were present to maximize differential partial pressures of O₂ and CO₂ in hypoxic and/or hypercapnic environments to promote better gas exchange along their respective partial pressure gradients. Lastly, Hetz and Bradley (2005, Bradley, 2006) presented the oxidative damage model which suggested that insects evolved DGE to lower the PO₂ in trachea, as a mechanism to limit oxidative occurring when tracheal PO₂ approaches aPO₂, by minimizing the O-phase while making use of a F-phase to promote limited O₂ entry resulting in lower tracheal PO₂. Recent literature has provided support for each hypothesis, however it is illogical to assume that DGE serves one evolutionary purpose as it has evolved many times in the insects. Nonetheless, the consensus supports the chthonic and oxidative damage hypotheses in most insects, while support of the hygric hypothesis is diminishing (Bradley, 2006; Chown et al., 2006; Quinlan & Gibbs, 2006; Bot, 2002; White et al., 2007). In addition, Chown and Holter (2000) proposed a non-adaptive hypothesis, the emergent properties hypothesis, which states that DGE was not the result of adaptive selection *per se*, but is rather the result of two competing and interacting sensory and regulatory systems, with one responding to oxygen levels and the other to carbon dioxide (Chown & Holter, 2000). However, DGE is not practised by all insects at all times and is only found in certain insect species at rest. This behaviour is discarded when O₂ demands increase, which is typically observed in aerobically demanding behaviours such as flight (Contreras & Bradley, 2010; Contreras & Bradley, 2009; Lehmann, 2001).

DGE is presented to highlight its importance in the literature when considering gas exchange in insects, as well as to give a conceptual example whereby insect are able to modify exchange of O₂ and CO₂ as well as reduce water loss. However, insects are not able to completely compensate for changes in atmospheric composition by modifying physiological traits governing gas exchange (Frazier et al.,

2001; Henry & Harrison, 2004). Altered aPO₂, such as hypoxia and hyperoxia, alters O₂ diffusion throughout the tracheal system (Lehmann, 2001; Dudley, 1998; Lehmann & Heymann, 2005; Van Voorhies, 2009) and can potentially affect performance and consequently reduce fitness in these environments. Although insects have been shown to modify their behaviour/physiology/morphology to maximize gas exchange or limit oxidative damage in hypoxia and hyperoxia, respectively, it does not allow them to completely compensate for changes in aPO₂. Nonetheless, studies examining the effects of hypoxia and hyperoxia have provided insight into regulation of the tracheal system, as well as the physiological/morphological responses that insects exploit to cope with hypoxia and hyperoxia.

Hypoxia

Where and how does hypoxia occur?

Although atmospheric O₂ content is relatively uniform for a given altitude, microhabitats can exist that exhibit substantial variations in atmospheric O₂ content (White et al., 2007). Low oxygen (i.e. hypoxia) can occur in habitats where O₂ is consumed faster than it is replenished via diffusion, convection, or localized O₂ production, leading to O₂ deficient microhabitats (e.g. burrow or small opening to ambient air) (Schmitz & Harrison, 2004). Hypoxia is also a natural consequence of living at high altitude, where barometric pressure and PO₂ are reduced compared to normobaric/normoxic conditions present at sea level (Schmitz & Harrison, 2004).

Why is hypoxia stressful?

Hypoxia induces stress to aerobic organisms by limiting the ATP production via aerobic metabolism. Consequently, if aPO₂ falls below the critical partial pressure of O₂ (P_{crit}) needed to maintain aerobic metabolism, the organism is left with only anaerobic metabolism to generate ATP, which is likely insufficient to satisfy its metabolic requirements. Aerobic organisms might respond by limiting energy expenditure (i.e. metabolic depression) or can increase their aerobic scope to generate more ATP despite limited aPO₂.

How do insects perceive hypoxia?

Insects facing hypoxia are able to perceive low aPO_2 via a variety of mechanisms. Although specific neural structures responsible for O_2 sensing have not yet been elucidated in insects, the metathoracic ganglion has been implicated as a site of O_2 sensing at the whole animal level (Woodman et al., 2008). Such structures have been described in actively ventilating species (Bustami & Hustert, 2000; Woodman et al., 2008). It has also been suggested that O_2 sensing may occur at the spiracular level (Hoogewijs et al., 2007; Burkett & Schneiderman, 1974). The exact neurological basis of O_2 sensing remains elusive, however insects are able to detect intra-tracheal pH as a means to determine PO_2 in respiratory structures (Chown & Nicholson, 2004). CO_2 is generated by aerobically consuming O_2 and diffuses through the tracheal system according to inverse pressure gradients in insects (Chown & Nicholson, 2004). As tracheal pCO_2 increases, an increasing amount of CO_2 is buffered in tracheal water where it disassociates to form bicarbonate and a proton, thereby lowering tracheal pH (Chown & Nicholson, 2004). As pO_2 is inversely linked to tracheal pH, insects are able to indirectly monitor pO_2 via this mechanism and induce spiracle opening to alter tracheal pO_2 as necessary (Chown & Nicholson, 2004). Since tracheal PO_2 directly correlates with cellular PO_2 , aerobic organisms have evolved O_2 -dependent cell signalling to alter gene transcription and respond to hypoxia.

HIF-pathway

The hypoxia-inducible factor (HIF) pathway is implicated in detecting hypoxia at a cellular level and initiating an O_2 -dependent signalling cascade. When O_2 is abundant, HIF α subunits are hydroxylated by HIF prolyl-hydroxylases allowing their recognition and ubiquitination by VHL E3 ubiquitin ligase where it is targeted for proteasomal degradation. However, when O_2 is limited, as is the case during hypoxia, HIF prolyl-hydroxylases are unable to hydroxylate HIF α subunits. This allows them to relocate in the nucleus where they are free to bind with HIF β and form a complex that is able to recognize HIF response elements and transcription of genes allowing the organism to respond to

hypoxia-induced stress (Hoogewijs et al., 2007). This mechanism is present throughout the animal kingdom, including insects.

Insect response to hypoxia

The HIF-pathway is activated in response to acute as well as chronic hypoxic stress in insects (Azad et al., 2009). Insects faced with hypoxia activate the HIF-pathway to reduce metabolic rate and upregulate genes, like heat shock proteins (HSP), that are implicated in preparing the organism to respond and cope with hypoxia and other acute stressors (Hoogewijs et al., 2007; Schmitz & Harrison, 2004; Zhou et al., 2008; Maxwell, 2008; Frei & Edgar, 2004; Berra, 2006). The HIF-pathway is also known to induce tissue remodelling in *Drosophila melanogaster*. For example, chronic hypoxia is known to reduce body size, initiate tracheogenesis and increase terminal branch sprouting as well as tracheal diameters in larvae (Hoogewijs et al., 2007; Morin et al., 2005; Romero et al., 2008; Gorr et al., 2006; Benizri et al., 2008; Maxwell, 2008; Mortimer & Moberg, 2009; Van Voorhies, 2009; Henry & Harrison, 2004). Similarly in adults, cell size and proliferation are thought to be controlled by the HIF-pathway, as individuals raised in hypoxia have smaller body-sizes and weights. Body size is thought to be limited in these individuals to meet the O₂ demands of tissues located at the periphery of the endoskeleton by limiting diffusion distance to enhance O₂ diffusion in hypoxia (Kaiser et al., 2007). These are examples of plastic/acclimatory changes in larvae induced by hypoxia, but adults also exhibit acclimatory (i.e. reversible) responses to hypoxia. Adults are able to remodel tracheoles by migrating the tracheal cells towards tissues with insufficient O₂. Cells experiencing hypoxia induce, via the HIF-pathway, the transcription, and translation of a *branchless* ligand that is secreted into the inter-cellular space. When the *branchless* ligand is bound to the *breathless* receptor located on tracheal cells, this induces their migration towards the highest concentration of *branchless* (Arquier et al., 2006; Jarecki et al., 1999; Romero et al., 2008).

Previous research has also provided insight into the adaptive changes that insects utilize when

faced with chronic multi-generational hypoxia. First, populations reared under hypoxia for multiple generations demonstrate reductions in body-size and weight are uniquely plastic responses and do not represent evolved differences (Klok et al., 2009). Additionally, tracheal parameters governing O₂ delivery and metabolic parameters such as maximum tracheal gas conductance (G_{\max}), P_{crit} , and basal metabolic rate (BMR), which are thought to be relevant to how insects maintain performance in hypoxia, do not respond to multi-generational selection in hypoxia (Klok et al., 2010). These hypoxia-evolved populations do not appear to employ a metabolic depression as a strategy to cope with limited O₂ availability as BMR remains unchanged. Furthermore, these populations do not appear to increase aerobic scope in hypoxia as indicators of oxygen flux also remain unchanged (Hochachka et al., 1983). Thus, the metabolic strategy that hypoxia-evolved insect utilize to match metabolic demand to ATP production remains unknown.

Hyperoxia

Where and how does hyperoxia occur?

Brief periods (e.g. hours or days) of hyperoxia occur when an excess of O₂ is produced by photosynthetic organisms. While this phenomenon is common in aquatic settings, atmospheric O₂ content is constant over short time scales making hyperoxia rare occurrences in terrestrial settings. However, atmospheric PO₂ has fluctuated over very large time scales (e.g. millennia), resulting in conditions hyperoxic to those that currently exist (Berner et al., 2007; Berner et al., 2003).

Why is hyperoxia stressful?

The oxidative damage from reactive oxygen species (ROS) observed at the whole-animal level is a consequence of damage incurred at the cellular level. Elevated aPO₂ values are associated with increased tracheal PO₂, which are correlated with increased ROS production (Harrison et al., 2006). Excess ROS production leads to cellular damage (Droge, 2002) and this can translate to the whole-

animal level (Joenje, 1989; Paget et al., 1987; Hermes-Lima & Zenteno-Savín, 2002), resulting in reduced performance and presumably fitness.

Reactive oxygen species

Hyperoxia generates increased levels of ROS when compared to normoxic conditions, presumably because increased aPO₂ leads to elevated mitochondrial PO₂ values (Freeman & Crapo, 1981). However, it is important to note that aerobic metabolism generates ROS regardless of mitochondrial PO₂ as a direct result of electron leakage at several sites of the mitochondrial ETC (Sanz et al., 2010). ROS produced at the ETC include, but are not limited to, the superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radical (·OH) (Halliwell & Gutteridge, 1999; Thannickal, 2009). These compounds are highly reactive with all types of cellular molecules (e.g. DNA, RNA, proteins and lipids) (Harman, 1956; Beckman & Ames, 1998). The theory of oxidative stress and aging predicts that damage resulting from ROS is cumulative and therefore over time can result in reduced lifespans (Harman, 1956; Balaban et al., 2005; Sohal, 2002). However, cells have antioxidant defences to protect against oxidative injury caused by ROS (Greshman et al., 1954). These include low-molecular weight antioxidants (e.g. glutathione, ascorbic and uric acid, tocopherols, etc.) and proteins including antioxidant enzymes [superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione-S-transferase (GST) and glutathione reductase (GR)] (Halliwell & Gutteridge, 1999; Hermes-Lima & Zenteno-Savín, 2002). Many studies examining the effects of ROS or hyperoxia on insects have done so in the context of ageing or antioxidant defence. The prevailing methods for inducing oxidative stress in these studies have been to expose organisms to ROS generating agents (e.g. paraquat) or severe hyperoxia (i.e. 100% O₂) (Bus et al., 1984; Missirlis et al., 2001). ROS generating agents are a poor substitute for naturally occurring ROS, as unlike naturally occurring ROS, they are not generated in the same cellular compartments and do not induce the production of all varieties of ROS (Bus & Gibson, 1984). Another method used to generate oxidative damage is 100% O₂ exposure.

Severe hyperoxia does not allow an increase of primary proteins catalyzing antioxidant reactions in house flies (*Musca domestica*) before they succumb (Sohal et al., 1993). Severe hyperoxia exposure has been criticized as biologically irrelevant as it is never naturally encountered and cannot be defended against by antioxidant defences (Harrison et al., 2006). It is important to note that oxidative damage only becomes deleterious to cellular processes if it surpasses the maximum protective potential of all antioxidant defences and cellular repair mechanisms (Droge, 2002). In order to avoid such situations, insects possess the ability to perceive elevated aPO₂.

How do insects perceive hyperoxia?

Even though insects do not habitually face hyperoxia, they are still able to detect it by monitoring tracheal PO₂ as previously described (Chown & Nicholson, 2004). Respiratory structures respond to high PO₂ by reducing spiracle opening in an attempt to limit diffusion of O₂ through the tracheal system (Lighton, 2004).

Insect responses to hyperoxia

Brief exposure to hyperoxia has been shown to cause a reduction in the mean lifespan of insects when antioxidant defence systems, such as SOD, catalase and glutathione, are overwhelmed by oxidative stress (from exogenous or endogenous sources), as predicted by the theory of oxidative stress and aging (Sohal et al., 1995; Mockett et al., 1999; Das et al., 2001). To mitigate the deleterious consequences of ROS, insects have been shown to utilize the '*hyperoxic-switch*' behaviour when exposed to hyperoxia (Lighton, 2004). This first line of defence attempts to reduce O₂ uptake, and ultimately tracheal PO₂, by reducing the spiracle opening (Lighton, 2004). This behaviour was initially described in *D. melanogaster* when they were exposed to severe hyperoxia (i.e. 100% O₂): Individuals responded by constricting the spiracle opening almost immediately and this behaviour continued for the entire hyperoxic exposure (Lighton, 2004). Thus, this behaviour can be experimentally utilized to quantitatively assess water loss via flow-through respirometry (Lighton, 2004).

Reductions in spiracle opening can only mitigate the cellular damage induced by ROS, which can ultimately lead to other effects including, but not limited to, reduced longevity and climbing ability, lethal mitochondrial phenotype, and upregulated transcription and translation of antioxidant defence proteins including HSP proteins (Klok et al., 2009; Feala et al., 2007; Henry & Harrison, 2004; Sohal et al., 1995; Mockett et al., 1999; Das et al., 2001; Gargano et al., 2005; Walker & Benzer, 2004; Hermes-Lima & Zenteno-Savín, 2002). Additionally, exposure to hyperoxia in *D. melanogaster* adults has been found to alter mitochondrial physiology, preventing normal oxidative respiration and resulting in lower RMR compared to their normoxic counterparts (Van Voorhies, 2009). Hyperoxia has been also shown to affect the phenotypes of *D. melanogaster* adults. When reared in a hyperoxia (40% O₂), increased PO₂ gradients facilitating O₂ diffusion help overcome the limitations associated with increased diffusion distance in larger insects. As a result, *D. melanogaster* adults reared in hyperoxia have increased body size and weight (Frazier et al., 2001). However, this trait has been shown to be a plastic and not an evolved response to hyperoxia (Klok et al., 2009). This is not to say that the evolutionary aspects of multi-generational hyperoxia exposure have not been considered.

While it is known that insects are generally tolerant of oxidative injury (Hermes-Lima & Zenteno-Savín, 2002) and employ several mechanisms to limit oxidative damage, it is not known whether they are capable of adapting to biologically relevant increases in oxidative stress, such as hyperoxia. It has been proposed (Monaghan et al., 2008) that a reduction in lifespan caused by increased ROS would be deleterious to the lifetime reproductive success of individuals. Thus, depending of the insect mating system, ROS could have evolutionary implications on the population dynamics of insects facing repeated or chronic ROS exposure (Dowling & Simmons, 2009). Previous studies have found that O₂ delivery and utilization parameters (i.e. G_{max}, P_{crit} and BMR) do not respond in hyperoxia-evolved populations, as previously mentioned with respect to hypoxia-evolved populations (Klok et al., 2010). These parameters do not appear to be directly relevant in limiting

tracheal PO₂ and ROS damage in hyperoxia-evolved populations (Klok et al., 2010). Lastly, aPO₂ has been shown to effect longevity in a non-linear fashion (Rascón & Harrison, 2010), with severe hypoxia and hyperoxia reducing longevity and mild hypoxia extending lifespan. However, it remains unknown if evolving under hyperoxic conditions would target sites of ROS generation and antioxidant defence systems individually or synergistically to limit oxidative damage (Das et al., 2001). Such changes could impact metabolic traits and possibly desiccation tolerance (Das et al., 2001). Additionally, it would be interesting to examine if hyperoxia-evolved populations could adaptively use the *hyperoxic-switch* as a mechanisms to limit oxidative damage.

Experimental rational

These examples demonstrate that hypoxia and hyperoxia are stressful environments for insects. Most previous research presented has focused the acclimatory responses of insects faced with hypoxia and hyperoxia (i.e. oxidative stress), without much consideration given to evolutionary consequences and adaptive changes selected for in these two specific aPO₂ environments, with the exception of body size and weight (e.g. Frazier et al., 2001; Harrison et al., 2006; Harrison & Roberts, 2000; Kaiser et al., 2007; Klok & Harrison, 2009; Henry & Harrison, 2004). Furthermore, no studies have examined whether hypoxia/hyperoxia acclimated or evolved populations exhibited increased performance in their respective rearing environment. Changes in performance arising from single or multi-generational hypoxia or hyperoxia exposure are directly relevant to lifetime reproductive success and other traits determining an individual's fitness (Monaghan et al., 2008). Moreover, increased performance in hypoxia or hyperoxia would suggest that individuals are able to overcome O₂ deficits or oxidative stress, respectively, by modifying the physiological systems underlying performance in such environments. Information regarding underlying physiological modifications caused by altered aPO₂ is limited (Harrison et al., 2006; Henry & Harrison, 2004), however recent studies do not address how

insects would manage or maintain performance levels in hypoxia or hyperoxia despite having reduced O_2 and reduced aerobic scope and facing increased levels of oxidative damage, respectively.

