Physiological and Genetic Mechanisms Underlying Variation in Anoxia Tolerance in *Drosophila Melanogaster*

by

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A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Approved May 2018 by the
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August 2018

ABSTRACT

The ability to tolerate bouts of oxygen deprivation varies tremendously across the animal kingdom. Adult humans from different regions show large variation in tolerance to hypoxia; additionally, it is widely known that neonatal mammals are much more tolerant to anoxia than their adult counterparts, including in humans. *Drosophila melanogaster* are very anoxia-tolerant relative to mammals, with adults able to survive 12 h of anoxia, and represent a well-suited model for studying anoxia tolerance. *Drosophila* live in rotting, fermenting media and a result are more likely to experience environmental hypoxia*;* therefore, they could be expected to be more tolerant of anoxia than adults. However, adults have the capacity to survive anoxic exposure times ~8 times longer than larvae. This dissertation focuses on understanding the mechanisms responsible for variation in survival from anoxic exposure in the genetic model organism, *Drosophila melanogaster*, focused in particular on effects of developmental stage (larval vs. adults) and within-population variation among individuals.

Vertebrate studies suggest that surviving anoxia requires the maintenance of ATP despite the loss of aerobic metabolism in a manner that prevents a disruption of ionic homeostasis. Instead, the abilities to maintain a hypometabolic state with low ATP and tolerate large disturbances in ionic status appear to contribute to the higher anoxia tolerance of adults. Furthermore, metabolomics experiments support this notion by showing that larvae had higher metabolic rates during the initial 30 min of anoxia and that protective metabolites were upregulated in adults but not larvae. Lastly, I investigated the genetic variation in anoxia tolerance using a genome wide association study (GWAS) to identify target genes associated with anoxia tolerance. Results from the

GWAS also suggest mechanisms related to protection from ionic and oxidative stress, in addition to a protective role for immune function.

ACKNOWLEDGMENTS

I would first like to thank my advisor, Jon Harrison, for his endless dedication, support and patience toward my graduate and future goals. We have spent what seems like an infinite amount of time together working on manuscripts, grant applications, and attending conferences, and I am eternally grateful for everything he has taught me. As my mentor, he has taught me more than I could ever give him credit for here.

I am grateful for the assistance of numerous undergraduate and graduate students. So, a huge thank you goes out to James Sargent, Paula Overby, Simon Werkhoven, Trevor Fox, Teddy Cogley, Alex Brantley, Hunter Smith for all of their time and effort assisting with rearing flies and collecting data.

Nobody has been more supportive of my endeavors than my family, and for this I am truly grateful. Most importantly, I would like to thank my loving wife, Leslie, for giving me continuous inspiration and never complaining about my long hours.

This research would not have been possible without the financial and technical support from various internal sources: the ASU School of Life Sciences, ASU Graduate College, the ASU Magnetic Resonance Resource Center. I would also like to thank the Company of Biologists for funding a travel award that allowed me to learn a new set of skills and collaborate on a project with Dr. Johannes Overgaard at Aarhus University in Denmark; a special thanks goes to Johannes for allowing me to visit and giving me space in his lab for a month-long research visit.

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THE BROAD IMPORTANCE OF OXYGEN AND THE PERILS OF ANOXIA: A REVIEW

INTRODUCTION

Oxygen is required for animals to synthesize ATP through the use of oxidative phosphorylation via the electron transport chain. In the absence of O_2, ATP supply can become limiting due to the reliance on anaerobic pathways and reduced levels of ATP can inhibit many behavioral and physiological functions such as locomotion, feeding, and growth (Gorr et al., 2010; Nilsson, 2010; Riedel et al., 2014). Additionally, decreasing ATP levels can lead to a disruption of pathways responsible for cellular homeostasis such as ion-motive ATPases, leading eventually to membrane depolarization and cell death.

As most research has involved model organisms and their translational potential for human health, research on anoxia has mostly focused on ischemia and reperfusion injury. Ischemia is a restriction in blood supply that results in the deprivation of O_2, metabolites, plasma ions and proteins in the affected tissues. Functional hypoxia is defined here as reduced levels of tissue O_2 relative to normal; anoxia is defined as the absence of O_2. When ischemia causes functional hypoxia, the decrease in circulation simultaneously reduces delivery of nutrients and removal of toxic end products. It is important to note that most research is this area has focused on the O_2 component and studies have shown that the cytoprotective mechanisms underlying hypoxia tolerance also provide tolerance against the full scope of ischemia in heart and brain tissue of various species (Dave et al., 2006; Kesaraju and Milton, 2009; Pamenter et al., 2012).

Additionally, reoxygenation occurs during recovery from an anoxic bout; reperfusion is the restoration of blood supply and/or O_2 to ischemic tissue. Reperfusion itself has consequences associated with increased inflammation and/or oxidative stress (Murphy and Steenbergen, 2008a). Ischemia and reperfusion injury are considered the primary causes of cell death in multiple pathologies, including acute myocardial infarction (Opie, 1991) and stroke (Bickler, 2004). Despite years of research dedicated to the treatment and prevention of ischemic/reperfusion pathologies, the full pathological scope has proven to be complex and elusive. Therefore, understanding the mechanisms and molecular pathways intrinsic to hypoxia/anoxia-tolerant animals is pertinent to the discovery of new therapeutic agents for the prevention and/or treatment of oxygen-mediated human disease pathologies.

OXYGEN AND METABOLISM DURING NORMOXIA, HYPOXIA AND ANOXIA

O_2 is essential for cellular respiration and ~90% of O_2 is used by mitochondria in producing ATP via oxidative phosphorylation (Rolfe and Brown, 1997). During oxidative phosphorylation, electrons from substrate oxidation are donated to the electron transport chain (ETC) and follow the gradient down the ETC to the final electron acceptor, O_2. Protons are pumped by the mitochondrial complexes—I, III, and IV—from the matrix to the inner membrane space, resulting in the generation of a transmembrane electrochemical gradient. This electrochemical gradient drives the transport of protons through complex V (the ATP synthase, or F_1F_0-ATPase), generating ATP from ADP and P_i (Papa et al., 2012).

The reduction of O_2 can involve potentially hazardous by-products (Galli and Richards, 2014). Although most O_2 is normally reduced to water, a small fraction of O_2 is incompletely reduced producing reactive oxygen species (ROS), particularly superoxide (O_2^-) and hydrogen peroxide (H_2O_2). During oxidative phosphorylation, leakage of electrons at complexes I and III from the ETC leads to partial reduction of O_2 to form O_2^-, which is then subsequently dismutated to H_2O_2 in the intermembrane space (Murphy, 2009). The superoxide radical is generally thought to not be directly responsible for the toxic effects of oxygen, but it can lead to the production of other highly toxic, reactive species (Buechter, 1988). ROS are continuously produced and eliminated under normal aerobic conditions and moderate ROS production is considered necessary for a variety of regulatory pathways (Dickinson and Chang, 2011; Pamenter, 2014). ROS act as important secondary messengers that regulate numerous intra- and extra-cellular pathways; they can modify target proteins via the oxidation of –thiol groups that result in an alteration of protein structure and function (Chandel and Schumacker, 2000). Mitochondria are the primary producer of ROS, however there are other mechanisms by which ROS are endogenously produced outside the mitochondria. For example, NADPH oxidases are present in various tissues and are thought to play important roles in oxygen sensing and host defense mechanisms (Orient et al., 2007); NADPH oxidases generate O_2^- by transferring electrons from NADPH inside the cell across the membrane coupled with O_2. Mitochondrial ROS production can be increased either by excessive or reduced oxygen levels (Chandel and Budinger, 2007). Since mitochondrial damage primarily occurs during oxygen deprivation or overload (Chandel and Budinger, 2007), the

increased ROS production following oxygen damage to the ETC causes complexes I and III of the ETC to become the major sources of ROS production (Chen et al., 2008).

One hypothesis regarding the hypoxic-mediated response of hypoxia-inducible factor (HIF) is that increases in ROS production during hypoxia prevents HIF from being degraded (Chandel et al., 1998)—the prevention of HIF degradation is required for transcription of various hypoxia-related genes (Semenza et al., 2006). Thus, this mechanism suggests that increased ROS production during hypoxia serves as one of the triggers for protective compensatory pathways that minimize pathologies associated with tissue hypoxia. However, excessive production of ROS that overcomes the buffering capacity of antioxidant defenses such as superoxide dismutase and glutathione, can lead to deleterious effects on cells and organelles (Galli and Richards, 2014; Pamenter, 2014). For example, elevated ROS levels can react with unsaturated fatty acids in membranes producing a variety of short chain alcohols, aldehydes, and alkanes (Behn et al., 2007). ROS can oxidize side chains of proline, arginine, lysine and threonine within proteins, producing carbonyls, and ROS can oxidize nucleic acids, particularly guanine (Cooke et al., 2003; Dickinson and Chang, 2011). Protein oxidation occurs very early in oxidative stress caused by oxygen deprivation, preceding cellular ATP depletion and subsequent cell death (Stadtman and Levine, 2000). Nucleic acid oxidation can alter gene expression patterns and cause telomere shortening (Evans and Cooke, 2004). Importantly, not only is the mitochondrion a primary producer of ROS, but it is also a primary target of ROS damage (Murphy and Steenbergen, 2008b). ROS-mediated lipid peroxidation of membranes may alter the fluidity of cellular membranes and their associated transport functions (Binkova et al., 1990; Wong-Ekkabut et al., 2007), and damage to

mitochondrial proteins and nucleic acids can have a large impact on the assembly and/or the function of the respiratory chain (Cui et al., 2012).

During oxygen deprivation, oxidative ATP production becomes limited eliciting a cascade of events termed the "hypoxic response" (Guillemin and Krasnow, 1997; Poellinger and Johnson, 2004). Elements of the mammalian hypoxic response include physiological responses to increase the capacity to deliver oxygen to the tissues—e.g. increased ventilation; physiological and/or behavioral responses to reduce oxygen need—e.g. suppression of protein synthesis and reduced locomotion; and the initiation of developmental changes that enhance oxygen delivery—e.g. angiogenesis.

Organisms exposed to hypoxia can decrease ATP turnover and/or switch to anaerobic ATP production (Staples and Buck, 2009); in some cases, anaerobic ATP production can completely replace aerobic ATP production (e.g. during sprinting in humans, and in highly hypoxia-tolerant organisms). In anoxia, aerobic ATP production stops completely, and all ATP must be produced anaerobically. Some organisms can do so at a relatively high rate and they also can perform essential functions such as locomotion and feeding at very low rates of ATP utilization—i.e. ghost shrimp (Holman, 2006) and crucian carp at or near 0˚C (Nilsson, 2001); but mammals and most vertebrates cannot produce ATP at sufficient rates to support normal functions and strong inhibition of functions such as locomotion and growth are seen (Nilsson, 2010).

Many anoxia-tolerant animals have particularly high capacities for anaerobic ATP production (Gorr et al., 2010; Müller et al., 2012). Some anoxia-tolerant animals possess the ability to use anaerobic pathways other than lactate production that may have additional benefits for ATP generation, pH maintenance, redox potential (i.e.