Additionally, with many elements common between stress responses, it has not been examined if increased hypoxia or hyperoxia tolerance would alter other stresses tolerances (e.g. desiccation).

Research goals

Therefore, it is the goal of this thesis to examine if acclimatory responses mirror evolutionary responses with respect to general effect and degree of change in performance traits. This includes when these increases in tolerance occur in the initial- or over multiple-generations of selection. Performance in hypoxia is assayed as the ability to remain righted upright despite progressively lower aPO_2 and as the time taken to return to an upright stance after succumbing to severe anoxia (referred to as anoxia tolerance and recovery, respectively). Performance in hyperoxia is assayed as the ability to maintain locomotor performance after oxidative insult (referred to as post-hyperoxia climbing ability).

Additionally, fitness traits, including productivity (a large component of lifetime fitness) and longevity under various aPO_2 's will be measured (Rascón & Harrison, 2010). Although it is clear that 100% O_2 is not a biologically relevant oxidative stressor to examine biological processes or systems during acute or chronic exposures, exposure to lower levels of oxidative stress, especially if applied over multiple generations, might shed light on which physiological parameters are altered as coping mechanisms.

Thus, the physiological traits underlying evolved differences in performance in hypoxia and hyperoxia will be examined, including routine metabolic rate (RMR) and mitochondrial density (measured via citrate synthase activity), to determine if hypoxia-evolved populations employ a metabolic depression strategy or show indications of increased mitochondrial machinery, possibly suggesting increased aerobic scope, as mechanisms to cope with low aPO_2 . Additionally, the *hyperoxic-switch* will be experimentally examined via water loss rates to determine if it can be employed as an evolutionary mechanism to potentially minimize oxidative damage in hyperoxia-evolved populations. Additionally,

as many physiological traits governing gas exchange may be altered to increase/limit O₂ uptake in hypoxia and hyperoxia, respectively, water loss and thus desiccation tolerance may be altered as an evolutionary consequence of these environments and will be examined in these evolved-populations. Lastly, aPO₂ has been shown to have an acclimatory but not an evolutionary effect on weight (Klok et al., 2009), so the acclimatory and evolutionary consequences of hypoxia and hyperoxia will be examined in these evolved-populations.

Experimental design – Experimental evolution and common gardens

D. melanogaster was selected as the study species as they are known to be both hypoxia and hyperoxia tolerant (Zhao, Zhou, Nizet, & Haddad, 2010; Zhou et al., 2007) and possess short generation times. Experimental evolution (Dallinger, 1887) was used in which twelve independent populations, derived from a common ancestor, were distributed among three environments — hypoxia (5% O₂), normoxia (21% O₂) and hyperoxia (40% O₂) — and were allowed to evolved for 34+ generations. These aPO₂ values were selected because the hypoxic environment approaches *D. melanogaster*'s P_{crit} value and the hyperoxic environment ensured moderate oxidative damage without causing severe damage seen at even higher O₂ concentrations (Van Voorhies, 2009; Sohal et al., 1995; Sohal et al., 1993), while still allowing indefinite propagation of *D. melanogaster* populations as demonstrated by previous studies (Frazier et al., 2001; Klok et al., 2009). The atmospheric balance in both these environments was composed of argon (Ar) as a previous study demonstrated that nitrogen had a negative behavioural effect on the chill recovery in *D. melanogaster* (Nilson et al., 2006). Therefore, to avoid possible confounding effects of nitrogen on behavioural responses, argon was used to complete the atmospheric content for both experimental treatments.

Adaptation to the treatment environments can be demonstrated by parallel evolution within a treatment. Parallel evolution provides strong evidence that natural selection is responsible because other mechanisms of evolutionary change, such as genetic drift, are unlikely to produce a concerted

response among populations in correlation with their environment (Endler, 1986; Schluter & Nagel, 1995). Evolved responses were assayed using a common garden approach in which flies were maintained for two generations in the ancestral (normoxic) environment prior to trait measurement. Standardizing the rearing environment removes any environmental differences, including acclimatory and plastic responses, among treatments. Environmentally-induced responses are indicated by differences among treatments immediately upon encountering these environments (i.e. in the first generation). Acclimation, evolution, and their interaction (i.e. the evolution of a different acclimatory response to an environment) can be differentiated via reciprocal transplant experiments in which all populations are raised in each of the three environments.

Predictions

Using this evolutionary protocol, I predicted that hypoxia-selected populations will demonstrate decreased weight and increased performance (anoxia tolerance and recovery) in hypoxia/anoxia. As to when changes in performance will occur (initially or after multiple-generations of selection) is unknown, however hypoxia has been demonstrated as a stressful environment (Zhou et al., 2007) and it is not unreasonable to expect that changes in performance could occur rapidly during experimental evolution. If increased performance is observed in hypoxia-evolved populations, I would predict that this would be the result of increased metabolic scope, suggested by increased citrate synthase activity (indicator of mitochondrial density), not metabolic depression, represented by decreased RMR. Increased performance in hypoxia should contribute to increased fitness in that environment, as observed by the increased productivity of male/female pairs compared to other treatments. Hyperoxia-evolved populations should evolve an increased tolerance of oxidative stress, which would underlie increases in performance, fitness and longevity in hyperoxia. Increased longevity should be present whether adults are exposed to severe (prolonged oxidative stress tolerance) or moderate (reciprocal longevity assay) oxidative stress, as selection on antioxidant defences could act to reduce both acute

and chronic oxidative stress. Hyperoxia-evolved populations should also display increased weight. The *hyperoxic-switch* should be utilized as an evolved trait to reduce oxidative damage and thus, water loss in these evolved populations should be reduced.

MATERIALS AND METHODS

Stock population

A laboratory stock population of *D. melanogaster* was originally founded from approximately 200 flies collected from multiple areas within a 5 km radius of Dundas, ON, Canada in the fall of 2005, and was supplemented by an additional collection of similar size from the same area the following year. This stock was maintained in two cages under controlled conditions (25°C, 70% relative humidity (RH), 12L:12D photoperiod) with overlapping generations in the laboratory of R. Dukas at McMaster University, Canada. In February of 2007, a large sample of this stock was transferred to the University of Ottawa, Canada and was maintained in 16 half-pint bottles containing standard laboratory corn-meal based media under controlled conditions (25°C, 50% relative humidity, 12L:12D photoperiod) with non-overlapping generations. Fifty generations elapsed under these conditions before individuals were randomly selected to establish the experimental populations described below.

Derivation and maintenance of experimental populations

Each of the 12 replicate experimental populations was independently founded from the stock via the random selection of approximately 400 individuals. These populations were assigned to one of three treatment environments that varied in atmospheric composition; hypoxia (5% O₂:95% Ar), normoxia (regular air containing approximately 21% O₂), and hyperoxia (60% O₂:40% Ar). This yielded four replicate populations within each treatment designated as follows: hypoxia, populations 1-4; normoxia, populations 5-8; hyperoxia, populations 9-12. These populations were maintained separately (i.e. with no gene-flow among them) via non-overlapping generations, with individuals within each population experiencing their respective treatment conditions throughout their entire lifecycle. The normoxic environment had the same atmospheric composition as experienced by the

stock from which the experimental populations were derived and therefore act as a control for the maintenance protocol; the hypoxia and hyperoxia environments differ from the control only in their atmospheric composition such that differences between these indicate responses to the atmospheric treatments.

Each population was maintained in a group of ten vials, each containing 10 ml of standard cornmeal-based food with live yeast sprinkled on top. Every generation, adult offspring from the ten vials were mixed and then approximately 30 adults were transferred to each of ten new vials. Adults laid eggs in these vials for two days, after which they were discarded. All populations were transferred contemporaneously on a 14-day cycle.

Atmospheric compositions were manipulated by housing the populations in 19 cm × 19 cm × 13 cm acrylic-plastic chambers with removable airtight lids. One chamber was used for each of the three atmospheric treatments and all four populations within a treatment were housed together within a particular chamber (i.e. four populations of ten vials per population). Chambers had an inlet located at the lower left corner of one vertical face and an outlet located in the upper right corner of the opposite vertical face. The inlet was connected via plastic tubing to a mixing system that delivered the appropriate atmospheric composition. Oxygen/argon mixes for the hypoxic and hyperoxic treatments were achieved using flowmeters (Gilmont Instruments Inc, Barrington, USA), with the appropriate O₂:Ar mixture determined using a FOXY coated fibre optic O₂ sensor (Ocean Optics, Dunedin, USA) that measured the O₂ partial pressure (PO₂) inside a vial within a chamber. The regular air that was delivered to the normoxia treatment was supplied from the building's compressed air lines. (Mixing O₂ and Ar to achieve a comparable mix to normoxia was cost prohibitive.) To maintain atmosphere consistency and to remove excess moisture released from the food, total flow into each chamber was maintained at approximately 200 ml/min. Relative humidity within each chamber fluctuated around

50% and did not exceed 70%. All three chambers were housed within a single Caron 6030 constant-temperature incubator set at 25°C with a 12L:12D light cycle.

After three generations of experimental evolution, all flies from all four of the hyperoxia (60% O₂) populations died, presumably due to the cross-generational accumulation of the effects of this environment (e.g., maternal effects). This treatment was subsequently restarted using flies from the same stock and employing the same protocol as before, but with an atmospheric concentration of 40% O₂: 60% Ar. These four populations have therefore evolved under these treatment conditions for five generations fewer than those in the other two treatments.

PO₂ were monitored in all treatments for the first five generations and corresponded to desired values of 5.0, 21.3 and 40.5 kPa for the hypoxic, normoxic and hyperoxic treatments respectively. However, the monitoring protocol was not maintained for subsequent generations and a check after 20 generations of experimental evolution revealed that the hypoxic treatment had increased to 10.1 kPa O₂. Subsequent measurements confirmed that this was stable. When and how this value increased over these generations is not known given no change in the calibration of the flowmeters. For simplicity throughout, we refer to the hypoxic treatment as 10% O₂, although in reality the environment varied between 5-10%.

Tracking trait evolution during experimental evolution

Several traits of individuals in the experimental populations were tracked over time during course of 39 generations. Because individuals in the different treatments were raised in their respective treatment environments, differences between treatments in mean trait values can therefore occur due to both environmental effects and evolved differences. Within treatments, however, consistent changes

across generations in the replicate populations suggest adaptation (i.e. parallel evolution). In all cases, these trait assays were performed on adults emerging directly from their respective experimental environments, collected using light CO₂ anaesthesia as 1 day-old non-virgin adults, and stored in groups of ten same sex individuals in vials containing 5 ml of cornmeal media.

Statistical analyses were specific to each assay to account for their different designs and are described below. In all cases, analyses were conducted to account for the fact that populations are the independent unit of replication relevant to test for environmental and evolved changes. Individuals (or groups thereof) within populations represented subsamples of the true unit of replication.

Wet weight — Flies destined for this assay were raised in vials that controlled for egg density. This was achieved by releasing flies into a cage and allowing them to lay for 24 h on an 11 cm diameter Petri-dish filled with standard cornmeal media dyed using green food colouring (McCormick Canada, London, Canada). The food colouring aids in subsequent counting of the eggs. Petri-dishes were then removed and sections of the media containing 100-110 eggs were excised and placed separately into vials containing 10 ml standard food. From these vials, 100 females and 100 males were collected from each population and stored for 2-5 days in vials containing 5 ml of cornmeal-based food with active yeast added on top. Eight hours prior to weighing, the flies were starved by transferring them to vials containing 5 ml of cornmeal-based media but lacking active yeast. Individuals were weighed to the nearest µg using a MX5 microbalance (Mettler Toledo, Columbus, USA) after anaesthetizing with CO₂.

The analysis of wet weight involved a partly nested split-plot design (Quinn and Keough 2002; (Chenoweth et al., 2008) fit separately by generation:

$$W = \text{constant} + S_i + T_j + P(T)_{k(jl)} + ST_{ij} + SP(T)_{k(ijl)} + \text{error}, \quad \text{Eqn.1}$$

where W is the wet weight of an individual of a given sex (S) from population (P) nested within treatment (T). Sex, treatment and their interaction (ST_{ij}) were fixed effects, while population and its interaction with sex ($SP(T)_{k(ijl)}$) were random effects. Significance of the ST_{ij} interaction was evaluated using the $SP(T)_{k(ijl)}$ interaction as the denominator in calculating the F-ratio (Quinn & Keough, 2002).

Acute anoxia tolerance — This assay used 2-3 day-old females or males that were placed in groups of ten same sex individuals in a narrow polystyrene vial (25 x 95mm) with a foam plug protruding 1.5 cm into the vial. Six replicate vials were placed together within a 600 ml beaker, with two vials originating from one population from each of the three treatments (Fig. 1). This generated four blocks, each representing a unique combination of the 12 populations: block 1 – populations 1, 5, 9; block 2 – populations 2, 6, 10; block 3 – populations 3, 7, 11; block 4 – populations 4, 8, 12. Three replicate beakers were performed per block and sex (Fig. 1), all using new groups of flies. Parafilm® was used to cover the opening of the beaker and a small hole was cut through which a piece of plastic tubing was passed. The tubing was tipped with a needle inserted into a foam plug to promote gas dispersion. Argon (purity 99.9%) was pumped into the beaker through the tube at a rate of 600 ml/min. A computer program (MAMER; Craig Riedl and Ken Dawson-Scully), was used to record the incapacitation times of individuals. A timer was started when the Ar was introduced into the system and a visual inspection provided the basis for the incapacitation times. Incapacitation was indicated by a number of small convulsions and a loss of adhesion of the fly to the surface.

Block means for each sex and treatment were created by averaging all the population subsamples (i.e. replicate vials and then beakers). The statistical analysis was conducted separately by generation and employed a non-additive mixed linear model for a factorial randomized complete block design:

$$A = \text{constant} + S_i + T_j + S \times T_{k(ij)} + B_l + B \times T_{k(lj)} + S \times B_{k(il)} + S \times B \times T_{k(ijl)}, \quad \text{Eqn. 2}$$

where A is the average incapacitation or recovery time of a group of individuals of a particular sex (S) from treatment (T) within block (B). Block is a random effect and represents the blocking of the experimental units (populations) into four unique sets of one population each from the hypoxic, normoxic, and hyperoxic treatments. Sex and treatment were fixed effects and their F ratios were constructed using the mean square of their respective interaction with block as the denominator (Newman et al., 1997; Quinn & Keough, 2002), and the sex \times treatment interaction was tested over the mean square of the three-way interaction. As with all unreplicated versions of such a design, there is no test of block effect or any of the interaction terms involving it (Quinn & Keough, 2002). A two-way analysis of covariance (ANCOVA) was used to detect changes in anoxia tolerance within a treatment over time (across generations), with sexes analyzed separately. Fixed and random variables remain as stated above. The interaction population(treatment) \times generation was used as the denominator when calculating the F-ratio for the treatment \times generation interaction.

Post-hyperoxia climbing ability — Immediately following their collection as adults, eight replicate vials of ten individuals from every population and sex were placed for 24 h in an airtight container (20 cm \times 20 cm \times 35 cm) saturated with 100% O₂. Following exposure, the flies were transferred to empty plastic vials and groups of six vials were combined side-by-side within a rectangular acrylic-glass support frame. Flies exhibit negative geotaxis and therefore innately climb the walls of the vial. Their climbing ability was assayed by striking the apparatus against the desktop, knocking all the flies to the bottom. Digital pictures of the vials were then taken after 4 s, from which the number of flies having climbed at least 4.8 cm (60% of the vial) were counted. All three treatments were represented in each apparatus generating four blocks, each containing unique combinations of populations as described in the acute anoxia tolerance assay. The assay was repeated separately by sex with each block replicated three times using separate flies. The analysis for this assay employed a

partly nested design fit separately by sex to accommodate the complex nesting as follows:

$$C = \text{constant} + B_i + R(T)_{k(ij)} + T_l + P(T)_{k(lm)} + \text{error}, \quad \text{Eqn. 3}$$

where C is the post-hyperoxia climbing ability of an individual of a given sex from population (P) nested within treatment (T) in beaker R , nested in a given block B . Block and treatment were fixed effects, while population and beaker were random effects (Fig. 1), with ‘apparatus’ being analogous to ‘beaker’ in the previous analysis. The F-ratio for the treatment effect was constructed using the mean square of block \times treatment as the denominator (Quinn & Keough, 2002).

Common gardens

To isolate and characterize the evolutionary response to these treatment environments after multiple generations of experimental evolution, a variety of traits were assayed on individuals from these populations after being raised in a common normoxic environment for two generations, thereby removing any environmental differences including cross-generational maternal effects. Consistent trait differences among treatments, treating populations as replicates (i.e. parallel evolution), therefore indicate adaptation because genetic drift is unlikely to produce concerted change, correlated with environment, in multiple, independent populations (Endler 1986; Schluter & Nagel 1995). Seven separate common gardens were performed at different times beginning after generation 10 (5 for hyperoxic flies) of experimental evolution (Table 2). Individuals for use in each common garden were randomly collected as non-virgin adults from the experimental populations and were allowed to lay eggs in vials following the same protocol as for the maintenance of the experimental populations. These vials were housed under environmental conditions that matched those of the stock population (25°C, 50% relative humidity, 12L:12D photoperiod), with all portions of the life cycle occurring in

this environment. Flies for use in the assays were collected using light CO₂ anaesthesia from the second generation offspring as 1 day-old non-virgin adults and stored in groups of ten same sex individuals in vials containing 5 ml of cornmeal media. As before, in all cases the analyses were conducted to account for the fact that populations are the independent unit of replication relevant to tests for evolved treatment effects. Tukey's post-hoc tests were employed when applicable to determine the differences between experimental treatments in the presence of a significant treatment effect overall.