NAD^+/NADH) and metabolic intermediates (Murphy and Steenbergen, 2007). In vertebrates, the primary anaerobic pathway is the conversion of glycogen to lactate (Bickler and Buck, 2007), with one unusual example of the crucian carp producing ethanol as an end product (Johnston and Bernard, 1983). In contrast, terrestrial insects are known to produce a wide array of products in hypoxia—lactate, alanine, sorbitol, succinate, glycerol, α-glycerol-3-phosphate, pyruvic acid and fatty acids (Hoback and Stanley, 2001). For example, some species of beetles produce equal amounts of lactate and alanine, and larval dipterans can utilize glycogen and amino acids to produce lactate, alanine, succinate, and acetate (Feala et al., 2007; Gäde, 1985; Hoback et al., 2000).

PHYSIOLOGICAL CONSEQUENCES OF ANOXIA

Although the exact mechanisms and causes of death in anoxia are unclear, the current knowledge points to multiple trends that occur during oxygen deprivation (Figure 1.1). During periods of environmental hypoxia or anoxia, gas exchange and oxidative phosphorylation become limited and oxidative ATP supply cannot meet demand (Bickler and Buck, 2007; Hochachka et al., 1996). As such, an organism must coordinate cellular function to increase supply through anaerobic pathways and/or reduce metabolic demand (Boutilier and St-Pierre, 2000; Hochachka, 1986; Storey and Storey, 1990). Anaerobic respiration generally yields $<1/10^{th}$ of the ATP produced per glucose by oxidative pathways (Hochachka and Somero, 2002). The reduced rate of ATP production coupled with depletion of fermentable substrates and accumulation of deleterious end-products may lead to limits on anaerobic ATP production (Boutilier, 2001). If ATP supply fails to match ATP demand, ATP levels fall during anoxia/hypoxia, with a variety of deleterious

consequences. Additionally, when organisms recover from an anoxic/hypoxic episode, reoxygenation can elicit lipid peroxidation, protein oxidation, and DNA damage due to increased ROS production (Hermes-Lima and Zenteno-Savín, 2002).

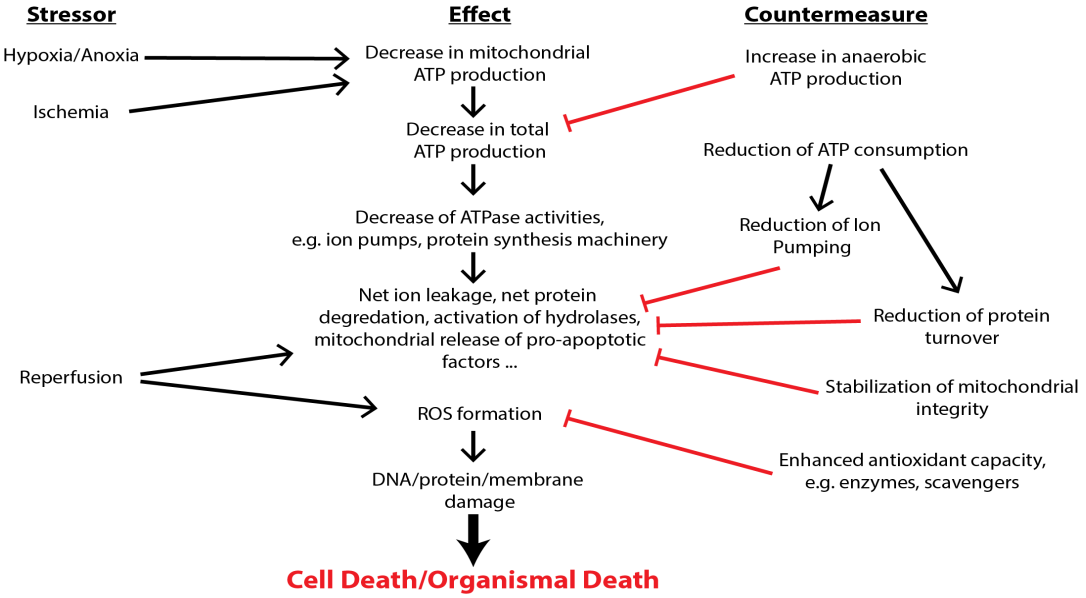

Figure 1.1: Series of events that transpire during cellular stress from oxygen deprivation and the physiological mechanisms used as countermeasures to maintain cellular homeostasis and prevent cell death. Black arrows indicate a positive relationship or activation while red lines represent an inhibition or negative regulation. Adapted from (Van Breukelen et al., 2010).

ATP depletion

As oxygen becomes limiting, mismatched ATP supply and demand can lead to a rapid depletion of ATP, initiating a cascade of events ultimately ending in cell death (Figure 1.1). Cell damage in anoxia is thought to be due to decrease in pH, altered calcium homeostasis, increased intracellular osmotic pressure, and/or mitochondrial damage all related directly or indirectly to decreased ATP levels (Hochachka and Somero, 2002). For example, changes in redox condition, pH, and ionic and osmotic balance are characteristic of anoxic conditions and can lead to inhibition or unfolding of

proteins (Krivoruchko and Storey, 2013); unfolded proteins aggregate causing cell membrane damage (Giffard et al., 2004). Depletion of high-energy phosphates leads to a failure of ion-motive ATPases; consequently, failure of Na^+/K^+- and Ca^{2+}-ATPases leads to reduced ion gradients that allows ions to readily diffuse through channels (Galli and Richards, 2014; Storey, 2007). Increased membrane permeability is followed by depolarization of membranes and unregulated influx of calcium through voltage-gated Ca^{2+} channels (Boutilier, 2001). The rise in free cytosolic intracellular calcium results in the activation of Ca^{2+}-dependent phospholipases and proteases that further accelerate the rate of membrane depolarization (Hochachka, 1986). The release of cytochrome *c* into the cytosol occurs under various cellular stress signals, including elevated Ca^{2+}, and is known to occur during oxygen deprivation (Zemke et al., 2004). As a result, cytochrome *c* binds to pro-apoptotic protein complexes initiating cell death (Jiang and Wang, 2004).

Reperfusion

Reperfusion, or the sudden reoxygenation of tissues after oxygen deprivation, is required for the survival of an aerobic organism after exposure to anoxia. During reperfusion, the sudden influx of oxygen cannot be metabolized fast enough by the previously inactive oxidative pathways (Ambrosio et al., 1987), leading to a sudden burst of ROS production. Additionally, the reduced mitochondrial redox state of oxygen-limited cells can also produce ROS during hypoxia that can amplify the cellular damage observed during reperfusion (Vanden Hoek et al., 1997). As noted above, ROS can damage lipids, proteins, and DNA, and lead to activation of cell death pathways (Galli and Richards, 2014). As of yet, we do not know the relative importance of oxygen

deprivation vs. reperfusion damage in causing pathologies due to anoxia (Penna et al., 2013).

OXYGEN SIGNALING PATHWAYS

In addition to the direct effects of O_2 deprivation on ATP and total adenylate levels, there are other signaling pathways responsible for regulating the response to oxygen deprivation. These include AMP kinases (AMPK), hypoxia-inducible factor (HIF), and cyclic GMP. AMPK is a dynamic regulator of energy homeostasis, which becomes activated by metabolic stressors that reduce ATP supply relative to demand (Hardie, 2011). AMPK is activated by increased ADP: ATP and AMP: ATP ratios via covalent modification. AMPK acts in a dual manner to up-regulate catabolic processes that generate ATP (e.g. glucose uptake, glycolysis, fatty acid uptake/oxidation) and down-regulate anabolic pathways that consume ATP (e.g. inhibiting fatty acid, protein and glycogen synthesis) (Carling, 2004). AMPK is best known for its role in regulating metabolism; however, it is clear that it regulates almost all aspects of cellular function including autophagy, maintenance of mitochondrial homeostasis, cell polarity, and cell growth and proliferation.

HIFs are heterodimers consisting of an O_2-labile α subunit and a stable β subunit. Under normoxic conditions, HIFα subunits are hydroxylated by prolyl hydroxylase domain-containing enzymes (PHDs) and marked for destruction by the von Hippel-Lindau protein complex (pVHL); however, during hypoxia, PHD activity is reduced and the accumulated HIFα proteins translocate into the nucleus where they bind to hypoxia response elements that can up- or down-regulate many genes (Majmundar et al., 2010).

HIF is involved with many aspects of cellular function; during hypoxic conditions, these include the reduction of cellular growth of somatic tissue and by indirectly reducing protein synthesis by inhibiting mitochondrial ATP production (Kaelin and Ratcliffe, 2008; Semenza, 2001).

The intracellular messenger, cGMP, is synthesized by guanylyl cyclases, which are heme-containing proteins that exist in two forms—membrane-bound or soluble. Soluble guanylyl cyclases are classically activated by nitric oxide (NO); however, mammals, *C. elegans*, and *Drosophila* have been shown to have atypical guanylyl cyclases that are activated by hypoxia, producing cGMP that may control neural sensation of hypoxia (Vermehren et al., 2006) (Vermehren et al., 2006). Similarly, nitric oxide-sensitive guanylyl cyclases have been shown to mediate behavioral responses (i.e. escape behavior) to oxygen deprivation in *Drosophila* (Wingrove and O'Farrell, 1999) (Wingrove and O'Farrell, 1999).

ANOXIA-TOLERANT VS. ANOXIA-SENSITIVE ANIMAL SPECIES

Matching ATP supply and demand.

The sequence of events leading to cell death is the same in anoxia-tolerant and anoxia-sensitive animals, with the difference being the time course over which events occur (Boutilier and St-Pierre, 2000). Two prevailing signatures of oxygen deprivation in anoxia-sensitive species are the rapid depletion of ATP followed by substantial loss of intracellular K^+ as the Na^+/K^+ pump begins to fail (Hansen, 1985; Krnjevic, 1993). In contrast, anoxia-tolerant species respond to similar levels of oxygen deprivation by protecting cellular ATP, reducing passive membrane K^+ efflux, and stabilizing membrane

electrochemical potential (Buck and Hochachka, 1993; Buck et al., 1993; Chih et al., 1989; Donohoe et al., 2000; Krumschnabel et al., 1997; Lutz et al., 1984; Nilsson, 2010). Currently, it appears that cell survival during anoxia is not related to a superior ability of anoxia-tolerant organisms to withstand ionic disruptions or sustained energy imbalances (Boutilier, 2001); instead, the capacity to survive anoxia seems to be related to the ability to initiate mechanisms to suppress ATP demand in a manner that preserves the ionic integrity of cell membranes (Boutilier and St-Pierre, 2000). Current theory suggests that the main factor explaining differences in anoxia tolerance among animals is the ability to match ATP supply and demand. However, in theory, animals might also differ in their capacities to tolerate the downstream effects of ATP depletion (e.g. membrane depolarization, cellular swelling, protein aggregation). Figure 1.2 shows a theoretical cascade of events that follow a mismatch of ATP supply and demand due to anoxia; it is important to note that this figure focuses on ion homeostasis disruption as a contributor to cell death but does not include all possible mechanisms.

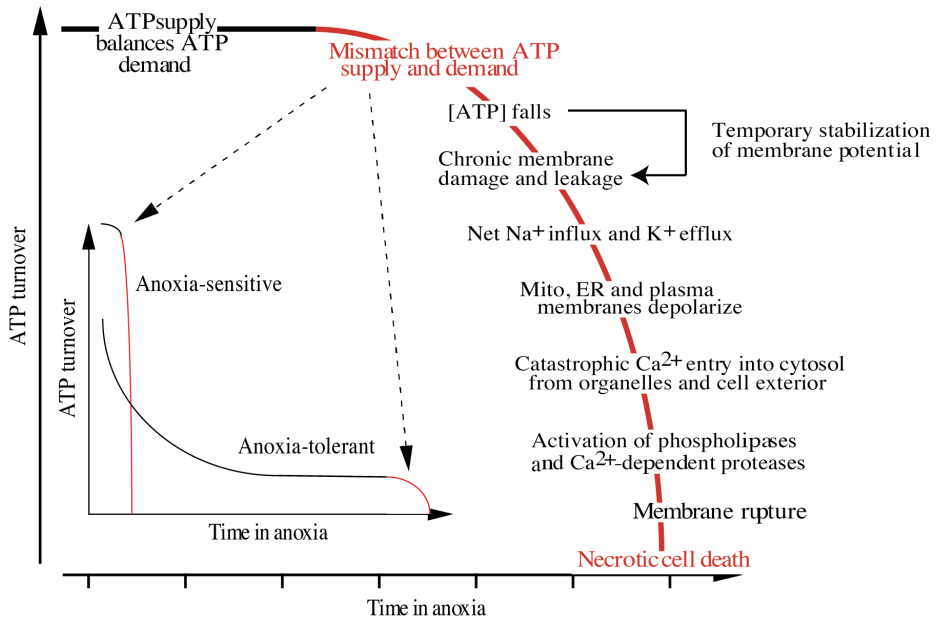

Figure 1.2: ATP turnover rates of cells as a function of time in anoxia. The figure shows a theoretical timeline of events that occur as a result of mismatched ATP supply and demand. The inset demonstrates differences in the initiation of the timeline between anoxia-tolerant and anoxia-sensitive animals. Adapted from Boutilier (2001).

Metabolic depression

Anoxia almost always causes metabolic depression. Metabolic rate decreases rapidly in response to anoxia, sometimes to as low as 10% of resting metabolic rate (Storey and Storey, 1990). Short-term responses to oxygen-deprivation often cause increases in ventilation (Barros et al., 2004; Harrison et al., 2006) and behavioral responses such as escape (Wingrove and O'Farrell, 1999; Wood, 1991). However, some animals exhibit the ability to function for short periods of anoxia without stopping locomotory behavior (Nilsson, 2001), while others transition quickly to a comatose-like state and remain paralyzed until oxygen returns (Nilsson and Lutz, 2004). In the long term, many organisms possess the ability to compensate for bouts of anoxia by altering

their morphology and biochemistry to enhance their chances of survival (Centanin et al., 2010; Majmundar et al., 2010).

At the cellular level, protein synthesis and ion-motive ATPases account for more than 90% of oxidatively coupled ATP consumption of resting rat muscle (Rolfe and Brown, 1997) and up to 66% of ATP turnover in rat thymocytes (Buttgereit and Brand, 1995). As such, decreases in ATP demand of these process are thought to be largely responsible for the observed metabolic depression during oxygen deprivation (Hand and Hardewig, 1996; Hochachka et al., 1996). As the largest consumers of ATP during normoxic conditions, protein synthesis and ion-motive ATPases represent the primary targets for metabolic depression under oxygen-limited conditions (Boutilier, 2001). Vertebrate studies confirm this notion by showing protein synthesis is largely inhibited during anoxia (Buc-Calderon et al., 1993; Land and Hochachka, 1994; Land and Hochachka, 1995). Another theory is that the reduction of the most energy-demanding processes will allow for the spared ATP to be reallocated to more critical functions, interpreted as a "hierarchy" with processes most important to cell survival being able to continue even at critically low levels of ATP (Buttgereit and Brand, 1995).

Of these essential processes, ion-motive ATPases have been implicated as the most important process for energy conservation in response to oxygen deprivation (Bickler and Buck, 2007; Hochachka et al., 1996). The maintenance of intracellular homeostasis requires the utilization of ATP-dependent pumps to redistribute ions moving through channels across membranes towards their electrochemical gradient. Na^+/K^+-ATPases aid in the maintenance of membrane potential, transport and cellular volume by exchanging 3 Na^+ ions out and 2 K^+ ions into the cell, facilitated by ATP hydrolysis, to

maintain low intracellular Na^+ and high K^+ intracellular concentrations. There is a large transmembrane electrochemical gradient of Ca^{2+} that drives calcium into the cell, and the Ca^{2+}-ATPase is required to actively pump Ca^{2+} out of the cell to maintain low intracellular concentrations needed for proper cell signaling. Additionally, plasma membrane V-ATPases use ATP hydrolysis to transport protons across cell membranes and is necessary for maintaining proper intracellular pH and aiding in secondary active transport. Active transmembrane ion transport has been shown to constitute as much as 20-80% of a cell's resting metabolic rate (Buck and Hochachka, 1993), and the rapid depletion of ATP during oxygen deprivation leads to failure of these ATP-dependent pumps, loss of ion gradients across membranes, and cell death in anoxia-sensitive animals. One critical difference between anoxia-tolerant and anoxia-sensitive animals is that the latter show no decrease in the ATP demand of ion-motive ATPases in response to anoxia; in contrast, anoxia-tolerant animals have been shown to exhibit large scale reductions in Na^+/K^+-ATPase activity without disruptions to electrochemical potentials, cellular ion levels or ATP concentrations in response to anoxia (Bickler and Buck, 1998; Lutz and Nilsson, 1997; Perez-Pinzon et al., 1992). This widespread defense strategy used by hypoxia/anoxia-tolerant animals to depress metabolic rate is referred to as "channel arrest" (Hochachka, 1986). A decrease in membrane ion permeability in anoxia-tolerant cells is seen as part of a coordinated process to conserve energy, wherein the net result is that cell membrane permeabilities are reduced (Buck and Hochachka, 1993). A reduced cell membrane permeability means less energetic costs of maintaining transmembrane ion gradients (Hochachka et al., 1996). Figure 1.3 shows a theoretical timeline of events that occur in the anoxia-tolerant turtle, *Chrysemys picta* that allow for

the metabolic depression necessary for cell survival during anoxia. In contrast, anoxia-sensitive organisms elicit less of a decrease in total energy demand of ATPases in response to oxygen deprivation. Anoxic-mammal cells show a more non-adaptive response corresponding to increased channel leak; for example, increases in amiloride-sensitive Na^+/H^+ and Na^+/Ca^{2+} exchange are observed in hypoxic mammal nerve cells (Haddad and Jiang, 1997) that leads to an increase in Na^+ cycling. This in turn, raises the ATP demand of ion-balancing ATPases to a critical point, leading to deleterious Ca^{2+} overload and ultimately cell death (Hammarstrom and Gage, 1998).

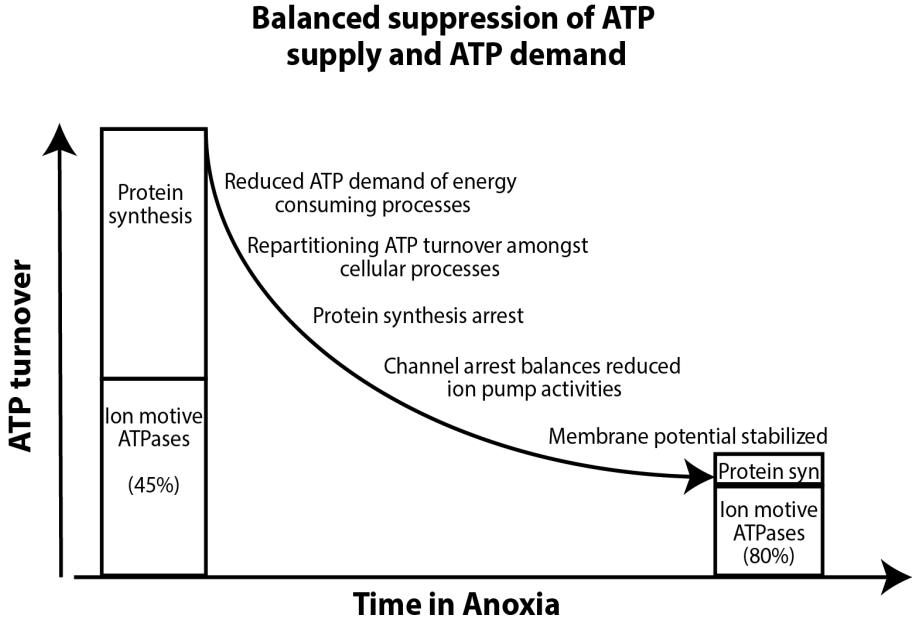

Figure 1.3: ATP turnover rates of cells as a function of time in anoxia. Bars show the relative contribution that protein synthesis and ion-motive ATPases make to total ATP turnover in normoxia and after a prolonged period of O_2 lack. Adapted from Boutilier and St-Pierre (2000).

Upregulation of anaerobic pathways

In anoxia, all ATP must be produced anaerobically (Bickler and Buck, 2007). Anoxia-tolerant organisms tend to be particularly capable of increasing anaerobic ATP

production to compensate for the decrease in aerobic metabolism during hypoxia/anoxia (Hochachka and Lutz, 2001)—this switch in metabolic pathways is often termed the "Pasteur Effect" (Pasteur, 1861). One direct mediator of anaerobic pathways is AMPK; this aids in the upregulation of ATP generating pathways by increasing glucose uptake, glycolysis, and fatty acid uptake and oxidation (Carling, 2004; Hardie, 2011). Most anoxia-tolerant vertebrates rely mainly on the conversion of glycogen to lactate to produce ATP during anoxia (Nilsson, 2010). The painted turtle decreases glycolytic flux after the first hour or so (Storey, 2007), while the crucian carp shows no decrease in glycolytic enzyme activity throughout 20 hours of anoxia (Johansson et al., 1995). It appears that exhaustion of glycogen stores is the limiting factor determining the duration of anoxia that can be survived by the western painted turtle and crucian carp (Nilsson, 2010). During prolonged anoxic exposure, an upregulation of genes required for glycolytic enzymes and a downregulation of genes for the citric acid cycle (TCA) occurs, supporting the Pasteur effect (Hochachka et al., 1996).

Invertebrates such as mollusks and some worms are able to use a wide variation of anaerobic end-products, including succinate, acetate, and propionate (Müller et al., 2012). These can accumulate during hypoxia/anoxia and during burst activity. For insects their ability to utilize anaerobic pathways is much more diverse than vertebrates, relying not only on glycogen but also on other carbohydrates (i.e. trehalose) and amino acids (Wegener, 1993). Terrestrial insects are known to produce a wide array of products in hypoxia—lactate, alanine, sorbitol, succinate, glycerol, α-glycerol-3-phosphate, pyruvic acid and fatty acids (Hoback and Stanley, 2001). For example, lactate has been reported as the primary end-product of drowning Collembola (Zinkler and Russbeck, 1986).

However, equal amounts of lactate and alanine account for total heat production in two species of tiger beetles exposed to anoxia (Hoback et al., 2000), similar to observed patterns in larval mosquitos (Redecker and Zebe, 1988). Severely hypoxic *Drosophila* accumulate twice as much alanine as lactate, in addition to significant amounts of acetate (Feala et al., 2007). Chironomid larvae have been shown to anaerobically convert glycogen to mostly ethanol with ample amounts of lactate, alanine, and succinate when exposed to anoxic water (Redecker and Zebe, 1988; Wilps and Zebe, 1976); the larval midge *Chaorobus crystallinus* anaerobically converts malate to succinate during anoxia (Englisch et al., 1982); and dipteran *Callitroga macellaria* larvae convert glycogen and amino acids to lactate, alanine, and polyols (Gäde, 1985). However, the genetic and biochemical mechanisms responsible remain poorly known.