Wet weights — In the second generation of the common garden, flies destined for this assay were raised in density-controlled vials (100-110 eggs per vial) and individual wet weights of 100 males and 100 females from every experimental population were measured as previously described.

Stress tolerance

Acute anoxia tolerance and recovery — Incapacitation times under acute anoxic stress (i.e. 99.9% Ar) were measured and analyzed as described above. Recovery times were also recorded by leaving the flies in the anoxic environment for 15 min and then removing the Parafilm® covering the beaker and placing the vials on the counter, allowing them to equilibrate rapidly with the ambient air. Individual recovery times were recorded visually as the point at which a fly righted itself on all six legs. The statistical analysis used for both assays employed the model previously described for the anoxia tolerance assay. Analyses were conducted separately on incapacitation times and recovery times.

Post-hyperoxia climbing ability — Climbing ability after acute hyperoxia was measured and analyzed as described previously.

Tolerance of prolonged oxidative stress — This was assayed as lifespan of individuals held in

100% O₂. Individuals were collected 24 h post-emergence using light CO₂ anaesthesia and held separately by sex in groups of ten individuals in vials containing 5 ml of standard cornmeal media. All populations were assessed simultaneously twice daily by visual inspection, with each vial scored for deaths until all flies had expired. A proportional hazard test was used to determine differences among treatments and sex as described previously.

Desiccation tolerance — Survival times under acute desiccation stress were measured by confining groups of ten 1 day old, same sex flies to the upper half of a vial divided into two chambers using a 1.75cm foam plug that was placed in the middle of the vial, effectively dividing it into two compartments. The upper compartment contained the flies and the lower compartment contained 9 g of desiccant (Drierite®, W. A. Hammond DRIERITE Co. LTD., Xenia, USA). Parafilm® was placed over the open end of the vial to create an airtight seal. Flies were assessed visually every 30 min for death and observations continued until all individuals had succumbed. Ten replicates per population per sex were set-up and all were assayed simultaneously. Statistical analysis employed a proportional hazard test (Rowell & Markham, 1984) to detect differences of lifespan among treatments and between the sexes. Sex, treatment and population nested within treatment were fixed effects (proportional hazards test does not allow for random effects). Risk-ratios were generated for every comparison within every level of treatment and sex. In this case, the risk ratios use the survival curves to indicate the risk of a given level (the numerator) as compared to another level of the effect (the denominator). For example, a risk-ratio >1 indicates an increased risk of death for the numerator compared to the denominator, and a risk-ratio <1, indicates that the denominator has an increased risk of death compared to the numerator. Reciprocal comparisons between all levels of sex and treatment were made.

Fitness components

Longevity — A reciprocal transplant assay was conducted in which all 12 populations were assayed in each of the three treatment environments (hypoxia, normoxia and hyperoxia). For each population, eight replicate vials of each sex were collected as described previously and placed in acrylic-plastic cages to which was delivered one of the three environmental gas mixtures matching those used during experimental evolution. Vials were scored for death once daily until all flies had expired. A proportional hazard test was used to determine differences among treatments and sex as described previously.

Productivity — A reciprocal transplant assay was used to count the number of adult offspring produced by replicate male-female pairs when raised in each of the three treatment environments. Productivity therefore represents a combined measure of the fecundity of a particular female and the egg-to-adult survivorship of her male and female offspring. The assay was conducted in two blocks, each consisting of 50 replicate pairs from every population raised in each environment. Gases were mixed as described previously in the maintenance protocol for the evolution experiment. Day-old male-female pairs were collected from each experimental population via light CO₂ anaesthesia and were immediately placed together in a vial containing 10 ml of standard cornmeal media for two days, after which they were discarded. Mirroring the maintenance protocol of the populations, fourteen days after setting-up the male-female pairs the number of adult progeny was counted.

The statistical analysis of productivity were estimated separately for each sex and population and analyzed via a partly nested split plot design:

$$PR = constant + B_i + T_j + P(T)_{k(l)} + E_m + S_n + T \times E_{k(jm)} + P(T) \times E_{k(ljm)} + T \times S_{k(jn)} + P(T) \times S_{k(ljn)}$$

$$+ E \times S_{k(mn)} + T \times E \times S_{k(jmn)} + P(T) \times E \times S_{k(ljmn)}, \quad \text{Eqn. 4}$$

where PR is the productivity of individual male-female pair from population (P) nested within treatment (T) within environment (E) of a given sex (S) from a given block (B). Block, Treatment, environment and sex were fixed effects and population was a random effect. The F ratio for the treatment effect was constructed using the mean square of population nested within treatment as the denominator. The F ratios for the environment and environment \times treatment effects employed the mean square of environment \times population(treatment), while and sex and sex \times treatment employed the mean square of sex \times population(treatment) as the denominator. The F ratios for the three way interaction and the sex \times environment term used sex \times environment \times population(treatment) as the denominator (Quinn & Keough, 2002). In all experimental populations the distribution of fitness was non-normal because certain male-female pairs failed to produce offspring. Because this fraction was small (5.3% of pairs set-up) and the exact cause of failing to produce offspring is unknown (but may include infertile individuals, refusals to mate, and experimenter error), analysis was restricted to pairs producing offspring. Results do not change qualitatively when these individuals are included and a non-parametric analysis is performed.

Metabolism

Metabolic rate — Routine metabolic rate was measured via two temporally separate assays employing slightly different protocols. In the first, flies were assayed at days 3-6 post-emergence in groups of 20 same sex individuals placed together in a 10 ml glass respirometry chamber (Sable Systems International, Las Vegas, USA). Two replicates per population and sex were randomly sampled consecutively over five days at 25°C. The chamber was covered with an opaque plastic cover that blocked all light. All CO₂ and H₂O were removed from the incurrent ambient air with Ascarite®

and Drierite®, respectfully. The inlet flow to the chamber was 15 ml/min and the chamber was connected to a Foxbox (Sable Systems International, Las Vegas, USA) that measured excurrent CO₂.

The second assay also involved groups of 20 same sex individuals held within a 10 ml glass chamber covered with an opaque cover. Seven samples were assayed at one given time and were randomly chosen from a pool totalling 8 replicates per sex and population. Assays were conducted at 25°C over a period of three days. The inlet flow to the chamber provided ambient air at 50 ml/min. The chamber was connected to a LI-7000 analyzer (LI-COR Biosciences, Lincoln, USA) that recorded excurrent CO₂ and H₂O. A reference channel on the analyzer was used and differential values between the reference and measurement chambers were used to calculate CO₂ and H₂O production rates. A Multiplexer v3 (Sable Systems) and FlowBar-8 (Sable Systems) were placed upstream of the chambers and were used to measure eight chambers sequentially. Measurements were taken over a period of 15 min and a baseline measurement was performed before each measure. Each sample of 20 flies was anaesthetized with CO₂ and subsequently weighed with a MX5 Microbalance (Mettler Toledo, Columbus, USA). Recorded CO₂ and H₂O emission rates in Pa were converted in ml/h/mg and µl/h/mg, respectfully (Lighton, 2008). The statistical analysis followed a partly nested split-plot design as used in the analysis of wet weight.

Mitochondrial citrate synthase activity — CS activity was measured according to Pichaud *et al.*, (2010), with minor modifications. Briefly, day-old males were collected after light CO₂ anaesthesia and weighed in groups before being frozen at -80°C. After 20 days, samples were processed and kept on ice unless otherwise indicated. Flies were finely cut in progressively smaller pieces for 2 min with dissection scissors and then a 19:1 ratio v/w of homogenization buffer (25 mM Tris-HCl, 2 mM EDTA, Triton X-100 0.5% v/v, pH 7.4 at 4°C) was added and the tissue was homogenized a total of three times, each a duration of 10 s, with 30 s rest in between each pulse using a PT-DA1307

homogenization generator (Kinematica, Luzernerstrasse, Switzerland). Samples were then sonicated with a multi-tip VC-505 sonication probe (Sonics & Materials, Inc, Newtown, USA) at low intensity for a single 5s pulse. Homogenized samples were then centrifuged at 5500 x g for 10 min at 4°C and the resulting supernatant was transferred to new receptacles where they were further diluted 100X. In a 96 well plate, 10 µl of the diluted sample was combined with 230 µl of reaction solution (50 mM Tris-HCl, 0.54mM oxaloacetate, 0.109 mM DTNB, pH 8.0 at 25°C) and read at 412 nm by a Synergy 2 Multi-Mode Microplate Reader (Biotek, Winooski, USA) before and after the addition of 10 µl acetyl-CoA (7.5 mM) to each well. Assays were performed in triplicate and control rates without substrate were determined for each assay. Activity was calculated by subtracting $\Delta A_{412}/\text{min}$ before the addition of acetyl-CoA from $\Delta A_{412}/\text{min}$ after the addition of acetyl-CoA (Robinson et al., 1987). A control rate was not subtracted because none was present. CS activity ($\mu\text{mol min}^{-1} \text{g}^{-1}$) was calculated as follows: activity = $(\Delta\text{OD}/\text{min})(\epsilon \times l)^{-1}(\text{dilution factor})$; whereby $\epsilon = 13.6 \text{ OD}/\mu\text{mol}$, $l = 0.705 \text{ cm}$, dilution factor = 5×10^4 . All chemicals used were purchased from Sigma. The statistical analysis used a nested design where population was a random effect nested within the fixed effect of treatment.

RESULTS

Traits measured during experimental evolution

During experimental evolution, wet weight, anoxia tolerance and post-hyperoxia climbing ability were all measured on flies directly taken from the experimental treatments. Divergence in these traits between experimental treatments may therefore include an environmental as well as a genetic component. As noted in the Methods, the hyperoxic treatment was started five generations after the other treatments [i.e. 6(1)].

Wet weights at generation 25(20) did not differ between experimental treatments in males ($F = 1.87$; $df = 2$; $p = 0.12$) or in females ($F = 2.74$; $df = 2$; $p = 0.21$; Fig. 2). Hypoxic flies exhibited a greater tolerance to acute anoxia at several time points, regardless of gender, as compared to controls ($F = 134.2$; $df = 2$; $p < 0.001$), with the exception of the third generation of males (Fig. 3). After the first generation, flies from the hyperoxia treatment had a lower tolerance to acute anoxia when compared to controls ($F = 106.5$; $df = 2$; $p < 0.001$), with the exception of the first generation. However, acute anoxia tolerance did not vary between sexes ($F = 3.14$; $df = 1$; $p = 0.17$; Fig. 3). To determine if anoxia tolerance within treatments changed throughout generations, we performed a two-way ANCOVA, which demonstrated the normoxic-controls did not change throughout time ($F = 1.34$; $df = 8$; $p = 0.57$), irrespective of sex. Tolerance of female flies from the hypoxia treatment also did not significantly change over time in females ($F = 0.87$; $df = 5$; $p = 0.61$) or males ($F = 1.27$; $df = 5$; $p = 0.37$). Hyperoxic conditions caused acute anoxia tolerance to decrease in females ($F = 6.37$; $df = 2$; $p < 0.001$) and in males ($F = 5.94$; $df = 2$; $p < 0.001$; Fig. 3) during the first five generations under selection. Exposing adults, irrespective of sex, to pure O_2 for 48 h reduced their climbing ability when eliciting a negative geotactic response (t -ratio > 2.99 ; $p < 0.01$ in both sexes; Fig. 4). Experimental treatments did differ significantly in post-hyperoxia climbing ability throughout time (i.e. generations) ($F = 35.9$; $df = 2$; $p < 0.05$; Fig. 5), however the effects were inconsistent and showed no obvious trend through time.

Evolved responses

At various times during experimental evolution, adults from every experimental treatment were placed in a common normoxic environment for two generations, after which evolved differences in traits related to body size, stress tolerance, fitness and metabolism were assayed. The specific traits were wet weight, anoxia tolerance and recovery, post-hyperoxia climbing ability, prolonged oxidative stress tolerance, desiccation tolerance, longevity, productivity, routine metabolic rate, water loss and citrate synthase activity (Table 2).

Wet weight and stress tolerance

Wet weight differed significantly between the sexes at all time points ($F = 123.4$; $df = 1$; $p < 0.001$; Fig. 2). In contrast, wet weights between experimental treatments were not significantly different at any time point ($F = 3.20$; $df = 2$; $p = 0.08$). There was an evolved difference in anoxia tolerance between the experimental treatments ($F = 99.7$; $df = 1$; $p < 0.01$), but not between the sexes ($F = 3.52$; $df = 1$; $p = 0.16$; Fig. 6). Post-hoc analysis revealed that hypoxia-evolved flies were more tolerant to anoxia than the normoxic flies and that the hyperoxic flies exhibited the lowest tolerance to anoxia. To determine if anoxia tolerance within treatments evolved throughout successive generations, a two-way ANCOVA examining the treatment by generation interaction was performed. The analysis revealed no modification of anoxia tolerance in either sex in any treatment throughout generation number ($F = 0.32$; $df = 6$; $p = 0.85$), suggesting that these traits did not further diverge after their initial measurements at generation five.

Recovery time from anoxia was measured as the time to re-establish an upright position after succumbing to anoxia, however there were no significant differences between treatments and no treatment \times sex interaction ($F = 0.17$; $df = 2$; $p = 0.85$; $F = 4.10$; $df = 2$; $p = 0.08$; Fig. 7), but the sexes were significantly different ($F = 12.26$; $df = 1$; $p < 0.05$; Fig. 7).

At generation 15(10), post-hyperoxia climbing ability did not differ between experimental treatments in males ($F = 0.95$; $df = 2$; $p = 0.64$) or females ($F = 4.65$; $df = 2$; $p = 0.06$; Fig. 8). Similarly, survival to prolonged oxidative stress did not significantly differ among experimental treatments in males ($\chi^2 = 1.59$; $df = 2$; $p = 0.19$; Fig. 9) or females ($\chi^2 = 1.59$; $df = 2$; $p = 0.45$). However, female had a lower survival rate than males ($\chi^2 = 50.29$; $df = 1$; $p < 0.001$; Table 3).

Males were less tolerant of desiccation stress, as indicated by a reduced survival rate, in comparison to females ($\chi^2 = 400.1$; $df = 1$; $p < 0.001$; Fig. 10; Table 4). In males, the hyperoxia-evolved lines had significantly lower survival rates to desiccation stress than the hypoxia treatment and normoxic-controls ($\chi^2 = 274.9$; $p < 0.001$) whereas in females, the hyperoxia- and hypoxac-evolved lines had a significantly reduced survival when compared to normoxic-controls ($\chi^2 = 9.89$; $df = 2$; $p < 0.01$).

Fitness components

When assessing longevity in a reciprocal transplant at generation 35(30), males had significantly reduced longevity compared to females ($\chi^2 > 3.78$; $df = 1$; $p < 0.05$; Fig. 11; Table 5). In the hypoxia environment, the males from the normoxia and hypoxia-evolved treatments exhibited a significantly higher longevity compared to the hyperoxia treatment ($\chi^2 = 24.09$; $df = 2$; $p < 0.001$) and females from the normoxia treatment had an increased longevity compared to the hypoxia and hyperoxia treatments ($\chi^2 = 38.35$; $df = 2$; $p < 0.001$). These differences were also present when the populations were all raised in the normoxic environment, for males ($\chi^2 = 11.45$; $df = 2$; $p < 0.01$) and females ($\chi^2 = 40.99$; $df = 2$; $p < 0.001$). There were no significant differences between treatments in the hyperoxic environment in males ($\chi^2 = 4.45$; $df = 2$; $p = 0.11$) or females ($\chi^2 = 1.82$; $df = 2$; $p = 0.40$).

Productivity differed significantly between the evolved treatments ($F = 6.02$; $df = 2$; $p < 0.05$; Fig. 4), with a post-hoc analysis revealing that the hyperoxia-evolved lines had a significantly lower

productivity overall compared to the other two treatments. Environment also had a significant effect on productivity ($F = 58.76$; $df = 2$; $p < 0.0001$; Fig. 4), with a post-hoc test showing significant differences in productivity in all three environments, with hypoxia and hyperoxia reducing productivity by 51% and 34%, respectively, compared to normoxia. The treatment \times environment interaction was non-significant, providing no evidence that the treatment response varied among environments ($F = 1.73$; $df = 2$; $p = 0.1863$; Fig. 4).