Protective mechanisms downstream of ATP depletion

Despite the many mechanisms utilized by anoxia-tolerant animals to match ATP supply and demand, recent work has shown a variety of protective mechanisms can be activated after ATP is depleted. These include increases in heat shock proteins (HSP), anti-apoptotic factors, and antioxidants. Interestingly, many of these not only protect against anoxic damage, but also aid in reducing oxidative stress during reperfusion.

HSPs are one of the first lines of defense during cellular stress aiding in the prevention of cell death (Krivoruchko and Storey, 2010a). Although their specific role in anoxia tolerance is not yet clear, HSPs increase in a number of animals and tissues during exposure to anoxia. Their roles in other environmental stresses (e.g. extreme temperatures, desiccation) suggest that HSPs play an important role in preventing protein unfolding and degradation (Feder and Hofmann, 1999; Krivoruchko and Storey, 2010b).

Additionally, it has been demonstrated that HSPs may provide cytoprotection by inhibiting cell death pathways (Giffard et al., 2008). Another proposed mechanism in determining cell fate is a delicate equilibrium between stress proteins and the apoptotic pathway (Beere, 2001), most notably in the levels of Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic). In anoxia tolerant turtles, the Bcl-2:Bax ratio is maintained, while the anoxia-sensitive mammalian brain increases Bax and decreases Bcl-2 that promote the initiation of apoptosis (Feldenberg et al., 1999). Not only do these mechanisms protect cells from damage during anoxia, but also protect against the detrimental effects of ROS production during reperfusion. The mammalian brain shows a drastic increase in ROS during reperfusion (Hashimoto et al., 2003), while the turtle brain appears to suppress ROS upon reoxygenation (Milton et al., 2007; Pamenter et al., 2007). Some anoxia-tolerant animals increase levels of antioxidants during anoxia, while others have constitutively high levels before anoxia (Bickler and Buck, 2007), allowing the organisms to combat the increased levels of ROS.

ANOXIA-TOLERANT VS INTOLERANT DEVELOPMENTAL STAGES

O_2 deprivation can have detrimental effects on development in most vertebrates. Infant monkeys exposed to shorts bouts of hypoxia exhibit impairment of the nervous system that is linked to the development of epilepsy (Sloper et al., 1980). In general, hypoxic exposure to adult vertebrates has various effects implicated in cardiovascular disease, inflammatory disease, diabetes, and cancer (Brahimi-Horn and Pouysségur, 2007). Most vertebrate embryos have minimal capacities to withstand bouts of oxygen deprivation. Severe hypoxia causes frog and chicken embryos to exhibit developmental

defects associated with decreased survival rate, delayed development, development abnormalities, and early hatching (Altimiras and Phu, 2000; Bradford and Seymour, 1988; Grabowski, 1961). However, it was noticed years ago that fetal and neonatal mammals can tolerate much longer durations of anoxia when compared to adult counterparts (Adolph, 1969; Fazekas et al., 1941; Kabat, 1940). In the large majority of vertebrates, anoxia tolerance is greatest in the earliest stages of development and is lost throughout the developmental process. This is especially true for the annual killifish, *Austrofundulus limnaeus*; early embryos are among the most anoxia-tolerant vertebrates, capable of surviving 30-40 days of anoxia without irreversible damage (Podrabsky et al., 2007). However, this survival rate diminishes throughout development and once embryonic development has finished individuals don't survive 24 hours of anoxia. Similar developmental patterns in anoxia tolerance have been observed for most animals studied thus far (Singer, 1999).

Animals that undergo metamorphosis, including amphibians and many invertebrates, experience dramatic morphological and physiological changes throughout development (Mueller et al., 2015). These ontological changes allow animals to specialize life stages for particular needs; for example, holometabolous insect larvae are often specialized to feed, digest, and grow on a specific diet, while the adults are more specialized for mating and dispersal. Holometabolous insects often inhabit different niches and have different locomotory capabilities that expose them to different oxygen environments and anoxic risks. Many larval insects live in hypoxic environments; for example, chironomid larvae often live in severely hypoxic waters (Doke et al., 1995), beetle larvae live in frequently water-logged soil (Hoback et al., 1998; Hoback et al., 2000), and parasitic *Gastrophilus*

larvae live in hypoxic horse intestines (Keilin and Wang, 1946). Larval caddisflies that live in frequently flooded wetlands are indeed more than 10x more tolerant to hypoxia than pupae (Cavallaro and Hoback, 2014). In contrast, the adult forms of these insects are terrestrial and are likely to only experience hypoxia/anoxia when trapped in water. These examples suggest that the ontogeny of holometabolous insects may be related to differential tolerance of hypoxia/anoxia and in their strategies for dealing bouts of oxygen deprivation. Studying holometabolous insects through ontogeny may yield useful insight into new paradigms in understanding anoxia tolerance.

DROSOPHILA AS A MODEL FOR STUDY OF ANOXIA TOLERANCE

Although substantial amounts of research involve the incredible tolerance of some lower vertebrates (e.g. some turtles and carp) to anoxia (Bickler and Buck, 2007), insects as a whole exhibit greater capacity to withstand oxygen deprivation than vertebrates and represent efficient systems to study the underlying mechanisms involved in anoxia tolerance. The ability for insects to survive bouts of anoxia also seems to be related to their capacities to survive drowning, freezing, and many pesticide treatments (Hoback and Stanley, 2001; Schmitz and Harrison, 2004). *Drosophila melanogaster* is an interesting and promising model for studying anoxia tolerance given its astonishing tolerance to anoxia (Krishnan et al., 1997), the power of its genetic and molecular tools (Azad and Haddad, 2009), and the benefits of using laboratory adapted inbred strains that significantly reduce phenotypic variation (Van Voorhies, 2009). Genes are mostly conserved from *D. melanogaster* to humans (Haddad and Ma, 2001) and flies share 65-70% of disease genes present in humans (Azad et al., 2009). Furthermore, the use of *D.*

melanogaster in human disease models has helped to establish relationships of many genes to particular diseases (Doronkin and Reiter, 2008; Fortini et al., 2000).

Drosophila melanogaster adults are capable of flying and thus can escape temporary anoxic conditions; whereas, larvae must possess the ability to cope with temporary anoxic exposure in their growth media. Adults possess a more developed and complex tracheal system than larvae; adults have 8 spiracles on each side of the body and air sacs enabling convective ventilation, while larvae have only 2 pairs of spiracles and no air sacs. Larvae normally position the posterior spiracles upward pointing out of the media and into the air for use like a snorkel. Thus, adults have a larger capacity for convective ventilation and oxygen reserves. Like most adult insects, when given an acute anoxic stress, adults are paralyzed within 30 seconds (Figure 1.4A, inlay); in contrast, larvae exhibit an escape behavior for up to 30 minutes in anoxia (Figure 1.4A). Adult resting metabolic rates are higher than for larvae, but both adults and larvae can withstand reduced oxygen levels down to ~1 kPa before exhibiting decreased activity and CO_2 production, which suggests similar capacities to match oxygen delivery to tissue needs during hypoxia (Klok et al., 2010). In response to anoxia, larvae and adults similarly reduce metabolic rates to ~3% of normoxia during 30-120 minutes of exposure (Figure 1.4C). Adult *D. melanogaster* have been shown to accumulate large amounts of alanine, lactate, and some acetate in severe hypoxia (Feala et al., 2007). Metabolic rates of larval *Drosophila* in anoxia can be fully accounted for by lactate production, but lactate production in adults only accounts for ~30% heat production in anoxia (Figure 1.4B); thus, larvae and adults utilize different anaerobic pathways in anoxia. Survival from anoxic exposure is also stage-dependent; adults can survive up to 8 hours of anoxia,

while larvae do not survive more than 90 minutes of anoxic exposure (Figure 1.4D), and embryos can survive >36 hours of anoxia with near 100% survival (DiGregorio et al., 2001).

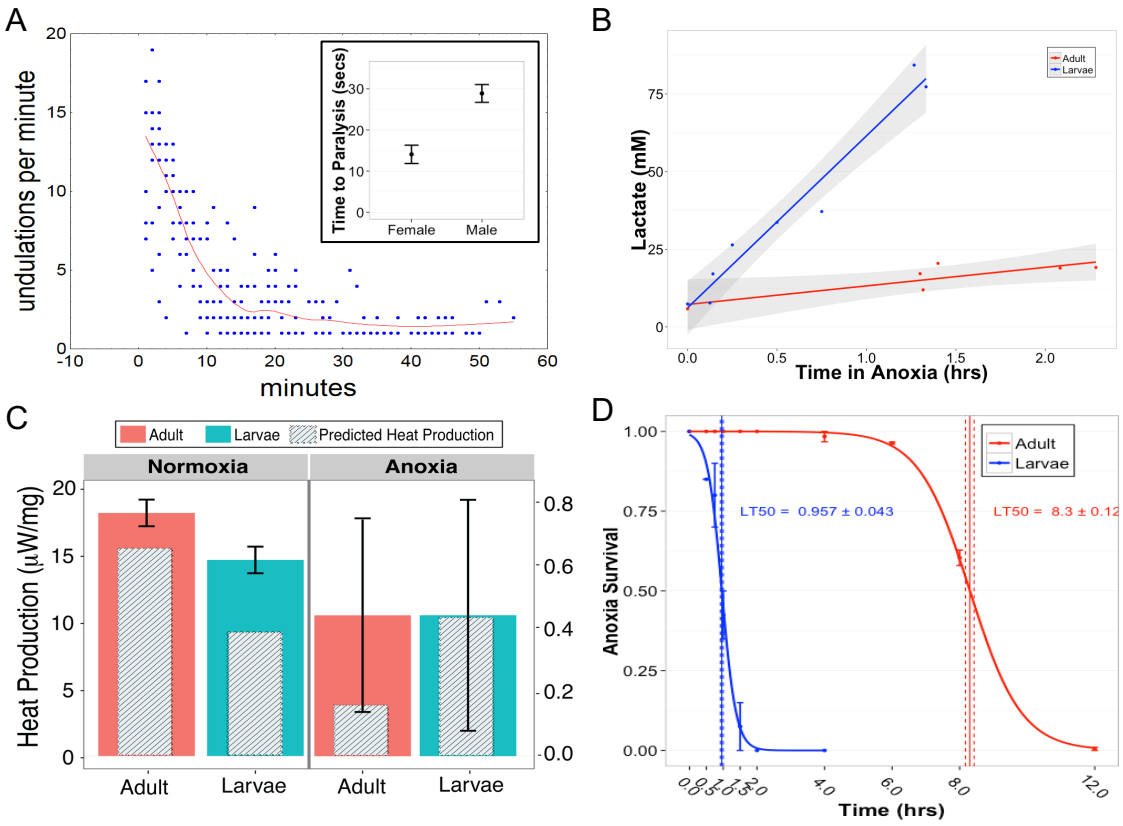

Figure 1.4: A) Time to paralysis for larvae and adult (inlay), B) Lactate production, C) measured and predicted metabolic rates from calorimetry, and D) Anoxia survival for larvae and adults (Callier et al., 2015).

While anoxia tolerance of larval *Drosophila* has yet to be thoroughly investigated, there is a substantial amount of research involving mechanisms of anoxia tolerance in adults. *Drosophila* can recover from hours of oxygen deprivation without evidence of cell damage (Ma et al., 2001). Adults do not survive more than 12 hours of anoxia, with a rapid decrease in survival after >4 hours. This increase in mortality also coincides with

large increases in protein aggregations—increasing over 4-fold after 4 hours of anoxia (Chen et al., 2002). Two mechanisms linked to anoxia survival are related to the prevention of protein unfolding, accumulation of HSPs and trehalose (Azad et al., 2009; Chen et al., 2002). HSPs are a group of molecular chaperones that have been implicated in a wide variety of environmental stressors including desiccation, extreme temperature tolerance and oxygen deprivation (King and MacRae, 2015). Flies exposed to hypoxia exhibit increased levels of HSP70 and HSP23, and flies with overexpressed HSPs had substantial increases in survival (Azad et al., 2009). Overexpression of trehalose-6-phosphate synthase (*tps1*), which synthesizes trehalose, increased levels of trehalose and increased tolerance to anoxia (Chen et al., 2003). As both HSPs and trehalose are protective against protein stress, it appears as though these to mechanisms contribute significantly to anoxia tolerance. Another important mechanism found to contribute to anoxia tolerance is a family of genes involved in RNA editing. Disruption of the *Drosophila* adenosine deaminase (dADAR) results in unedited sodium, calcium, and chloride channels and renders mutants sensitive to anoxia (Ma et al., 2001; Palladino et al., 2000); dADAR also plays a role in ROS metabolism by regulating expression of genes encoding important ROS scavengers (Chen et al., 2004), which can also be important in hypoxia/anoxia tolerance. Furthermore, studies have demonstrated that *Drosophila* neurons become hyperpolarized during anoxia, thus allowing for a reduction in neuron excitability and energy demand that may be an important contributor to anoxia tolerance (Gu and Haddad, 1999). Gene families overrepresented in flies exposed to severe hypoxia include those involved in metabolic processes, immune responses and protein unfolding (Azad et al., 2009; Gleadle and Ratcliffe, 1998; Liu et al., 2006).

However, the causal mechanisms that are most important to survival are not yet apparent, making it difficult to determine the relative importance of various responses.

SIGNIFICANCE

The perils of oxygen deprivation are key to many pathologies of human disease. Heart infarction, stroke and cancer are the most common diseases in the 21st century and all of these share one common feature in that oxygen availability contributes to the development of these pathological conditions (Michiels, 2004). Hypoxia, due to impaired blood flow, has hazardous effects on organ structure and function, especially in the case of stroke (cerebral ischemia) and heart infarction (myocardial ischemia). Hypoxia also plays a role in regulating tumor growth and metastasis. Understanding the pathways underlying the hypoxic response is pertinent to the development of novel therapeutic treatments of oxygen-mediated human disease.

There have been extensive studies on cells, tissues, and animals that experience oxygen deprivation, and many pathways have been discovered that can lead to or prevent anoxic-injury in mammals. However, the degree of hypoxia that mammals can tolerate is narrow. Any experimental attempts to extend anoxic survival in mammals by blocking or boosting a particular mechanism are likely to be hampered by failures of other functions (Nilsson and Lutz, 2004). Thus, mammalian systems are not a well-suited for studying defense mechanisms against anoxia. Hypoxia-tolerant organisms offer a convenient tool to study the global metabolic shift and regulation of metabolism that occurs during oxygen deprivation (Hochachka, 1980; Hochachka, 2003). Additionally, a powerful strategy for understanding the regulation of tolerance to hypoxia is through the use of

model organisms and their translational power to understand oxygen-mediated pathways in human disease. It should be recognized that some of the major discoveries in physiology and medicine have been made in model systems over the last 60 years (Haddad, 2006)—these include Nobel Prizes for the discovery of the basis of action potential generation in the giant squid axon (Hodgkin et al., 1951), genes that control programmed cell death in *C. elegans* (Ellis and Horvitz, 1986), and the discovery of the K^+ channel structure in bacteria (Doyle et al., 1998). Model organisms, like *Drosophila*, offer multiple benefits for approaching tolerance to anoxia; for example, the extensive library of genetic tools allows one to measure and manipulate genes related to anoxia tolerance on large numbers of individuals that can be controlled for genetic variation.

This dissertation includes experiments designed to test several hypotheses for how adult *Drosophila* survive much longer in anoxia than larvae. Firstly, I tested the most common mechanism for variation in anoxia tolerance among animals—ATP supply and demand—and the effects of anoxia on ionic homeostasis. Next, I used a combination of $^{+}$H-NMR based metabolomics and biochemical assays to investigate if differences in carbohydrate levels, anaerobic metabolism, and/or protective osmolytes contribute survival differences between larvae and adults exposed to anoxia. Lastly, I used a genome wide association analysis to investigate the genetic variance underlying differences in anoxia survival between larvae and adults. Together, these three multifaceted experiments shed light on potential alternative mechanisms responsible for variation in tolerance to oxygen deprivation in a model organism. Understanding the mechanisms responsible for anoxia have not only broad physiological importance but are also pertinent to the discovery of novel therapeutic treatments for human pathologies.

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CHAPTER 2

PARALYTIC HYPO-ENERGETIC STATE FACILITATES ANOXIA TOLERANCE DESPITE IONIC IMBALANCE IN ADULT *DROSOPHILA MELANOGASTER*

INTRODUCTION

Animals differ tremendously in their ability to tolerate oxygen deprivation. With a few noteworthy exceptions (e.g. killifish embryos, turtles, and carp), vertebrates can only tolerate minutes of anoxia at body temperatures of 35°C or higher (Nilsson and Lutz, 2004; Podrabsky et al., 2007). Many of the champions of anoxia tolerance are found among the invertebrates that can tolerate hours or days of anoxia even at high temperature (Hoback and Stanley, 2001). This tolerance is even more impressive given that invertebrates are quite small and are characterized by high resting metabolic rates in normoxia. Additionally, ontogenetic variation in anoxia tolerance occurs in most animals, with tolerance often decreasing as development progresses (Singer, 1999). Despite the importance of anoxia tolerance to stress-resistance, the physiological mechanisms responsible for the great variation in anoxia-tolerance across animals are poorly known.

Cell damage in anoxia is thought to be caused by decreased pH, disrupted calcium homeostasis, increased intracellular osmotic pressure, and/or mitochondrial damage. All of these pathologies are related directly or indirectly to decreased ATP levels (Hochachka and Somero, 2002). Depletion of high-energy phosphates leads to reduced activity of Na^+/K^+- and Ca^{2+}-ATPases, leading to dissipation of ionic gradients (K^+ in particular)

responsible for membrane polarization (Galli and Richards, 2014b; Storey and Storey, 2007). Cessation of membrane ion pumps also causes intracellular ionic and osmotic imbalances that lead to unfolding and aggregation of proteins (Giffard et al., 2004). Eventually, a series of detrimental apoptotic and necrotic cascades are initiated that cause cell death (Murphy and Steenbergen, 2008b). In theory, tolerance to anoxia might involve the capacity to conserve ATP; alternatively, anoxia tolerance could be related to mechanisms downstream of ATP depletion—i.e. the prevention or tolerance to ionic disturbances.

Variation in anoxia tolerance among vertebrates is tied to their ability to match ATP supply and demand to preserve cellular ATP (Boutilier and St-Pierre, 2000). For animals to preserve ATP during anoxia, they must suppress metabolic rate and/or upregulate anaerobic metabolism (Staples and Buck, 2009). Anoxia almost always causes metabolic depression, often to levels less than 10% of resting metabolic rate (Bickler and Buck, 2007; Hoback and Stanley, 2001). To suppress energy consumption, animals often reduce protein synthesis (Boutilier, 2001; Buc-Calderon et al., 1993; Land and Hochachka, 1994; Land and Hochachka, 1995), and activity of ion-motive ATPases. Additionally, many anoxia-tolerant animals have large glycogen stores and considerable capacity for sustained anaerobic ATP production (Gorr et al., 2010; Müller et al., 2012). Accordingly, it is the combination of anaerobic ATP production and cessation of non-essential ATP use that allows preservation of cellular ATP in anoxia-tolerant vertebrates such as crucian carp and painted turtle (Jackson, 2000; Lutz et al., 1984). Furthermore, these species have the ability to reduce membrane ion permeability during anoxia (Buck and Hochachka, 1993; Hochachka et al., 1996), which helps to defend extracellular

potassium concentration ($[K^+]_o$) and membrane potential during prolonged periods of anoxia (Chih et al., 1989a; Nilsson et al., 1993).

Many invertebrates (e.g. adult insects) and one notable vertebrate (killifish embryos) can survive long periods of anoxia with low levels of ATP, demonstrating that some animals can avoid or recover from the damage that normally ensues from low cellular ATP levels (Hoback and Stanley, 2001; Podrabsky et al., 2007; Wegener, 1993). Embryos of annual killifish can survive up to 100 days of anoxia during development (Podrabsky et al., 2007); yet, ATP levels decline by 80% in the first 12 h of exposure (Podrabsky et al., 2012). Similarly, the majority of adult insects show a rapid depletion of ATP, yet they show a great tolerance to anoxia (Hoback and Stanley, 2001; Wegener, 1993). Therefore, protective mechanisms downstream of ATP depletion must aid in surviving anoxic exposure. For example, protein aggregations increase during anoxia and two mechanisms linked to survival involve the accumulation of heat shock proteins (HSP) and trehalose to prevent protein unfolding and the accumulation of protein aggregates (Azad et al., 2009; Chen et al., 2002). These protective mechanisms that prevent damage associated with ATP depletion indicate that surviving despite low ATP is a key factor in enduring anoxic exposure in invertebrates, and perhaps in vertebrates that also experience rapidly declining ATP during oxygen deprivation.

As in many vertebrates, there are strong developmental differences in anoxia tolerance in *D. melanogaster*. Adults can survive anoxic exposure times ~8 times longer than larvae (Callier et al., 2015) and adults can recover from hours of oxygen deprivation without evidence of cell damage (Ma et al., 2001a). In response to anoxia, larvae and adults similarly reduce metabolic rates to ~3% of normoxia during 30-120 minutes of

exposure, so differences in anoxia tolerance cannot be explained by capacities to suppress metabolism (Callier et al., 2015). Nevertheless, adults are paralyzed within 30 sec and larvae exhibit escape-like behavior for almost 30 min (Callier et al., 2015) so it is possible that survival differences are related to the different behavioral responses and ATP use during the first 30 min of anoxia.

Here I present whole body ATP, along with extracellular [K^+] and pH in adult and larval *D. melanogaster* exposed to varying durations of anoxia. I test whether the anoxia tolerance of *D. melanogaster* can be explained by the ability to maintain ATP levels, maintain extracellular ionic levels, or to tolerate low energetic state and ionic disruptions, and how this varies across developmental stages (adult vs. late-stage 3rd instar larvae).

METHODS

Insects and rearing conditions and experimental design.

All flies used here were from the same population of Samarkand strain *D. melanogaster*, maintained as described in (Callier et al., 2015). All flies were maintained on the standard malt-based cornmeal diet (Fly Food Mix B, Lab-Express) at 25˚C in 300 mL plastic bottles. Late third instar larvae (LL3) were collected by choosing individuals that lacked colored food in the gut and were found wandering on the walls of the vials. For adults, vials were cleared and individuals were collected 24 hours later; adults were then sexed under CO_2 anesthesia, separated into groups of 20, and then allowed to age until 3-4 days old. All flies were given a minimum of 24 hours of recovery after exposure to CO_2 anesthesia before experimental treatments. All experiments were conducted at 25±1˚C.