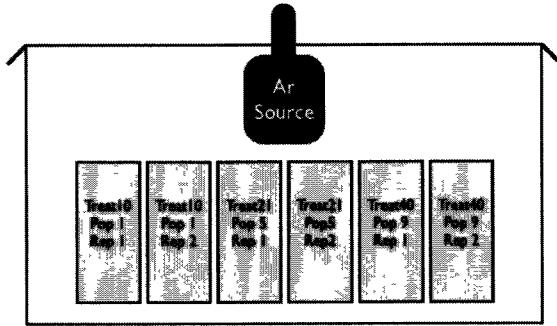
Metabolism

At generation 17(12), routine metabolic rate (RMR) was significantly higher in males than females [$F = 35.4$; $df = 1$; $p < 0.001$; Fig. 13(A)]. In females, hypoxia-evolved lines had a higher RMR than other treatments ($F = 6.9$; $df = 2$; $p < 0.01$). In males, the hypoxia experimental treatments had a higher RMR than hyperoxia-evolved lines ($F = 4.79$; $df = 2$; $p < 0.05$). At generation 29(24), RMR was higher in males than females [$F = 28.7$; $df = 1$; $p = 0.3167$; Fig. 13(B)]. However, RMR at generation 29(24) was not different between experimental treatments ($F = 30.6$; $df = 1$; $p = 0.34$). The rate of water loss under normoxia was significantly lower in the hyperoxia treatment compared to other treatments ($F = 4.6$; $df = 2$; $p < 0.05$; Fig. 14). Citrate synthase activity differed between treatments, with a post-hoc test revealing that the hypoxia-evolved treatment had a significantly higher activity level when compared to other treatments ($F = 7.3$; $df = 2$; $p < 0.01$; Fig. 15).

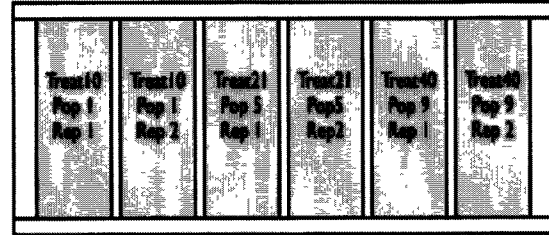
FIGURES AND TABLES

Figure 1. Experimental and statistical design of acute anoxia tolerance and post-hyperoxia climbing ability. A) Depicts a beaker of the anoxia tolerance experimental, B) depicts an apparatus of the post-hyperoxia climbing ability assay. C) depict the statistical hierarchy of the factors for the acute anoxia tolerance assay and the assay of climbing ability after acute hyperoxia.

A



B



C

Model Design

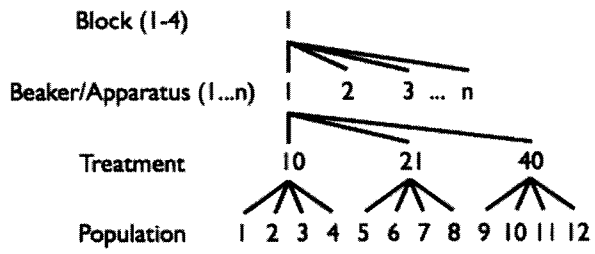


Figure 1

Figure 2. Environmental and evolved effects of multi-generational exposure to normoxia (circles), hypoxia (squares) and hyperoxia (triangles) on the wet weights of male (open symbols) and female (filled symbols) *D. melanogaster*. The hyperoxia treatment was started five generations after the other treatments (i.e. 6(1)). Flies from generation 15(10) were raised in a common normoxic environment (common garden) for two generations prior to conducting the assay and at generation 25(20) environmental response was assessed in flies taken directly from their experimental environments (acclimation). All flies emerged from density-controlled vials. Population means \pm SEM are displayed. Females were significantly heavier than males ($p < 0.001$) in all assays. There was no significant treatment effect in males ($p = 0.12$) or in females ($p = 0.21$) within assays.

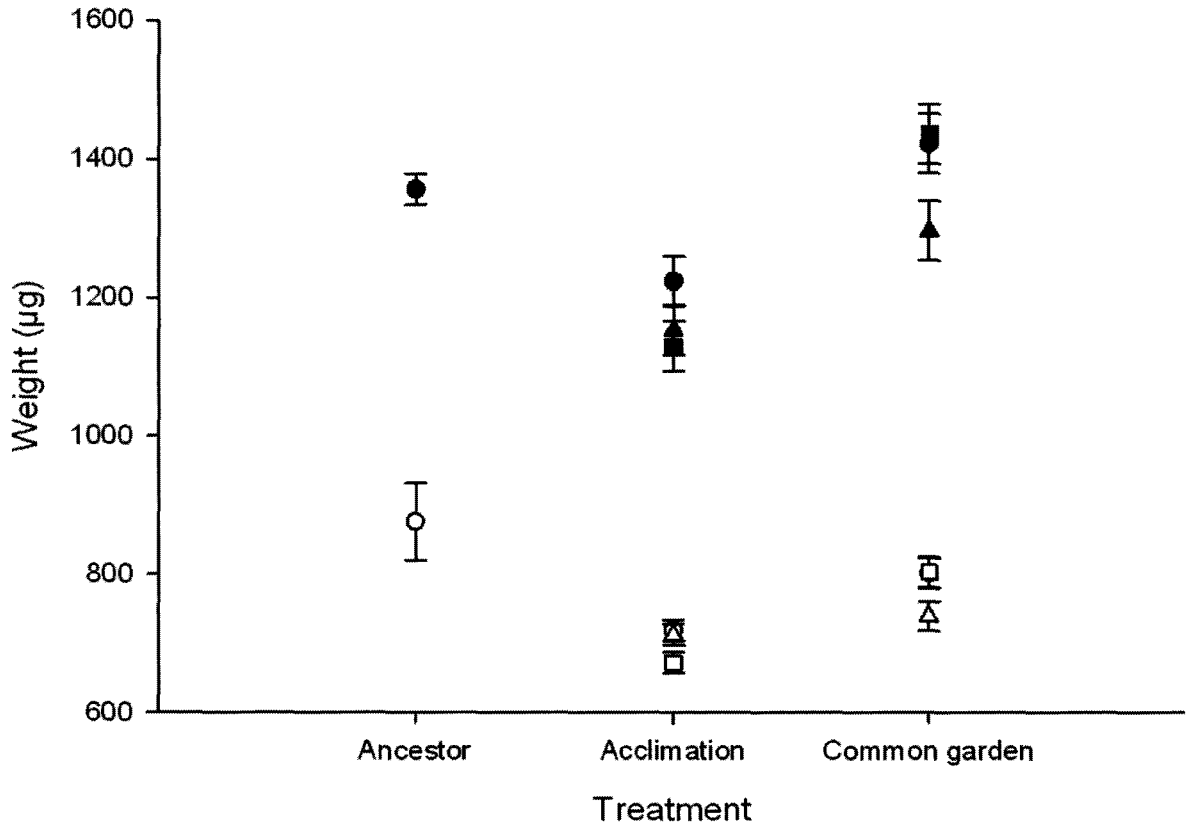
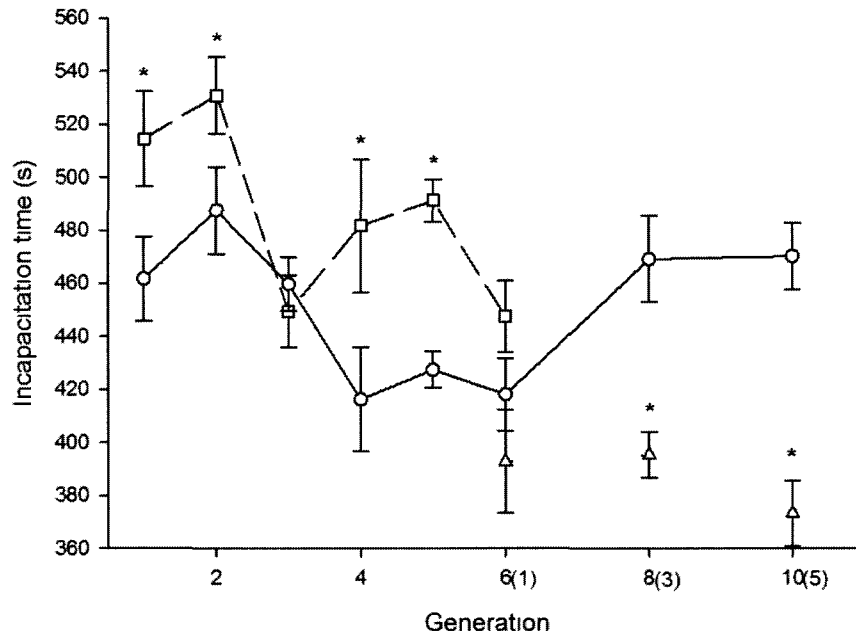


Figure 2

Figure 3. Differences in anoxia tolerance of A) male and B) female *D. melanogaster* after multi-generational exposure to normoxia (circles, solid line), hypoxia (squares, dashed line) and hyperoxia (triangles, dotted line). All flies were taken directly from their experimental environments prior to estimating anoxia tolerance. The hyperoxia-exposed flies were started five generations after the other treatments (i.e. 6(1)). The main focus of this assay was to track the initial divergence in the hypoxia and hyperoxia treatments compared to the normoxia treatment. Population means \pm SEM are displayed. Within generations, hypoxia acclimated flies displayed a greater tolerance to acute anoxia when compared to controls ($p < 0.001$), with the exception of the third generation of males ($p = 0.24$). The hyperoxia acclimated flies had a lower tolerance to acute anoxia when compared to controls ($p < 0.001$), with the exception of the first generation ($p = 0.37$). These significant differences are indicated with asterisks. There were no significant difference between sexes ($p = 0.17$). To determine if anoxia tolerance within treatments changed throughout generations, we performed a two-way ANOVA that showed that the hypoxic- and normoxic-treatments did not change throughout time ($p = 0.37$). Tolerance of hypoxic-flies significantly decreased over time ($p < 0.001$).

A



B

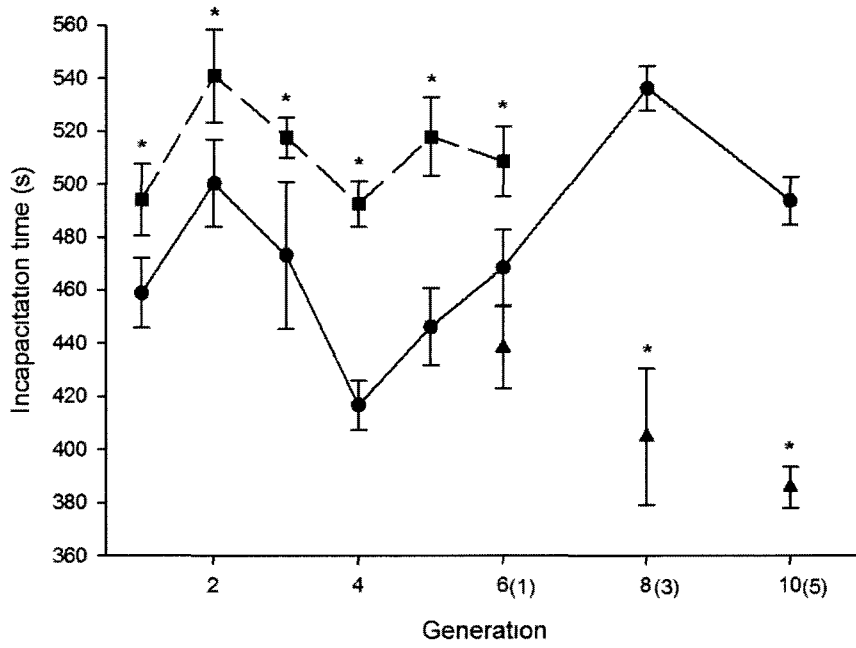
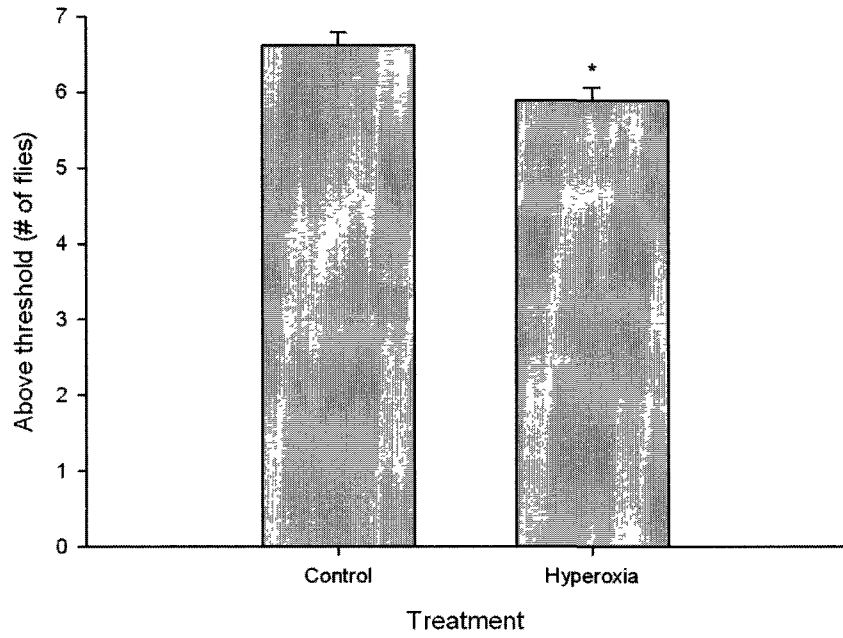


Figure 3

Figure 4. Post-hyperoxia climbing ability of A) males and B) female *D. melanogaster*. All flies were taken directly from the ancestral stock population raised in normoxia. Hyperoxia flies were exposed to 100% O₂ for 24 h, while control flies were kept at normoxia. Climbing ability was assessed by recording the number of flies that successfully climbed over a height threshold (4.8 cm). Treatment means ± SEM are displayed. Exposure to pure O₂ for 48 h significantly reduced the climbing ability in both sexes (denoted by asterisk; p<0.01).

A



B

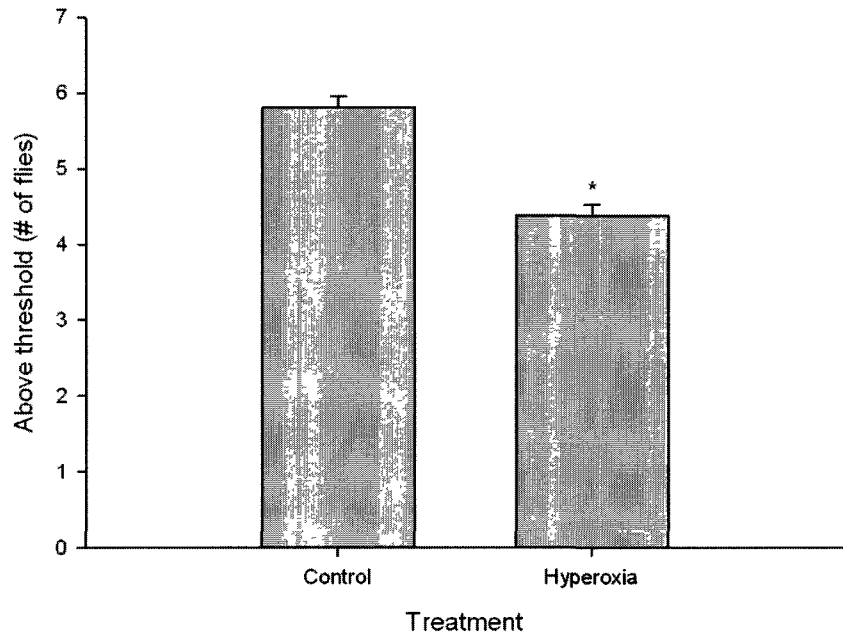
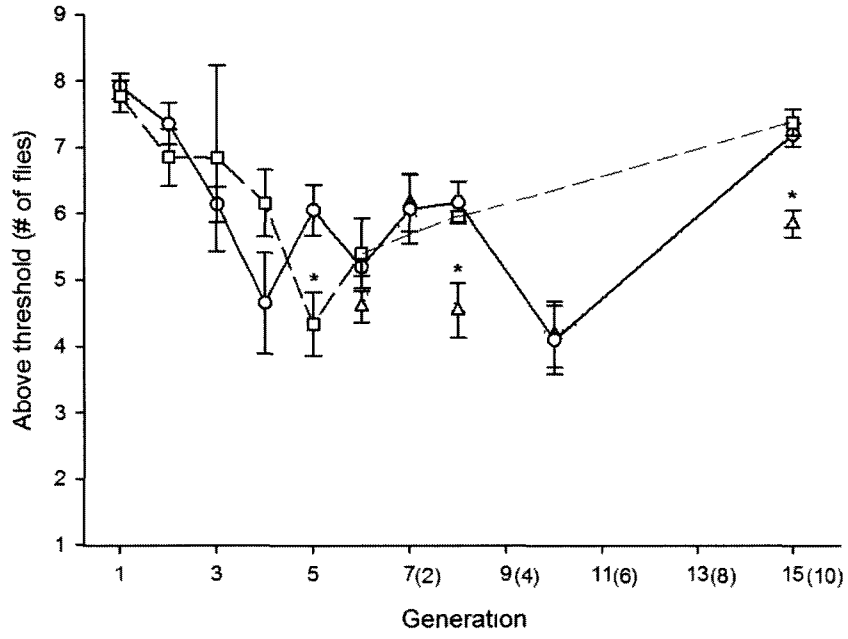


Figure 4

Figure 5. Differences in post-hyperoxia climbing ability of A) male and B) female *D. melanogaster* after multi-generational exposure to normoxia (circles, solid line), hypoxia (squares, dashed line) and hyperoxia (triangles, dotted line). All flies were taken directly from their experimental environments prior to assessing climbing ability. Climbing ability was assessed by recording the number of flies that successfully climbed over a height threshold of 4.8 cm. The focus of this assay was to measure the initial response to hypoxia and hyperoxia. Consequently, the hypoxia treatment was measured for six generations. The hyperoxia-exposed flies were measured for the first five generations, but they were started five generations after the other treatments (i.e. 6(1)). Population means \pm SEM are displayed. Significant differences between treatments and normoxic-controls occurred (denoted by asterisk; $p < 0.05$), however there were no visible trend within treatments.

A



B

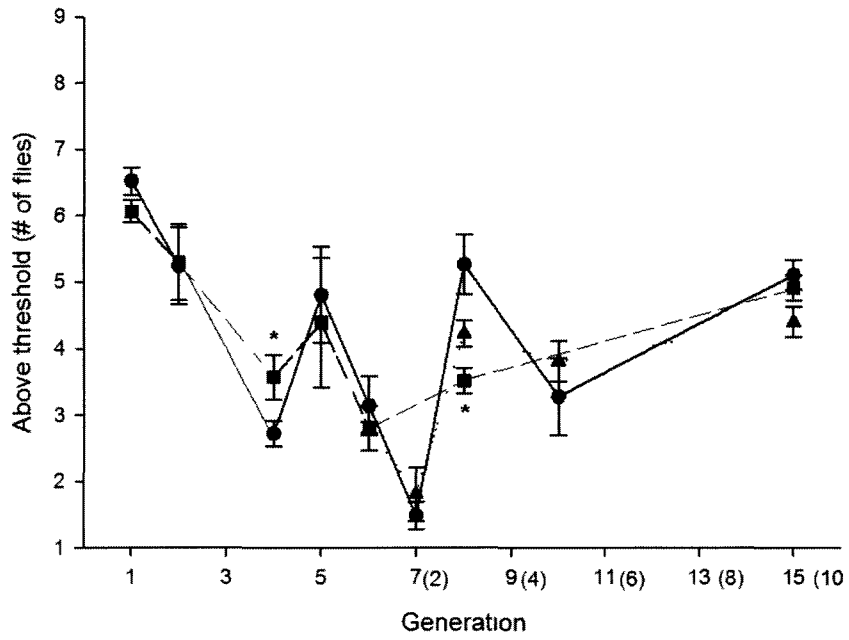
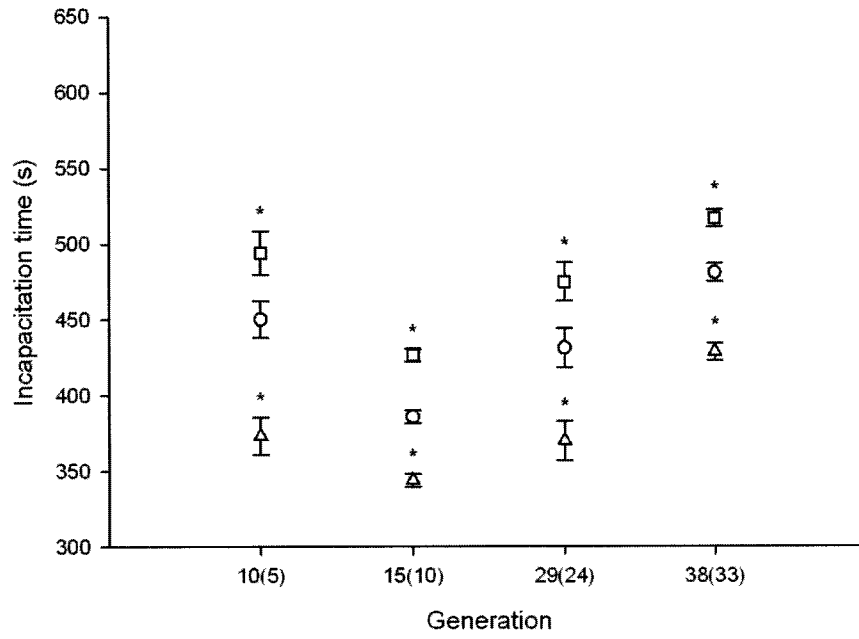


Figure 5

Figure 6. Evolved differences in anoxia tolerance of A) male and B) female *D. melanogaster* after multi-generational exposure to normoxia (circles), hypoxia (squares) and hyperoxia (triangles). All flies were raised in a common normoxic environment (common garden) for two generations prior to conducting the assay. Hyperoxia exposed flies were started five generations after the other treatments (i.e. 6(1)). Population means \pm SEM are displayed. The anoxia tolerance of hypoxic and hyperoxic flies were significantly different than controls in males and females for all generations (indicated by asterisk; $p < 0.01$), but the sexes were not significantly different ($p = 0.16$). There was no change in anoxia tolerance in hypoxia and hyperoxia treatments across generations when compared to normoxic-controls ($p = 0.85$).

A



B

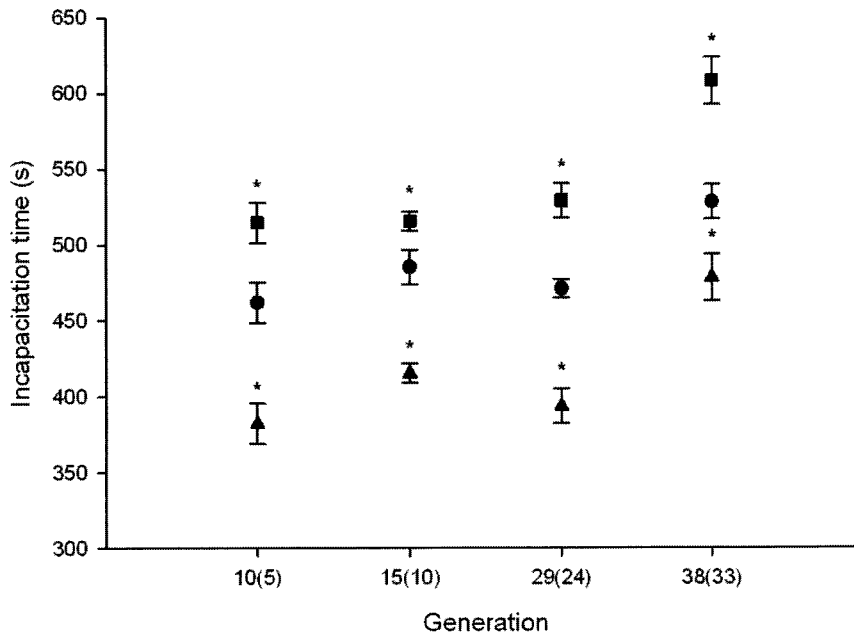


Figure 6

Figure 7. Evolved differences in recovery times to acute anoxia (time taken to right itself on all six legs) of male (open symbols) and female (filled symbols) *D. melanogaster* after multi-generational exposure to normoxia (circles), hypoxia (squares) and hyperoxia (triangles). Flies collected at generation 38(33), where hyperoxia exposed flies were started five generations after the other treatments, were raised in a common normoxic environment for two generations prior to conducting the assay. Population means \pm SEM are displayed. There were no significant differences between treatments and no treatment \times sex interaction was present ($p=0.85$; $p=0.08$), but the sexes were significantly different ($p<0.05$).

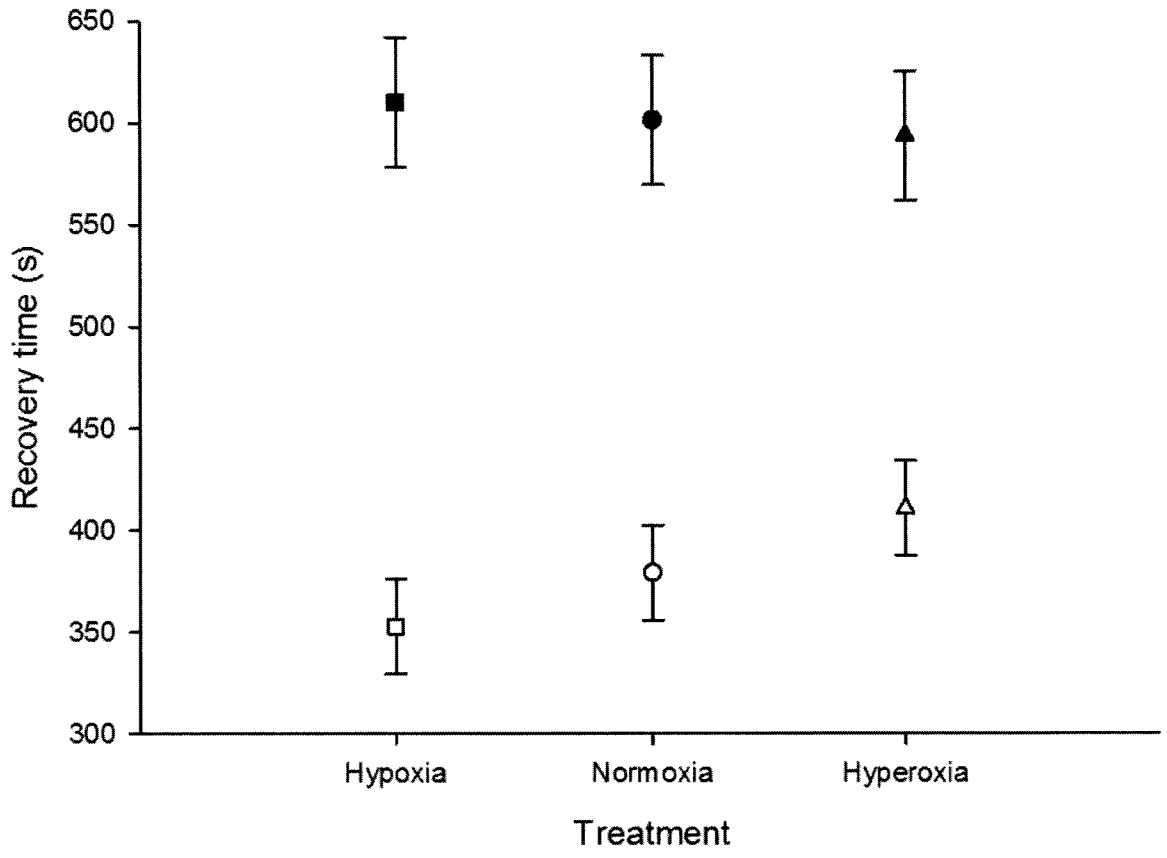


Figure 7

Figure 8. Evolved differences in post-hyperoxia climbing ability of male (open symbols) and female (filled symbols) *D. melanogaster* after multi-generational exposure to normoxia (circles), hypoxia (squares) and hyperoxia (triangles). Flies collected at generation 15(10), where hyperoxia exposed flies were started five generations after the other treatments, were raised in a common normoxic environment for two generations prior to conducting the assay. Population means \pm SEM are displayed. No significant treatment effect was present in (p=0.64) or females (p=0.06).

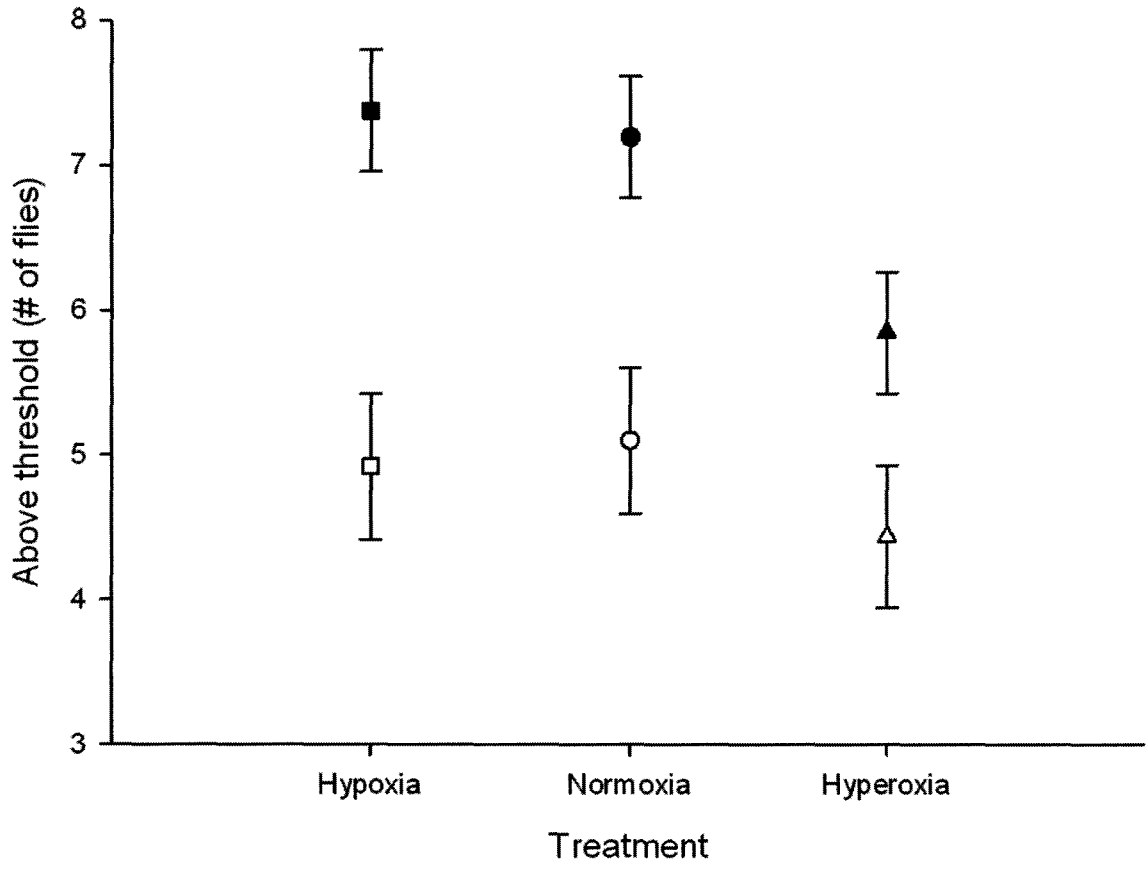
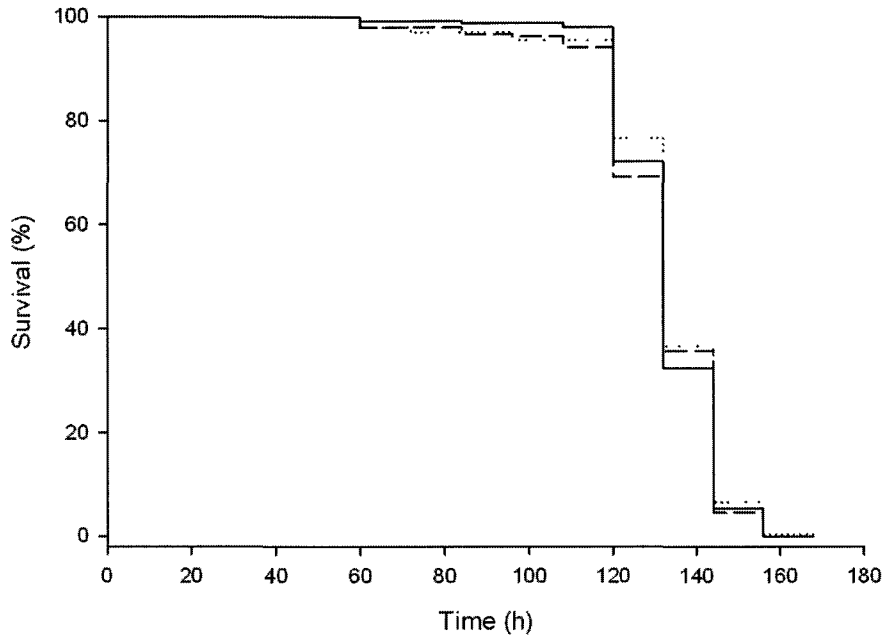


Figure 8

Figure 9. Evolved differences in survival rate when exposed to prolonged oxidative stress in A) male and B) female *D. melanogaster* after multi-generational exposure to normoxia (solid line), hypoxia (dashed line) and hyperoxia (dotted line). Populations had evolved in their respective treatment environments for 15(10) generations, where hyperoxia exposed flies were started five generations after the other treatments, and were then raised in a common normoxic environment for two generations prior to conducting the assay. Treatment means are shown. Males had a significantly higher survival rate than females ($p < 0.001$). There was no significant difference between treatments in males ($p = 0.19$) or females ($p = 0.45$). Symbols are omitted for clarity.