Experiments were designed based on previous studies in the same population of flies that exhibit large differences in anoxia tolerance between late 3rd instar larvae and adults (Callier et al., 2015) (Figure 2.1). For both larvae and adults, I measured whole body ATP levels, hemolymph $[K^+]$ ($[K^+]_o$) and pH across anoxia exposure times associated with 0% to 100% mortality. Thus, I measured these parameters across times ranging up to 2 hrs in larvae and up to 12 hrs on adults. To statistically compare life stages, I tested for life-stage effects with a factorial ANOVA. Additionally, I tested for significant effects of time within each life-stage using ANOVA across all times tested for that life stage. For $[K^+]_o$, I included samples taken after 15 minutes of recovery from anoxia to assess the ability to recover $[K^+]_o$. To compare the ability to significantly recover $[K^+]_o$, I used a factorial ANOVA for each life stage comparing recovery state (anoxia or 15 min of recovery) and time in anoxia. All data met assumptions of normality and homogeneity of variance for parametric tests. Main effects and post hoc tests were conducted at a family-wise alpha and Type I error rate of 0.05. All statistics were conducted using R software (Team, 2016) and various R packages [doBy (Højsgaard and Halekoh, 2016), ggplot2 (Wickham, 2009), lsmeans (Lenth, 2016), multcomp (Hothorn et al., 2008)].

Measurement of the effect of anoxic duration on whole-body ATP concentrations.

Individual adults or larvae were put in groups of ~20 into 25 x 95 mm plastic vials containing food. Vials were sealed with gas permeable cotton plugs and placed into an airtight 1-liter chamber with two ports. To create humid, anoxic conditions, N_2 gas flowed (4 l min^{-1} regulated by a Mass Flow Bar, Sable Systems, Las Vegas, NV) first through a glass flask of distilled water, then through the chamber and into an oxygen

sensor (FoxBox, Sable Systems, Las Vegas, NV). After a measured duration of anoxic exposure, animals were flash-frozen in liquid nitrogen immediately after anoxic exposure and stored at -80˚C until assays were performed.

The ATP assay was carried out using methods derived from Tennessen et al. (2014). Groups of 5 individuals were homogenized with a pellet pestle (Kimble Kontes, Rockwood, TN) in 200 µL of homogenization buffer [6 mol l^{-1} guanidine HCl, 100 mmol l^{-1} Tris (pH 7.8), 4 mmol l^{-1} EDTA] on ice and a 50 µL aliquot taken for protein assays. The remaining sample was boiled for 5 minutes and then centrifuged for 3 minutes at 12000 x g in a refrigerated centrifuge at 4˚C. Next, 10 µL of the remaining supernatant was transferred to a new centrifuge tube and diluted 1:10 with dilution buffer (25 mmol l^{-1} Tris (pH 7.8), 100 mmol l^{-1} EDTA). Then, 10 µL of the diluted samples and standards were added to each well of a white opaque 96-well plate (Corning Inc., Corning, NY, USA). To run the assay, 100 µL of the ATP reaction mix (Molecular Probes, Eugene, OR) was added to each well and luminescence was measured using a Wallac Victor 2 luminometer; the reaction mix contained 1.25 µg ml^{-1} of luciferase, 50 µmol l^{-1} D-luciferin, and 1 mmol l^{-1} DTT in the 1x reaction buffer per well. The weights of each sample were used to express ATP in µmol • g^{-1} wet mass.

Ion-selective microelectrodes for measurement of effects of anoxic duration on hemolymph [K^+] and pH.

Hemolymph [K^+] and pH were measured on larvae or adults (n=12-14 for $[K^+]_o$, n=4-9 for pH) at each timepoint. Larvae and adults were placed individually into air-tight glass chambers (20 mm diameter x 70 mm length) and connected to a multiplexer (Sable Systems) regulating the flow of humidified nitrogen to each chamber, allowing for the

duration of anoxia for each chamber to be individually-controlled. After anoxic exposure, individuals were quickly removed from the anoxic chamber and hemolymph was collected as quickly as possible, usually within 60 seconds. I also measured $[K^+]_o$ for adults and larvae allowed to recover for 15 min in air after their assigned duration of anoxia.

Adult hemolymph was extracted as previously described (MacMillan and Hughson, 2014). Briefly, adult flies were placed head-first into 10-µL pipette tips fit to a system of tubing connected to lab air supply; positive pressure was applied to the pipette tip forcing a portion of the head to be exposed. I removed the first segment of the antennae with forceps to allow a droplet of hemolymph to flow out. The droplet was placed into hydrated paraffin oil for immediate measurement of $[K^+]_o$. For larval hemolymph collection, I submerged animals under hydrated paraffin oil and used a fine glass pipette to puncture the epidermis near the head; hemolymph then pooled around the larvae under the oil, and I collected the hemolymph with the pipette by capillary action. I took care to not rupture the gut and any gut perforation was visible by the presence of cloudy fluid; therefore, only samples with clear hemolymph were used. The hemolymph was then transferred from the glass pipette into a secondary dish of hydrated paraffin oil setup for measurement of $[K^+]_o$. Although hemolymph volumes were not measured during this experiment, volumes were large enough for easy insertion of a reference and ion selective electrode; similar methods were able to extract ~50 nl of hemolymph for adults (MacMillan and Hughson, 2014) and ~170 nl for larvae (Piyankarage et al., 2008).

Hemolymph potassium concentrations and pH were measured using ion-selective microelectrodes prepared as described by (MacMillan et al., 2015). Borosilicate glass

capillaries (TW150-4, World Precision Instruments, Sarasota, FL, USA) were pulled to a ~3-5 µm tip and silanized at 300˚C with *N,N-dimethyltrimethylsilylamine* (Sigma Aldrich, St. Louis, MO, USA) vapors for 1 hour. Immediately before use, $[K^+]$ microelectrodes were filled with 100 mmol l^{-1} KCl solution, while pH microelectrodes were filled with 40 mmol l^{-1} KH_2PO_4, 15 mmol l^{-1} NaCl, 23 mmol l^{-1} NaOH, pH 7.0 (Lee et al., 2013). Next, the ion-selective ionophore was added to the tip of the ion-selective electrode (K^+ ionophore I, cocktail b; H^+ ionophore I cocktail b; Sigma Aldrich). To prevent the displacement of the ionophore by paraffin oil, the tip of the electrode was quickly dipped in a solution consisting of 10mg of polyvinylchloride (Sigma Aldrich) dissolved in 3 ml of tetrahydrofuran (Sigma Aldrich). The ion-selective electrode was inserted into the hemolymph drop using micromanipulators, with the electrode tip observed with a dissecting microscope. For both $[K^+]_o$ and pH, a 2mm OD glass reference electrode with filament (1B200F-4, WPI) pulled to a long thin tip (~1 µm) was filled with 0.5 M KCl and inserted into the hemolymph to complete the circuit. The voltage difference between the two electrodes were measured with a FD223a (WPI) differential electrometer, a MP100A data acquisition system and AcqKnowledge software (Biopac Systems, Goleta, CA). I calibrated the ion-selective electrode before and after each measurement using solutions differing 10-fold. Standards used for $[K^+]_o$ measurements were 10 and 100 mmol l^{-1} [KCl] solutions; the 10 mmol l^{-1} KCl solution also contained 90 mmol l^{-1} LiCl to balance osmolarity. A three-point calibration using standard pH buffer solutions ranging from 6-8 were used to determine hemolymph pH. Voltage from the ion selective electrode was converted to $[K^+]$ or pH using:

$$[h] = [c] \times 10^{\frac{\Delta V}{S}}$$

where $[h]$ is the ion concentration in the hemolymph, $[c]$ is the concentration of one calibration solution, ΔV is the difference in voltage between the calibration solution and hemolymph, and S is the slope of the difference in voltage between the two calibration solutions. For pH calculations, $[H^+]$ was converted to pH by multiplying $[h]$ by –log10. The slopes of the ion selective electrode averaged 53.85 ± 2.50 mV per 10-fold difference in ion concentration, close to the expected Nernst relationship of 58 mV per 10-fold difference.

RESULTS

Whole body ATP levels during anoxia.

Time in anoxia and the interaction significantly affected whole body ATP levels (Time: $F_{9,34}$=141.87, p<0.001; Interaction: $F_{4,26}$=16.41, p<0.001), while the main effect of life stage was not different (Life Stage: $F_{1,34}$=3.25, p=0.080). Levels of whole body ATP dropped near 100-fold for larvae after 2 hours of anoxia (0.51 ± 0.059 to 0.005 ± 0.0001 μmol g^{-1} wet mass) and adults after 12 hours of anoxia (1.00 ± 0.044 to 0.009 ± 0.0006 μmol g^{-1}wet mass); Normoxic ATP levels were higher in adults (Figure 2.2A). Adult ATP decreased significantly to 35% of resting values after 0.25 h of anoxia and to ~3% of anoxic values after 0.5 h of anoxia; however, then ATP remained stable until another significant drop at 8 h (Figure 2.2A). Larval ATP levels fell to 49% and 21% of resting levels after 0.25 and 0.5 h of anoxia, reaching less than 10% of normoxic values after 1 h and to <1% of normoxic values after 1.5-2 h of anoxia (Figure 2.2A). Adults survived at much lower levels of ATP than larvae during anoxia (Figure 2.2B; Larvae:

0.058 ± 0.010, Adult: 0.011 ± 0.006; Likelihood Ratio Test, χ^2=10.21, p=0.006). Thus, unlike larvae, adults maintain low but survivable ATP levels for long durations in anoxia.

Hemolymph [K^+] and pH during anoxia and recovery.

Contrary to ATP, larvae tended to maintain $[K^+]_o$ better than adults during anoxic exposure. The main effects for life stage and time in anoxia, and their interaction all significantly affected $[K^+]_o$ (Time: $F_{7,148}$=64.12, p<0.001; Life Stage: $F_{1,148}$=32.44, p<0.001; Interaction: $F_{4,148}$=3.73, p=0.006). Larvae had higher resting hemolymph potassium levels than adults in normoxia; however, adults reached levels of $[K^+]_o$ similar or higher to those of larvae by 30 min or more of anoxic exposure (Figure 2.3). Adults increased $[K^+]_o$ by almost 2-fold after 2 hours of anoxia and by 4-fold after 12 hours of anoxia (Figure 2.3A); in contrast, larvae only increased $[K^+]_o$ by ~50% after 2 hrs of anoxia with no significant changes from normoxic values until after 1 h of exposure (Figure 2.3B). Adults survived much higher proportional increases in $[K^+]_o$ than larvae (Figure 2.3C; LT50 for larvae: 45.89 ± 1.19, and for adult: 78.71 ± 0.30; Likelihood Ratio Test χ^2=10.87, p=0.004). Thus, the greater anoxia tolerance of adults relative to larvae is due to greater tolerance of elevated $[K^+]_o$ rather than a better ability to maintain $[K^+]_o$ homeostasis.

Larvae and adults showed similar strong (approximately 1 pH unit) decreases in hemolymph pH during anoxia (Figure 2.4A). There was no significant interaction between time and life stage ($F_{4,44}$=1.69, p=0.17), and no effect of life stage on hemolymph pH during anoxia ($F_{1,44}$=0.79, p=0.38). However, the main effect of time in anoxia was significant ($F_{4,44}$=80.21, p<0.001); hemolymph pH dropped from 7.4 to ~ 6.7 within the first 15 min of anoxia and remained stable until the 2 h mark, at which it

dropped to 6.4 (Figure 2.4A). Although the pattern of decreasing hemolymph pH was similar between life stages, adults were more capable of surviving the radical drop in hemolymph pH than larvae (Figure 2.4B, LT50 for larvae = 6.52 ± 0.18; Likelihood Ratio Test, χ^2=14.70, p<0.001).

For adults, the capacity to restore $[K^+]_o$ to resting levels in normoxia was a good indicator of survival across various durations of anoxia. Adults quickly restored $[K^+]_o$ after 0.5-4 hours of anoxic exposure, but not with longer anoxic exposures (Figure 2.3A; Table 2.1). In contrast, the larvae showed no significant capacity to lower $[K^+]_o$ levels during 15 min of normoxia after 0.25-2 h of anoxia (Figure 2.3B; Table 2.1).

DISCUSSION

D. melanogaster adults maintain and tolerate a hypo-energetic state that seems key to their remarkable ability to survive long periods of anoxia. *D. melanogaster* adults exposed to anoxia for 0.5-4 h exhibited a low, non-zero set-point for ATP at approximately 3% of those measured for resting flies; and over this duration, fly survival was near 100%. With longer periods of anoxia, ATP levels in adults fall to 1% of resting levels, correlating strongly with lethality. In contrast, *D. melanogaster* larvae exposed to anoxia exhibit steadily falling ATP levels that reach 1% of resting levels within 1-2 h, when survival falls to 0%.

I set out to investigate two non-alternative mechanisms that can explain the extraordinary (at least relative to most vertebrates) anoxia tolerance in *D. melanogaster* adults. Based on the leading hypothesis in anoxia tolerance for vertebrates, I first tested if adults are able to attain a regulated state where ATP supply is matched to demand.

Despite the observation that ATP levels fall dramatically in both adults and larvae during anoxic exposure, this hypothesis was partially supported; albeit with the observation that ATP levels in adults are regulated at 3% of resting levels for long periods of anoxia. A second hypothesis was that the anoxia-tolerant adults might be better-endowed with capacities to preserve acid base or ionic homeostasis. For extracellular ionic status this is clearly not true, as $[K^+]_o$ rose faster in adults than larvae during anoxia (Figure 2.3), and hemolymph pH fell at a similar rate (Figure 2.4A). Instead, a key aspect of the survival of adult *D. melanogaster* in anoxia is that they are able to tolerate and recover from dramatic departures from normal homeostatic conditions—tolerating ATP levels at 3% of resting, $[K^+]_o$ levels >4x resting, and hemolymph $[H^+]$ >10x resting.

Examination of the literature indicates that ATP patterns in anoxia are highly variable among anoxia-tolerant animals. With the exception of annual killifish embryos, anoxia-tolerant vertebrates maintain ATP near normal during anoxia while most invertebrates survive long periods of anoxia with low levels of ATP (Bickler and Buck, 2007; Hoback and Stanley, 2001). Unlike anoxia-tolerant vertebrates, the majority of insect species studied show patterns similar to anoxia-sensitive mammals in that ATP is usually substantially depleted in anoxia (Hoback and Stanley, 2001; Wegener, 1993). However, the extent and timeline of ATP depletion varies tremendously between species and tissues. For example, migratory locusts show minimal survival after 4 h of anoxia while whole body ATP levels are depleted to 1/3 of normal and ATP levels in the brain and flight muscle are depleted to ~6% and 40% of normal, respectively (Hochachka et al., 1993; Wegener, 1987; Wegener, 1993). Whether the pattern of maintenance of ATP concentrations at a low, non-zero level occurs commonly in anoxic insects is unclear;

while this has not been previously reported, I did not notice this pattern until after plotting log-transformed ATP levels, which most studies have not done.

Plausible mechanisms allowing adults but not larvae to maintain a prolonged, low ATP state that is linked to better survival of anoxia.

Maintenance of ATP levels (even at 3% of normoxic levels) requires a matching of ATP supply to demand. Adults may have lower ATP use during the first 30 min of anoxia, when they are paralyzed and larvae are attempting escape. Anoxia causes adult flight muscles and neurons to lose excitability (Gu and Haddad, 1999; Krishnan et al., 1997), suggesting a response similar to the classical vertebrate channel and spike arrest wherein depolarization causes a slow-inactivation of voltage-gated channels that limits ionic flux and neuronal activity thereby preserving ATP (Lutz and Milton, 2004; Rodgers et al., 2010). Larvae likely do not exhibit this paralysis (at least initially) in anoxia because they have been selected to attempt escape from anoxic, semi-liquid media into air. The cellular mechanisms underlying the differential behavioral responses of adults and larvae to anoxia would be very interesting to explore. In adults, paralysis occurs in less than a minute (Callier et al. 2015), when, at the whole-body level, ATP levels have fallen by less than 50% (Figure 2.2).

Calorimetric measurements demonstrate that larvae and adults have similar metabolic rates during 30-120 min of anoxia; thus, a better ability of adults to suppress metabolism is unlikely to completely explain the superior ability to regulate a hypo-energetic ATP level in adults (Callier et al., 2015). The prolonged maintenance of ATP at 3% of normoxic levels shows that adults are more able to sustain a match of anaerobic ATP production to ATP use. One possible explanation for this is that adults may use a

more diverse array of anaerobic pathways with reduced negative impact of intracellular acid-base status. Lactate accumulation (3x higher in larvae than adults) accounts for nearly all heat production in anoxic *D. melanogaster* larvae while only accounting for ~1/3 in adults (Callier et al., 2015). Adults accumulate high levels of acetate and alanine, potentially improving ATP generation from glycogen with less H^+ production (Feala et al., 2007a; Feala et al., 2009). If larvae experience a higher rate of intracellular H^+ accumulation, this could compromise cellular functions such as glycolysis sooner, inhibiting anaerobic ATP production and survival. Another possibility is that larvae might more quickly exhaust their fuel stores (such as glycogen, trehalose and glucose) due to possession of lower stores or during their initial high-intensity locomotory response to anoxia. Finally, it is conceivable that adults are more capable of defending or repairing cellular damage such as aggregations of unfolded proteins during anoxia. Adult *D. melanogaster* upregulate heat shock proteins (Azad et al., 2011), and have high levels of trehalose that protect protein denaturation (Chen et al., 2004); and it is possible that such protective mechanisms are lower in larvae.

Higher survival in anoxia in Drosophila adults is associated with tolerance to extreme ionic imbalance.

According to most classical models of anoxia tolerance, a key factor is prevention of substantial elevation of $[K^+]_o$ that depolarizes membranes (Erecinska and Silver, 1989; Katsura et al., 1994; Knickerbocker and Lutz, 2001). However, hemolymph $[K^+]_o$ increases more rapidly in adults than larvae, and adults survive much higher levels of $[K^+]_o$ than larvae (Figure 2.3). For hemolymph pH, the changes are similar in larvae and adults (Figure 2.4). Thus, the higher anoxia-tolerance of adults is associated with a

greater tolerance of extracellular ionic disruption rather than better extracellular ionic regulation (Figure 2.3).

In both larval and adult *Drosophila*, $[K^+]_o$ increases in a nonlinear fashion during anoxia, wherein changes in $[K^+]_o$ are at their highest early on and begin to slow as exposure continues. For adults, during hour 1, when ATP levels fall dramatically, $[K^+]_o$ rises 3x, while during the next six hours when ATP is statistically constant, $[K^+]_o$ rises only by 50%. This pattern is likely due to the dynamic changes in passive and active forces on K^+ balance. As ATP declines, Na^+/K^+ ATPase activity and passive reuptake decrease leading to increased extracellular K^+; this change in $[K^+]_o$ disrupts the electrochemical gradient and leads to further loss of intracellular K^+. Furthermore, editing of ion channels by adenosine deaminase improves hypoxia survival in adult *Drosophila*, likely by reducing transmembrane ion flux (Ma et al., 2001a).

Adult hemolymph $[K^+]$ recovery facilitates rapid recovery of behavior after anoxia.

Following a detrimental disruption of ion homeostasis and the loss of neuronal activity, successful recovery from anoxia requires the ability to clear excess $[K^+]_o$ and recover ion gradients (Rodgers et al., 2007). Fitting with their higher anoxia-tolerance, adults demonstrate higher capacity to restore ionic homeostasis after anoxia. Within 15 min of reoxygenation (after 2 h of anoxia), adults return $[K^+]_o$ to normal levels (Fig. 2.3A). Only at the anoxia durations of 8 h or more, are adults unable to make a significant recovery of $[K^+]_o$, coinciding with the rapid decline in survival (Callier et al., 2015). The rate of recovery of $[K^+]_o$ correlates well with reported times for flies to behaviorally recover from anoxia. Adult flies exposed to less than 2 h of anoxia exhibit the first signs of movement within 15 min of recovery, and a 4 h exposure of anoxia extends mobility

recovery time to 60 min (Krishnan et al., 1997). However, larvae exhibit very little recovery of $[K^+]_o$ when returned to normoxia after anoxic exposures. This may be partly due to the fact that the changes in $[K^+]_o$ were smaller than in adults, making it more challenging to observe recovery.

Speculations on the links between ATP, extracellular ionic changes and survival

It seems difficult to believe that preservation of ATP at 3% of normoxic levels could be essential to sustaining life during anoxia exposure in adults; however, the tight correlations between ATP, $[K^+]_o$, and survival (Figures 2.2, 2.3) are certainly suggestive. Possibly *Drosophila* Na^+/K^+ATPases are able to sustain some level of transport at very low ATP, slowing the rate of ionic fluxes that eventually lead to cell death. In support of this possibility, larvae have ~3x the hemolymph volume as adults (larvae: 0.178 µL, adult:0.064 µL; Folk and Bradley, 2003; Folk et al., 2001; Piyankarage et al., 2008); assuming that hemolymph volume remains stable throughout anoxia, adults have ~4x lower rate of K^+ efflux to the hemolymph than larvae during anoxia (Figure 2.5), perhaps due to higher Na^+/K^+ATPase activity or differences in membrane conductance. This low level of ATP might also be able to sustain some extrusion of H^+ or Ca^{++} to prolong survival. Direct measurement of intracellular conditions, and intra- to extracellular ionic fluxes in anoxic larvae and adults will be necessary to test this hypothesis. It is also plausible that these low but non-zero ATP levels are sufficient for other protective functions such as translation of heat shock proteins, or synthesis of protective organic osmolytes.

Conclusion

When metazoans are broadly considered, it is clear that anoxia tolerance can be achieved without preservation of near-normoxic ATP levels, contrary to the paradigm for anoxia-tolerant vertebrates such as turtles and carp. Here I have identified that survival from anoxic exposure in *D. melanogaster* is associated with maintenance of extremely low but nonzero ATP levels, likely made possible by behavioral paralysis that reduces metabolic rate and use of diverse metabolic pathways to generate ATP anaerobically. Additionally, I show that surviving anoxia in *Drosophila* adults is related to the ability to tolerate large disturbances in extracellular ionic homeostasis. Given the many similarities in response to anoxia with mammals, identification of the molecular and cellular mechanisms that allow *Drosophila* to survive low energetic state and large ionic disruptions may be useful for advancing understanding variation in anoxia-tolerance in a biomedical context.