A



B

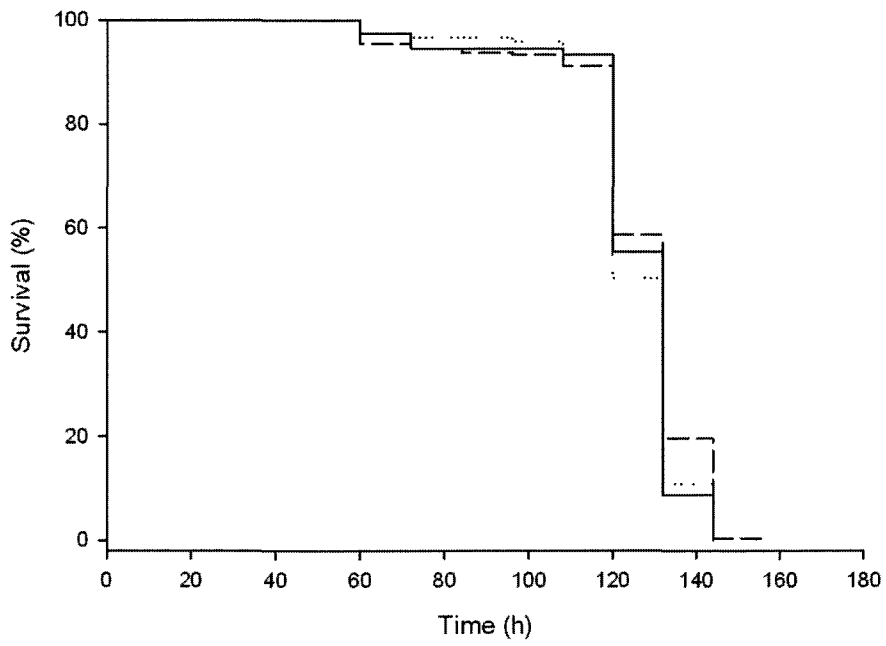


Figure 9

Figure 10. Evolved differences in survival rate under severe desiccation stress in A) male and B) female *D. melanogaster* after multi-generational exposure to normoxia (solid line), hypoxia (dashed line) and hyperoxia (dotted line). Populations had evolved in their respective treatment environments for 15(10) generations, where hyperoxia exposed flies were started five generations after the other treatments, and were then raised in a common normoxic environment for two generations prior to conducting the assay. Treatment means are shown. Females had a significantly higher survival rate compared to males ($p < 0.001$). In males, the normoxia and hypoxia treatments had a higher survival rate than hyperoxia-evolved flies ($p < 0.001$). In females, the normoxia treatment had a significantly higher survival rate than the hypoxia and hyperoxia-evolved lines ($p < 0.01$). Symbols were omitted for clarity.

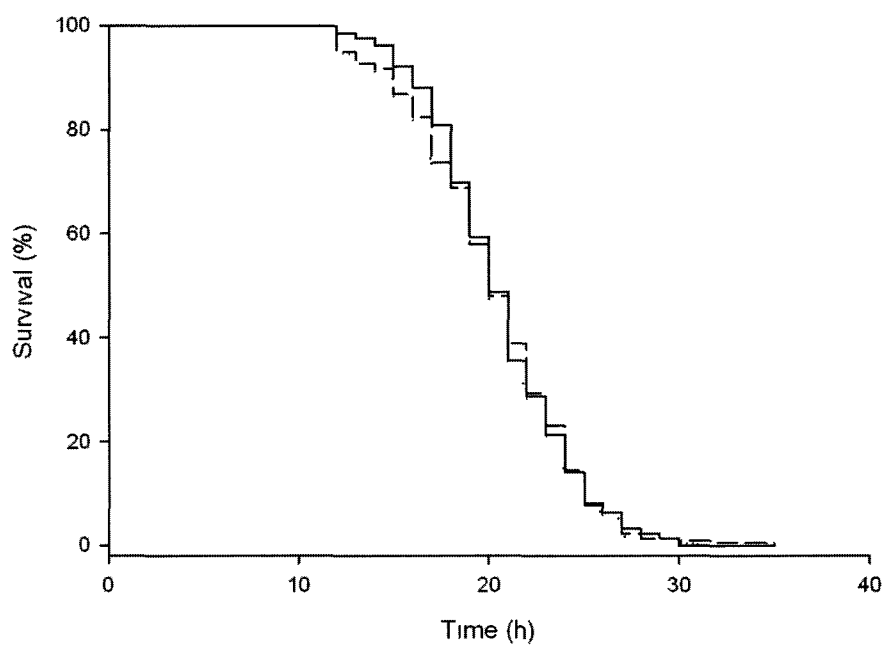
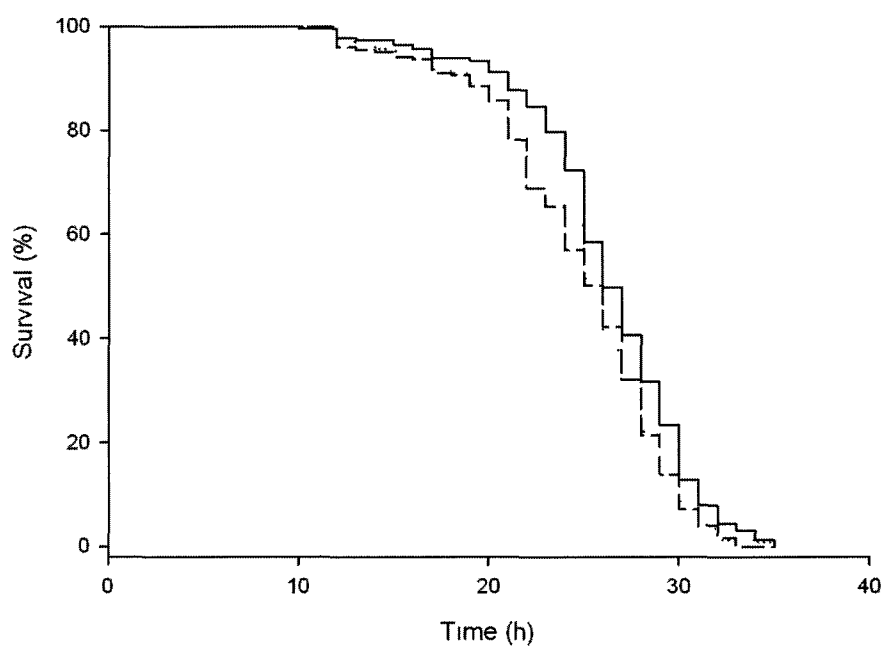
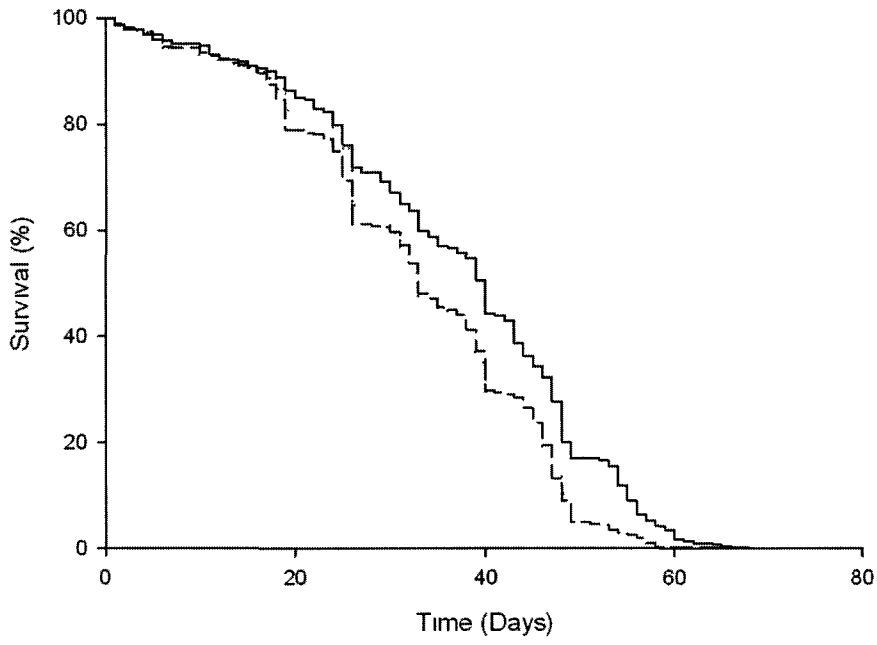
A**B**

Figure 10

Figure 11. Reciprocal transplant assay measuring longevity of evolved populations in the normoxic (line), hypoxic (dash) and hyperoxic (dot) treatments when held in the A) hypoxic, B) normoxic, and C) hyperoxic environments in male (1) and female (2) in *D. melanogaster*. Populations had evolved in their respective treatment environments for 35(30) generations, where hyperoxia exposed flies were started five generations after the other treatments, and were then raised in a common normoxic environment for two generations prior to conducting the assay. Treatment means are shown. Females had a significantly longer longevity compared to males in all environments ($p < 0.05$). In hypoxia and normoxia environments, males exhibited a longer longevity in the normoxia-evolved flies compared to the hypoxia and hyperoxia treatments ($p < 0.01$) and in females, the normoxia and hypoxia treatment had a longer longevity than the hyperoxia-evolved flies ($p < 0.001$). In hyperoxia, there was no difference between treatments ($p = 0.40$). Symbols are omitted for clarity.

A-1



A-2

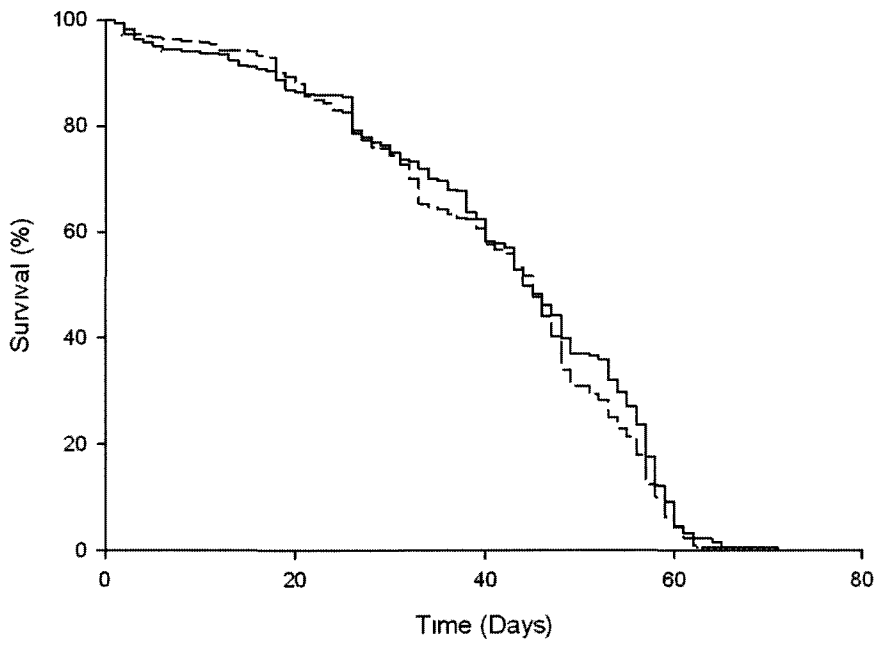
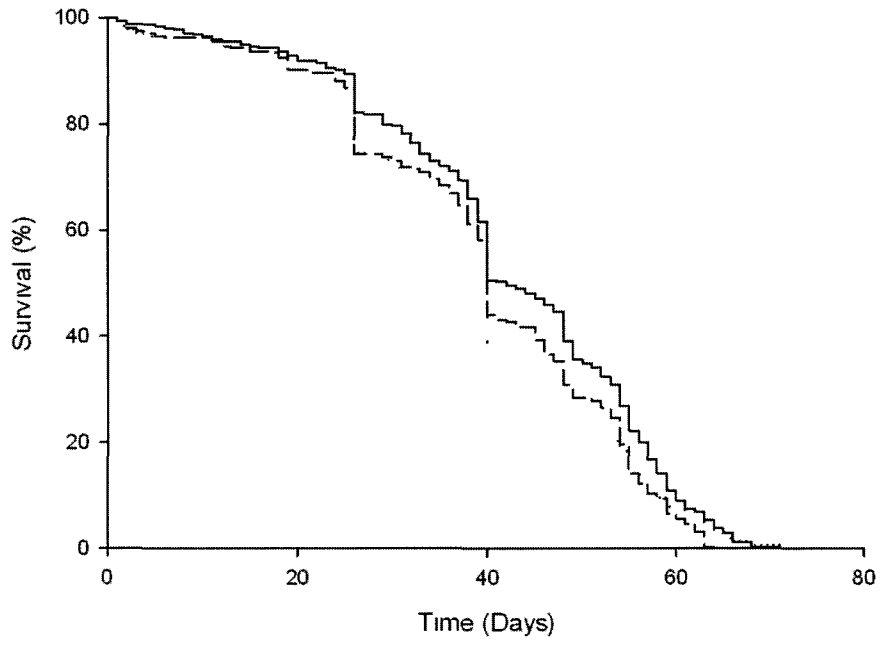


Figure 11

B-1



B-2

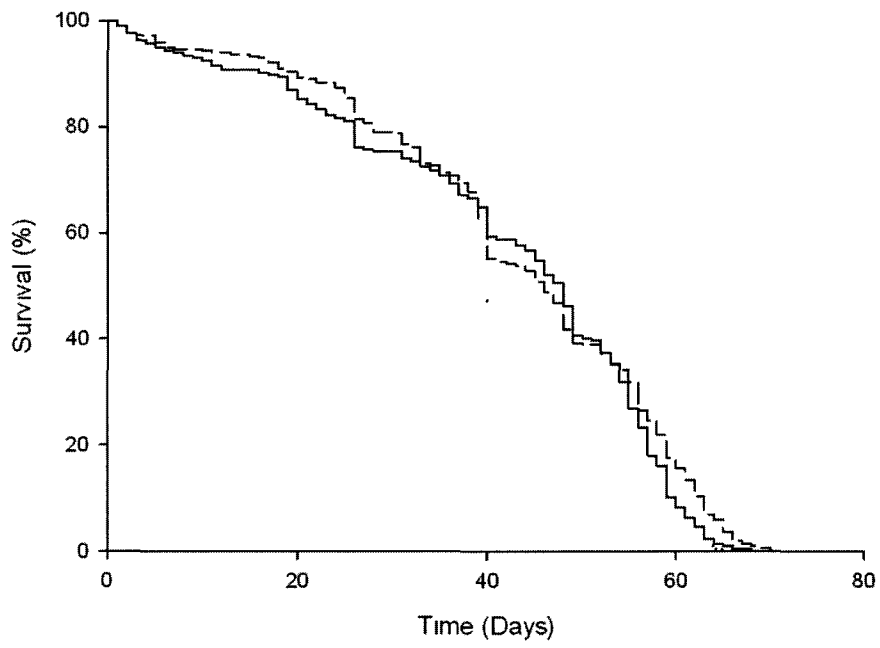
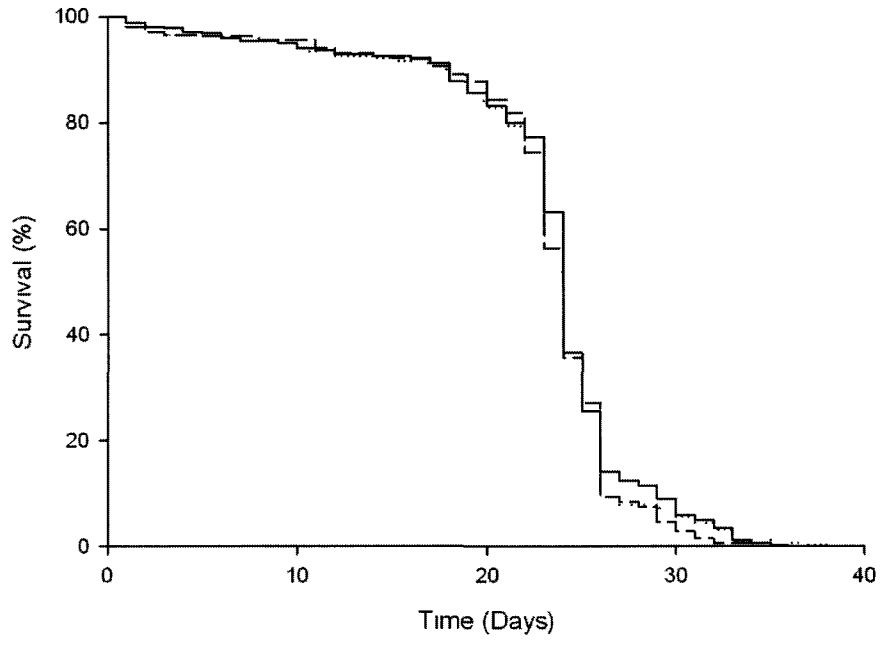


Figure 11

C-1



C-2

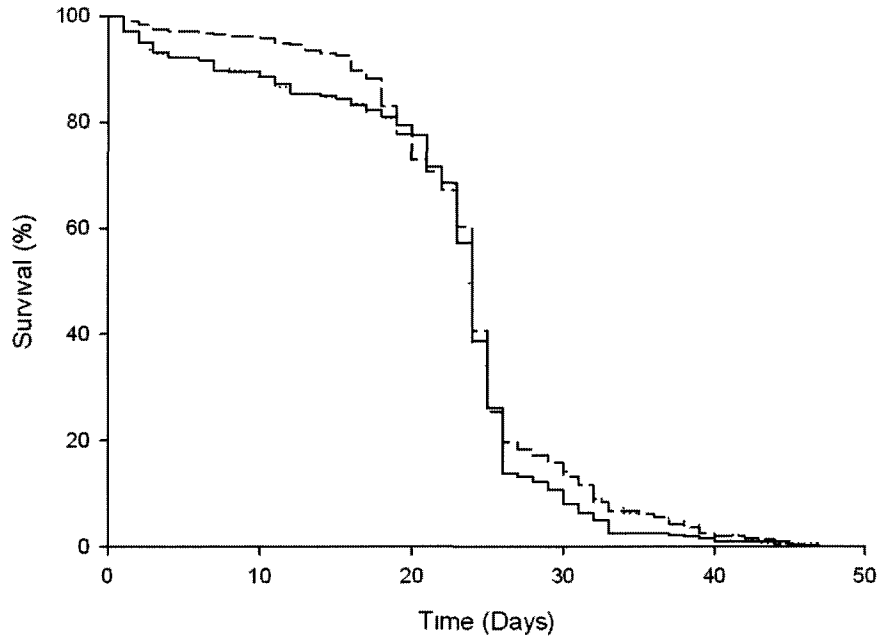


Figure 11

Figure 12. Reciprocal transplant assay measuring productivity of evolved populations of *Drosophila melanogaster* when raised in each of the three experimental environments. Populations had evolved in their respective treatment environments for 35(30) generations, where hyperoxia exposed flies were started five generations after the other treatments, and were then raised in a common normoxic environment for two generations prior to conducting the assay. Normoxia (circles, solid line), hypoxia (squares, dashed line) and hyperoxia (triangles, dotted line) treatments are displayed as population means \pm SEM. Productivity (as determined by the number of adult offspring produced by a male-female pair at 14 days) differed between environments ($p < 0.0001$), with a post-hoc test revealing all three environments were significantly different. Treatments were significantly different ($p < 0.05$), with a post-hoc test revealing that the hyperoxia treatment being significantly lower compared to the others. The treatment \times environment interaction was not significant ($p = 0.19$).

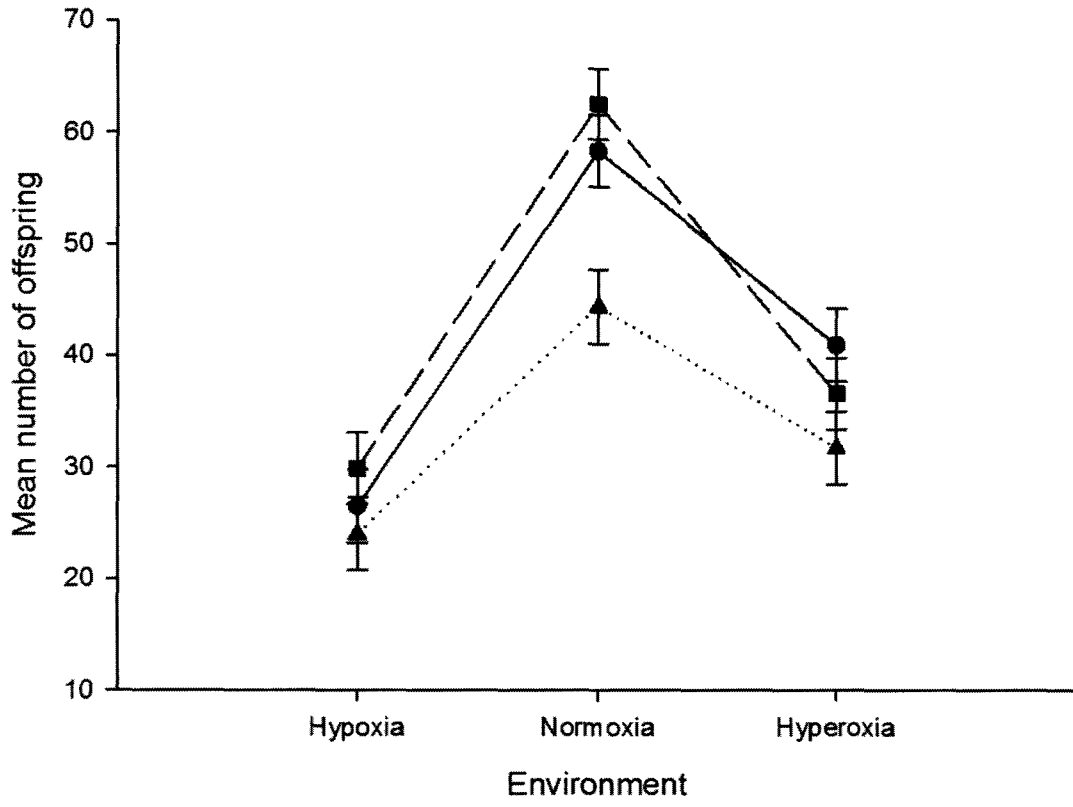


Figure 12

Figure 13. Evolved differences in CO₂ production (an index of routine metabolic rate) of male (open symbols) and female (filled symbols) *D. melanogaster* after multi-generational exposure to normoxia (circles), hypoxia (squares) and hyperoxia (triangles) environments at generation A) 17(12) and B) 29(24). Hyperoxia exposed flies were started five generations after the other treatments. All populations were collected and raised in a common normoxic environment for two generations prior to conducting the assay. The first assay used CO₂ scrubbed and desiccated incurrent air, while the later assay used ambient room air. Population means \pm SEM are displayed (error bars are omitted in one direction for clarity). Sexes were statistically different at generation 17(12) ($p < 0.001$) but not at generation 29(24) ($p = 0.3167$). Within sex, treatments not sharing a common letter are statistically different. At generation 17(12), hypoxic females were statistically different than their normoxic and hyperoxic counterparts ($p < 0.05$), while in males, the hypoxia and hyperoxia treatments were significantly different from each other ($p < 0.05$). At generation 29(24), experimental treatments did not differ from each other ($p = 0.3403$).

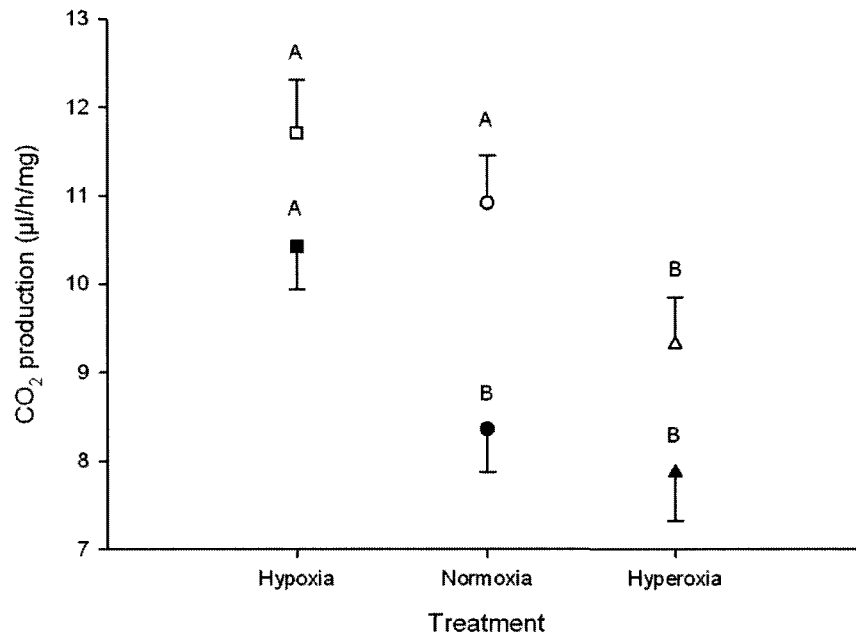
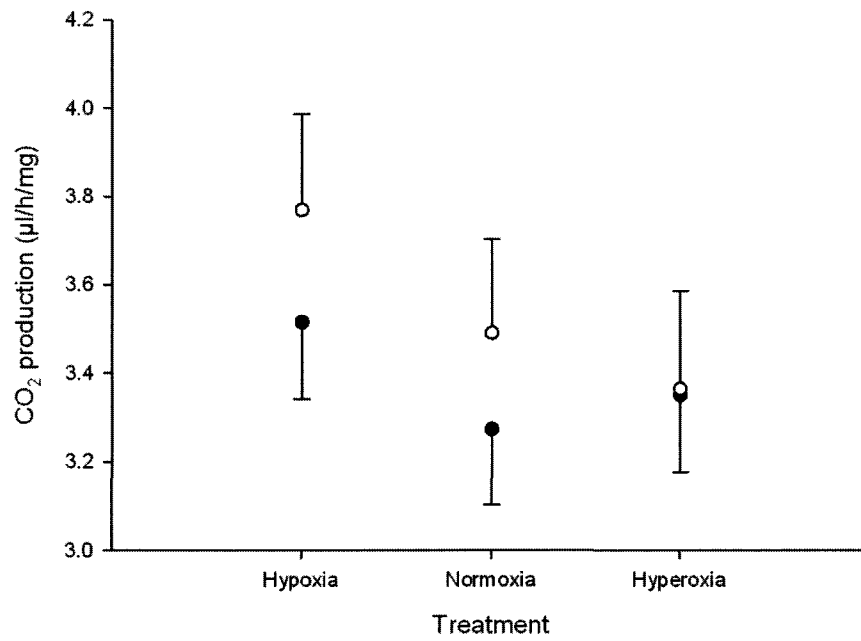
A**B**

Figure 13

Figure 14. Evolved differences in water loss of male (open symbols) and female (filled symbols) *D. melanogaster* after multi-generational exposure to normoxia (circles), hypoxia (squares) and hyperoxia (triangles) environments. Populations had evolved in their respective treatment environments for 29(24) generations, where hyperoxia exposed flies were started five generations after the other treatments, and were then raised in a common normoxic environment for two generations prior to conducting the assay. Population means \pm SEM are displayed. Within each sex, treatments not sharing a common letter are statistically different. The hyperoxia treatment was also significantly different from the hypoxia and normoxia treatments ($p < 0.05$).

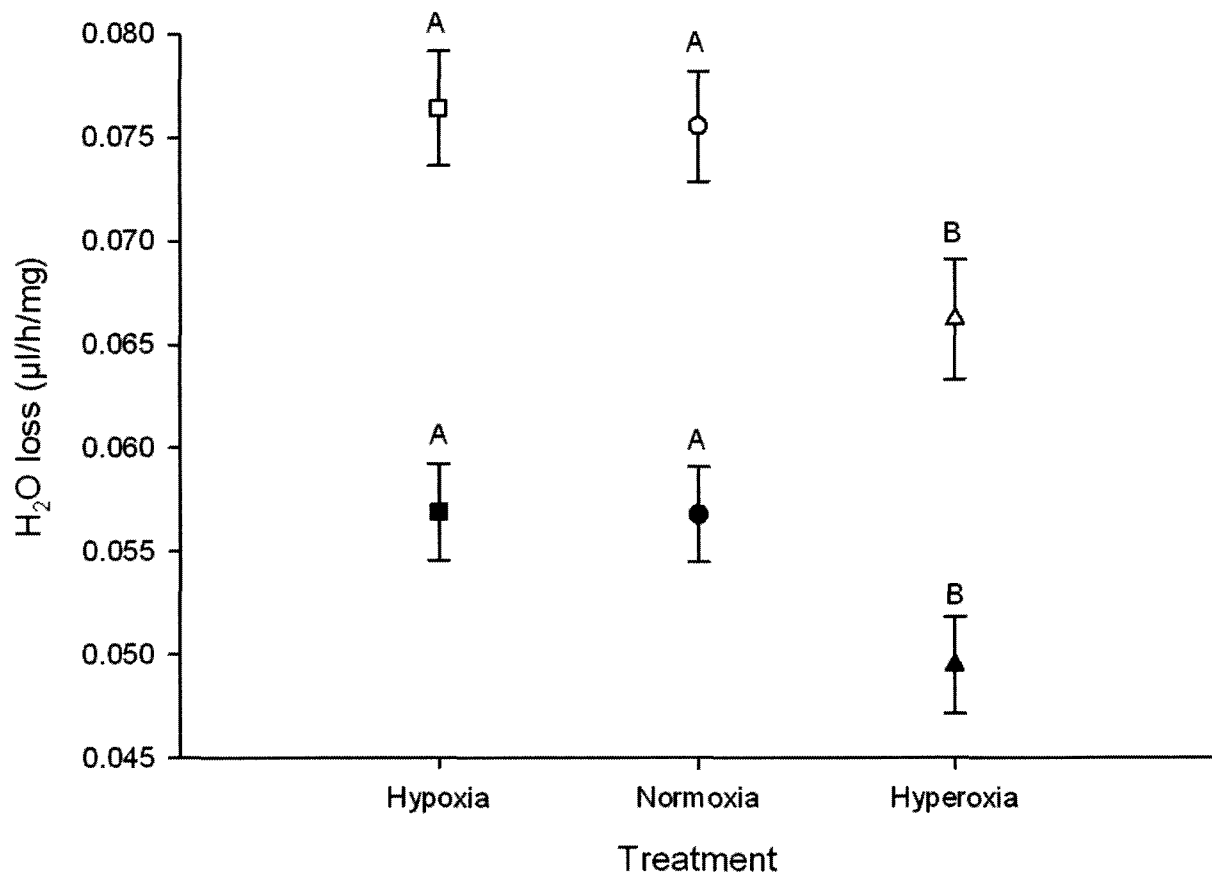


Figure 14

Figure 15. Evolved differences in citrate synthase activity of male (open symbols) and female (filled symbols) *D. melanogaster* after multi-generational exposure to normoxia (circles), hypoxia (squares) and hyperoxia (triangles). Populations had adapted to their respective treatment environments for 29(24) generations, where hyperoxia exposed flies were started five generations after the other treatments, and were then raised in a common normoxic environment for two generations prior to conducting the assay. Population means \pm SEM are displayed. The hypoxia treatment was significantly different from the normoxia and hyperoxia treatments (denoted with asterisk; $p < 0.01$).

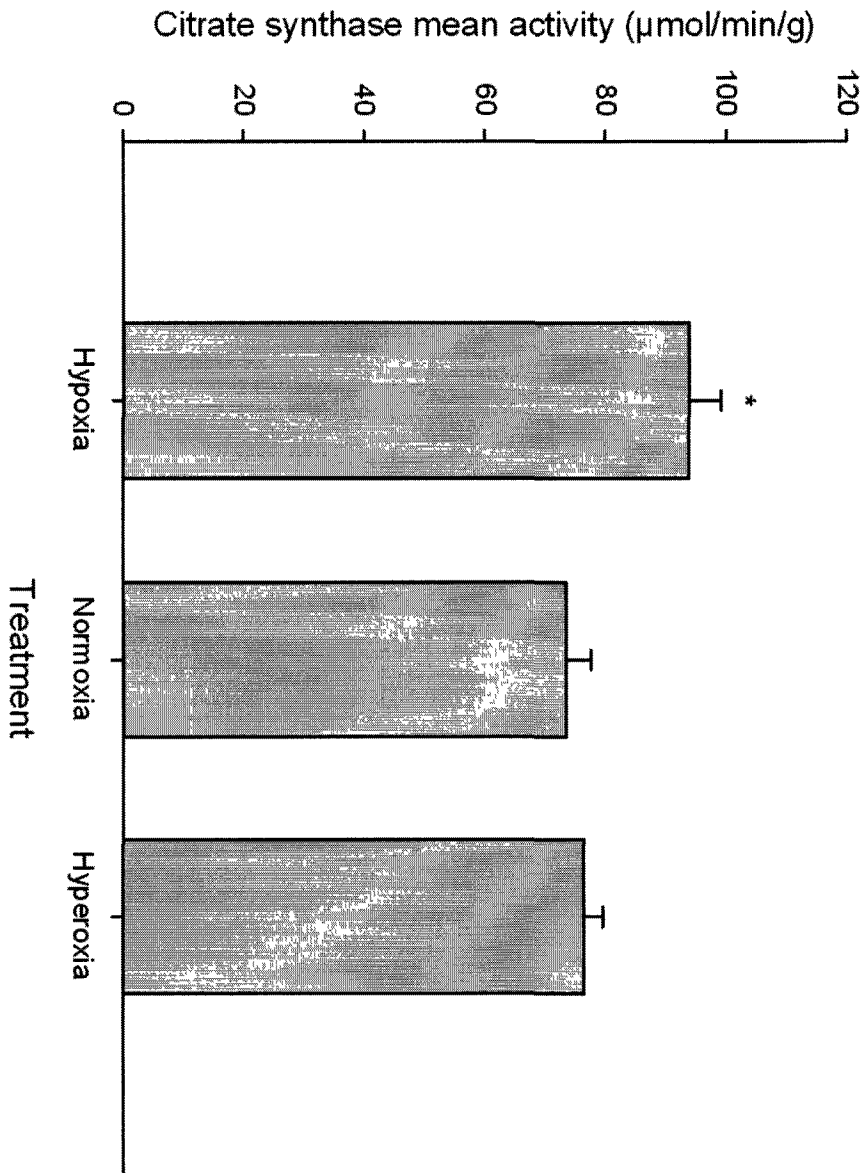


Figure 15

Table 1. ANOVA tables showing the construction of the F-ratios for the partially nested model used in the analysis of the multiple assays. Block and treatment are fixed effects while population and beaker are random effects nested as indicated. The term ‘beaker’ is used for the analysis of the anoxia tolerance and the recovery assays. The term ‘apparatus’ is used interchangeably with ‘beaker’ in the analysis of the post-hyperoxia climbing ability assay. This table is sourced from Quinn and Keough (2006).

Source	df	Test
Block	p-1	$MS_{\text{Block}}/MS_{\text{Beaker(Block)}}$
Beaker(Block)	p(q-1)	$MS_{\text{Beaker(Block)}}/MS_{\text{Residual}}$
Treatment	r-1	$MS_{\text{Treatment}}/MS_{\text{BlockXTreatment}}$
Population(Treatment)	s-1	$MS_{\text{Population(Treatment)}}/MS_{\text{Residual}}$
Block X Treatment	(p-1)(r-1)	$MS_{\text{BlockXTreatment}}/MS_{\text{Beaker(Block)XTreatment}}$
Beaker(Block) X Treatment	p(q-1)(r-1)	$MS_{\text{Beaker(Block)XTreatment}}/MS_{\text{Residual}}$
Residual	pqr(n-1)	

Table 2. Timing of the common gardens and the assays performed at various points during experimental evolution. Generation refers to the number of generations spent in the experimental environment (hypoxia, normoxia or hyperoxia).

CG ^a	Generation	Assay(s) Performed
1	5 ^b	Acute anoxia tolerance Post-hyperoxia climbing ability Wet weight
2	10(5)	Acute anoxia tolerance Post-hyperoxia climbing ability Wet weight
3	15(10)	Acute anoxia tolerance Desiccation Prolonged oxidative stress tolerance Post-hyperoxia climbing ability Wet weight
4	17(12)	Routine metabolic rate (Foxbox)
5	29(24)	Acute anoxia tolerance Routine metabolic rate (LI-7000)
6	35(30)	Longevity Productivity (Block 1)
7	36(31)	Productivity (Block 2)
8	38(33)	Acute anoxia tolerance Recovery from acute anoxia
9	39(34)	Mitochondrial citrate synthase activity

a - Common garden
b - Measurements performed on the hypoxia and normoxia experimental treatments

Table 3. Risk-ratios of evolved oxidative stress tolerance of experimental treatments, as determined in a proportional hazards analysis.

Comparisons	Risk Ratio	P-value
Male vs. Female	0.67	p<0.001
Normoxia vs. Hypoxia-evolved lines	1.08	p=0.45
Hyperoxia vs. Hypoxia-evolved lines	1.05	
Hyperoxia vs. Normoxia-evolved lines	0.97	

Table 4. Risk-ratios of desiccation tolerance of experimental treatments as determined in a proportional hazards analysis.

Comparisons	Risk Ratio	P-value
Male vs. Female	3.43	p<0.001
Normoxia- vs. Hypoxia-evolved lines	0.91	p<0.01
Hyperoxia- vs. Hypoxia-evolved lines	1.15	
Hyperoxia- vs. Normoxia-evolved lines	1.26	

Table 5. Risk-ratios of the reciprocal transplant assay measuring longevity of evolved populations when held in the hypoxic, normoxic, and hyperoxic environments as determined in a proportional hazards analysis.

Environment	Comparisons	Risk Ratio	P-value
Hypoxia	Male vs. Female	1.6	p<0.001
	Normoxia vs. Hypoxic-evolved lines	0.8	p<0.001
	Hyperoxia vs. Hypoxic-evolved lines	1.31	
	Hyperoxia vs. Normoxic-evolved lines	1.63	
Normoxia	Male vs. Female	1.09	p<0.05
	Normoxia vs. Hypoxia-evolved lines	0.78	p<0.001
	Hyperoxia vs. Hypoxia-evolved lines	1.21	
	Hyperoxia vs. Normoxia-evolved lines	1.55	
Hyperoxia	Male vs. Female	1.12	p<0.05
	Normoxic vs. Hypoxia-evolved lines	1	p=0.19
	Hyperoxic vs. Hypoxia-evolved lines	1.09	
	Hyperoxic vs. Normoxia-evolved lines	1.08	

DISCUSSION

The focus of the present study was to examine if altered aPO₂ would lead to adaptation in *D. melanogaster*, while studying the traits underlying these adaptations, thereby providing insight into the physiological basis of tolerance to atmospheric stress. To do so, an evolution experiment was conducted in which 12 replicate populations, derived from a common ancestor, were allowed to evolve under either hypoxic (5.0 -10.1 kPa O₂), normoxic (21.3 kPa O₂), or hyperoxic (40.5 kPa O₂) conditions for 34 (hyperoxia) to 39 (normoxia, hypoxia) generations. The hypoxic and hyperoxic environments had substantial negative impacts on productivity, reducing it by 52 and 44% respectively relative to the control populations (Fig. 12), demonstrating that these environments were stressful and suggesting that at least initially, the populations were not well adapted to them.

Adaptation to hypoxia

The four replicate populations allowed to evolve under hypoxic conditions exhibited increased anoxia tolerance. The parallel evolution of this trait, which is presumably indicative of increased performance under hypoxia, strongly argues that adaptation of these populations occurred in this environment. Surprisingly, this was not detectable as a significant increase in the fitness components tested; hypoxia-evolved flies had neither higher productivity nor greater longevity than control or hyperoxia-evolved populations when assessed under hypoxic conditions. There are two potential explanations for this apparent discrepancy. First, these assays may not have captured the relevant fitness components underlying increased performance in the hypoxic environment. Indeed, lifetime fitness is empirically difficult to measure in laboratory populations (Brommer et al., 2004; Rundle et al., 2007) and the current measure did not include all components that likely contribute to it. For example, reproductive success was not measured in this study and is generally considered a large component of male lifetime fitness. It is therefore possible that increased performance under hypoxic

conditions may have arisen from sexual selection on male precopulatory and/or postcopulatory reproductive success. Similarly, although productivity captures a substantial component of lifetime fitness, including female fecundity and the survival to emergence of both sexes, female mate choice (cryptic or overt) may also be important (Partridge, 1980) yet was prevented in this assay. In addition, fitness components were measured under conditions that did not exactly match those experienced during experimental evolution, involving differences in larval density and hence competitive environments. It therefore remains possible that the hypoxia-evolved population would outperform the others under these conditions. The beneficial acclimation hypothesis (BAH) postulates that acclimations that increase performance in a given environment should increase fitness in that same environment if adaptive (Wilson & Franklin, 2002). However, multiple tests of this hypothesis in insects thermal acclimation has failed to detect increased fitness in individuals demonstrating beneficial thermal acclimations (Hoffmann, 1995; Huey et al., 1996; Huey et al., 1999; Leroi et al., 2004). Thus, our results seem to confirm that increased fitness for a given environment in populations demonstrating beneficial acclimations (i.e. increased performance) are empirically difficult to demonstrate.

Assays that were performed on experimental populations monitored whole animal phenotypes, an approach that allows an integration of the various physiological systems that impart tolerance. This approach was proposed to study the physiological effects of low PO_2 rearing (Farahani & Haddad, 2003). However, while examining whole animal phenotypes is well suited to characterizing divergence in performance related traits between evolving/evolved populations, it does not readily provide a mechanistic basis to explain the evolved changes observed. Thus, while the hypoxia-evolved populations showed an increased tolerance to acute anoxia, using whole animal measurements did not allow for in depth analysis of the mechanistic basis for the increased performance. The one exception was the measurement of CS activity; the increase in CS activity in the flies evolving under hypoxic

conditions suggests an increase in mitochondrial density and thus offers a potential mechanism by which hypoxia-evolved organisms could tolerate such environments. However, with these data it is possible to postulate the mechanistic basis for evolved increase in anoxia tolerance in hypoxia-evolved populations.

The literature presents great support for metabolic scaling with mass at all hierarchical levels, with smaller organisms possessing larger mass specific metabolic rates (Damouth, 1981; Whitfield, 2004; Suarez et al., 2004). However, these populations display increased CS activity rates despite unchanged mass compared to controls and it is therefore unlikely that differences in CS activity levels could have been due to size differences alone.

An obvious challenge for organisms inhabiting perpetually hypoxic environments is maintaining levels of oxidative metabolism to fuel processes above those used to satisfy resting metabolic rate (i.e. aerobic scope). Maintenance of aerobic scope and performance was proposed as a mechanism to overcome the challenges related to hypoxia (Hochachka et al., 1983). It was proposed that aerobic scope could be maintained via increased O₂ flux through the electron transport chain (ETC), thereby increasing the rate of ATP production. Thus, it is proposed that the hypoxia-evolved populations have evolved such a mechanism to deal with low environmental PO₂. First, the increase in CS activity (and presumed elevation of mitochondrial density) is consistent with increased O₂ flux capacity and rates of ATP generation. Furthermore, an increased capacity for O₂ flux could be important in maintaining and re-establishing ATP homeostasis after anoxic insult, resulting in increased performance at low PO₂ and reduced recovery time from anoxia. Although this study provides no direct evidence for an increase in O₂ delivery to the tissues, prior studies suggest that the tracheal system has the potential to increase O₂ delivery at low ambient PO₂, via increased tracheal ramification and internal volumes when flies are reared in hypoxia (Henry & Harrison, 2004). As yet, there is no empirical evidence from evolution

experiments supporting this idea of increased tracheal ramification and volume, as our hypoxia-evolved lines showed no increase in recovery from anoxia and indeed data collected from hypoxia-evolved *D. melanogaster* showed that maximal conductance of O₂ was not altered by multiple generations of rearing under low PO₂ conditions (Klok et al., 2010). However, during acute periods of anoxia, increased tracheal volume could increase internal O₂ stores (Bradley et al., 2009), thereby permitting greater performance at low PO₂, improving anoxia tolerance. Therefore I suggest that increased anoxia tolerance still provides indirect evidence supporting increased tracheal branching as an evolved trait in hypoxia-evolved populations. Furthermore, I postulate that elevated mitochondrial density enables the hypoxia-evolved populations to achieve higher rates of O₂ flux through the ETC, which is delivered at an increased rate owing to alterations in tracheal morphology (i.e. increased tracheal ramification and tracheal volume), ultimately leading to the improved performance.

Data suggest that in addition to physiological adaptation to hypoxia, there are more complex behavioural components (i.e. activity) that contribute to hypoxia tolerance at the whole animal level. Increased CS activity does not induce a correlated increase in RMR. This study did not control for activity levels when measure metabolic rates, as done in previous studies (Klok et al., 2010). Therefore, it is proposed that activity levels of the flies could account for the discrepancy between RMR and mitochondrial CS activity. In accordance with the observed mitochondrial activity and RMR data, I propose that the hypoxia-evolved populations evolved lower activity levels, which would resemble control RMR levels when measured in normoxia. This hypothesis is consistent with previous work that found that multiple generations of altered aPO₂ did not have an effect on CO₂ emission rates in *D. melanogaster* (Klok et al., 2010).

Previous studies have shown that a reduction in wet weight is an environmentally induced developmental consequence of hypoxia (Frazier et al., 2001; Zhou et al., 2007; Klok et al., 2009).

However, in the present study, there was no change in wet weight in the hypoxic flies after a single-generation of hypoxic rearing. The difference between the present results and those of previous studies may have resulted from the different genetic backgrounds of the stocks used. In my study, flies reared for multiple generations in hypoxia also showed no change in wet weight, which agrees with previous work by the same research group (Klok et al., 2009).

Adaptation to hyperoxia

In contrast to the results for the hypoxia-evolved populations, direct evidence of increased performance of the hyperoxia-evolved populations in a hyperoxic environment is lacking. Hyperoxia-evolved flies also showed no increase in productivity or longevity relative to the other treatments when assayed in the hyperoxic environment. Nonetheless, a consistent reduction in performance (i.e. anoxia tolerance) of all four replicate hyperoxia-evolved populations under anoxic conditions was observed. Such decreased performance could have arisen in two ways. First, it may have resulted from a general reduction in fitness caused by inbreeding depression (Keller & Waller, 2002). Indeed, the hyperoxia-evolved lines exhibited an overall decrease in productivity that was independent of rearing environment, consistent with this hypothesis. The hyperoxic environment also decreased productivity to a greater extent than the hypoxic environment, meaning effective population sizes could have been reduced to a greater extent in this treatment both by strong selection and reduced census sizes, thereby increasing inbreeding. However, decreased performance of the hyperoxia-evolved populations was not detected in the performance measures conducted in other environments (i.e. hyperoxic and normoxic). In addition, census population sizes remained quite large throughout the experiment ($N > 1000$). This suggests the second possible explanation that decreased anoxia tolerance is an evolutionary cost (i.e. side-effect) of adaptation to hyperoxia. Although the hyperoxic performance assay failed to detect any

difference in climbing ability after acute hyperoxic exposure, despite clear evidence that 100% O₂ exposure reduces climbing ability (Fig. 4), this assay may have overwhelmed antioxidant defences. Alternatively, equal reduction in post-hyperoxia climbing ability would be achieved if 100% O₂ triggered the hyperoxic-switch (i.e. maximal closing spiracle opening) equally among all treatments, leading to equal levels of oxidative damage incurred in all treatments. Climbing ability was also highly variable within and among populations, resulting in large standard errors and making it difficult to detect treatments differences given limited replicates (n=4). In addition, as mentioned above the fitness assay might not have captured the relevant components underlying adaptation to hyperoxia.

In addition to having evolved reduced anoxia tolerance, hyperoxia-evolved populations evolved reduced rates of water loss while possessing similar RMR, suggesting that cuticular and/or respiratory water loss was diminished in these populations. However, this reduced rate of water loss did not translate into increased desiccation tolerance in the hyperoxia-evolved treatment. There exist two possible explanations for this discrepancy between rates of water loss and desiccation tolerance. First, subtle differences in the rate of water loss might not be distinguishable by survival in a severe desiccation environment (0% RH). Second, rates of water loss were measured 14 generations after the desiccation assay, thus it is possible that the decreased rate of water loss evolved after desiccation tolerance was measured.

High aPO₂ has been reported to modify traits; traditionally, elevated ambient PO₂ is thought to have contributed to insect gigantism, increasing ROS production leading to oxidative stress and modulating the open diameter of spiracles (Klok et al., 2009; Orr & Sohal, 1994; Wickens, 2001; Lighton et al., 2004). However, the novelty of the present data is that they reveal a decreased performance in anoxia of flies reared for a single or multiple generations in hyperoxia. Rearing in hyperoxia has been shown to decrease tracheal ramification and tracheal volume (Henry & Harrison,

2004), which could affect O₂ delivery or reserves and result in reduced hypoxia tolerance and increased recovery times in these evolved populations. Additionally, high environmental PO₂ is known to reduce the open diameter of spiracles in *D. melanogaster* (Lighton et al., 2004). Thus, respiratory water loss can be minimized by reducing the diameter of spiracle openings in the presence of high PO₂, which is thought to be the case in the hyperoxia-evolved populations. This phenomenon, termed the hyperoxic-switch, reconfirms the clear link between ambient PO₂, oxidative damage and rate of respiratory water loss as previously demonstrated (Lighton et al., 2004). Additionally, there is a link between cuticular hydrocarbons (CHCs) and water loss (Gibbs et al., 1997), but it has been suggested to account for a relatively small proportion of whole animal water movement (Gibbs et al., 2003). Therefore, it is proposed that hyperoxia-evolved populations have evolved a reduced rate of water loss because of an evolved reduction of the mean diameter of spiracle openings or an increased constriction of the lips governing spiracle opening, congruent with the hyperoxic-switch. Thus, these data would seem to provide an indirect example whereby populations could have evolved modified spiracle constriction, possibly leading to altered breathing patterns, as seen in DGE.

Contrary to previous findings (Frazier et al., 2001), hyperoxia-reared populations did not exhibit altered wet weight after one generation of exposure. This is not surprising considering that hyperoxia has a relatively small plastic effect on body size and hyperoxia reared flies did not exhibit changes in wet weight when reared in vials, as is the case here (Klok & Harrison, 2009; Harrison et al., 2010). Flies reared for multiple generations in hyperoxia also showed no change in wet weight, which agrees with previous work done by the same group (Klok et al., 2009).

Future studies

These experiments have provided many interesting results that should be followed with future experiments. For instance, we have found evidence that hypoxia-evolved populations have potentially

increased metabolic scope by increasing mitochondrial density in order to maintain performance level in hypoxia. However, increased metabolic scope would also necessarily entail a comparative increase in O₂ delivery in order to provide added flux to through the ETC to increase ATP generation in hypoxia. I have also suggested that increased tracheal branching could be occurring in the hypoxia-evolved populations, which could provide increased O₂ delivery. Thus, tracheal morphology in hypoxia-evolved adult *D. melanogaster* should be investigated to see if they compliment the increased mitochondrial density found in these populations and support the hypothesis of increased metabolic scope in these populations. In addition, the maximal activity rates of other mitochondrial enzymes should be investigated to see if they support added flux through the ETC.

Water present in the tracheal system can strongly influence gas diffusion with fluctuations in thickness in trachea and tracheoles. However, this parameter of tracheal water volume has never been examined in populations acclimatized to or adapted to hypoxia or hyperoxia. Thus, efforts should be made to experimentally determine if insect use tracheal water volume to influence O₂ diffusion in hypoxia and hyperoxia.

Hyperoxia-evolved populations possibly utilize the *hyperoxic-switch* to help reduce oxidative damage occurring in high aPO₂ environments. However, no direct measurement of oxidative damage or antioxidant capacity was performed in these populations. These biological parameters can be quantified by examining protein carbonyl levels and the reduction capacity of tissues.

CONCLUSION

This study has demonstrated adaptations in hypoxia evolved *D. melanogaster*. While clear increases in performance in the hypoxia evolved populations were observed, coupled with increased CS activity levels, direct evidence of adaptation to hyperoxia was lacking. However, it was observed that hyperoxia-evolved populations incurred a cost in terms of their performance in a hypoxic environment. No conclusive evidence could be provided as to which fitness component was responsible for maintaining these adaptive traits in the experimental populations. This study finds indirect experimental evidence to support the oxidative damage hypothesis, via the hyperoxic-switch as a method to reduce oxidative damage. Additionally, these results do not appear to support the BAH. Future experiments should focus on providing a clear demonstration of increased performance by the hyperoxia evolved populations in hyperoxic conditions, examining the activity levels of flies from all experimental treatments, as well as attempting to identify the fitness components that maintain the adaptive traits observed in the experimental populations.

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