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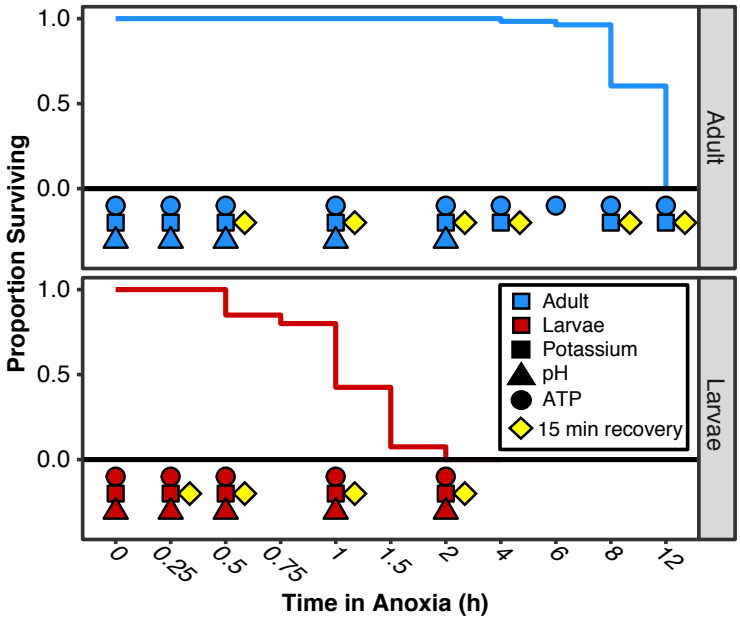

Figure 2.1: Anoxia survival and sample collection times. Survival from anoxic exposure of various durations for adult (top panel) and larvae (bottom panel) D. melanogaster from (Callier et al, 2015). Points below the zero line represent the times points at which samples were collected for whole body ATP, hemolymph [K^+], hemolymph [K^+] after 15 min of recovery from anoxia, and hemolymph pH. Note that the x axis is categorical on this and many graphs to allow ease of visualization of occurrences in the first hour.

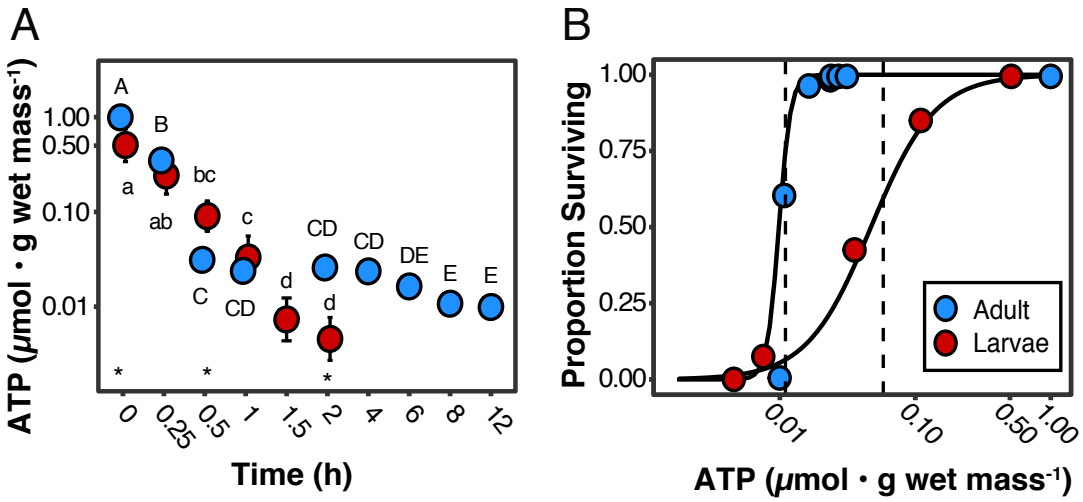

Figure 2.2: Whole body ATP levels throughout anoxia for larvae and adult. ATP was measured for up to 2 hrs for larvae (red squares) and 12 hrs for adults (blue circles). A) ATP in absolute concentration (µmols • g wet mass^{-1}) for the duration of anoxic exposure. Note data on x-axis are presented categorically and the y-axis shown on a log scale. Data shown are means ± 95% CI, * indicates a significant difference between life stages at that time point (Tukey post hoc test), and data with different letters—capital letters for adults and lowercase for larvae—represent time periods that have statistically different ATP levels within a life stage (Tukey). B) The relationship between normoxic ATP and survival shows that adults survive with lower levels of ATP than larvae. Vertical dashed lines represent LT50 for each life stage (Larvae: 0.058 ± 0.010, Adult: 0.011 ± 0.006). Note that the x-axis is on a log scale to help visualize the lower range of ATP

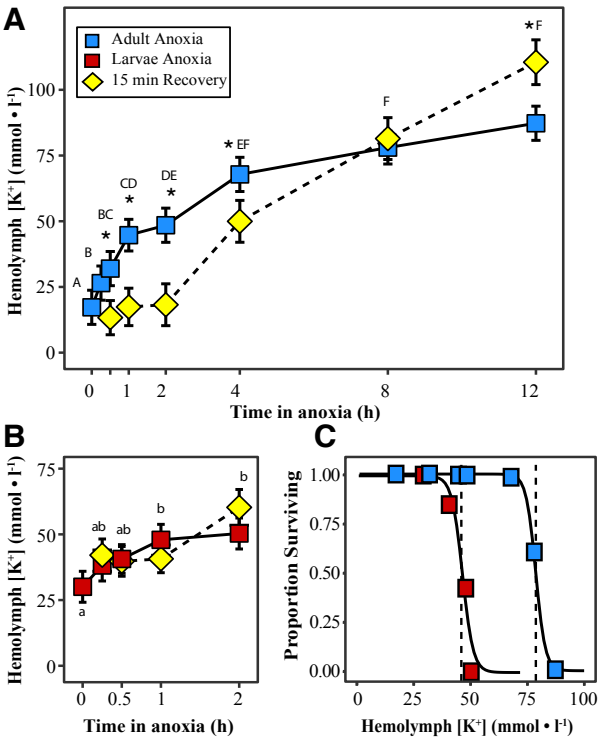

Figure 2.3: Hemolymph [K^+] during anoxic exposure for larvae and adults and after 15 minutes of recovery. Hemolymph [K^+] (mmol • l-1) during 12 hrs of anoxia and after 15 minutes of recovery (yellow) from anoxia for adults (A, blue) and larvae (B, red). * represent significant differences between anoxia and recovery [K^+] (ANOVA, Tukey, $p<0.01$), and data with different letters—capital letters for adults and lowercase for larvae—represent time periods that have statistically different hemolymph [K^+] levels within a life stage (Tukey). Data shown are means ± 95% CI. C) The relationship between the hemolymph [K^+] and anoxia survival shows that adults are more capable of surviving higher proportional changes in hemolymph [K^+]. Vertical dashed lines represent LT50 for each life stage (Larvae: 45.89 ± 1.19, Adult: 78.71 ± 0.30).

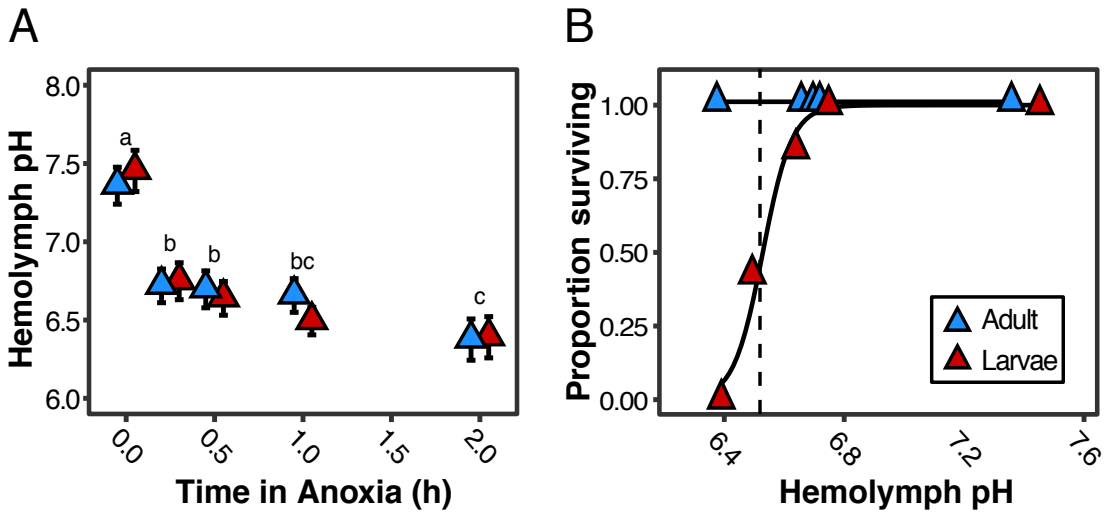

Figure 2.4: Change in hemolymph pH during 2 hrs of anoxic exposure. A) Hemolymph pH declined over time, but there were no statistically significant differences between larvae and adults. Data shown are means ± 95% CI, data with different letters represent time periods that have statistically different hemolymph pH levels (Tukey). B) The relationship between hemolymph pH and anoxia survival indicates that adults were more tolerant to the drop in pH. Vertical dashed line represents the LT50 for Larvae: 6.52 ± 0.18).

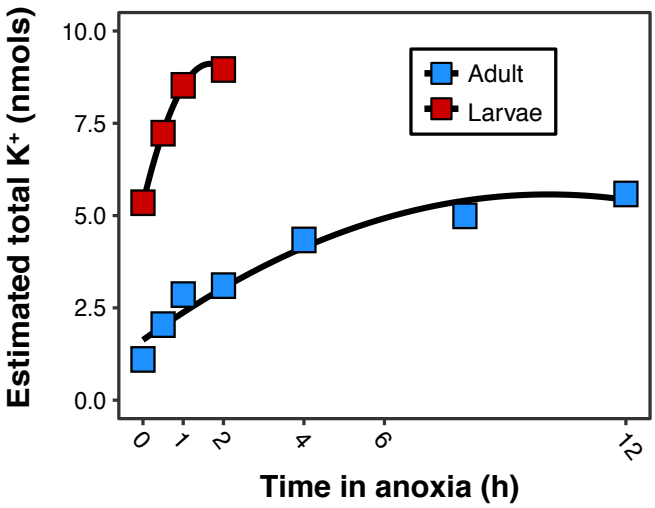

Figure 2.5: Estimated K^+ efflux (nmols) after correcting for differences in hemolymph volumes (adults: 0.064 µl, larvae: 0.178 µl; Folk and Bradley, 2003; Folk et al., 2001; Piyankarage et al., 2008).

Table 2.1: Results of factorial ANOVAs testing the effect of time in anoxia and recovery state on hemolymph $[K^+]_o$ in larvae and adults.

Life Stage		*df*	*SS*	*MSE*	*F*
Larvae	**Time**	4	5258.5	1314.6	12.42***
	Recovery state	1	15.4	15.4	0.15
	Time: Recovery state	3	935.5	311.9	2.95*
	Residuals	101	10693.9	105.9	
Adult	**Time**	7	107497	15356.7	118.29***
	Recovery state	1	4748	4748.2	36.58***
	Time: Recovery state	5	10056	2011.1	15.49***
	Residuals	138	17916	129.8	

Significance at p<0.001***, p<0.01**, p<0.05*

CHAPTER 3

CAUSES OF DEVELOPMENTAL VARIATION IN ANOXIA TOLERANCE OF *DROSOPHILA*: METABOLOMIC EVIDENCE AGAINST SUBSTRATE LIMITATION AND FOR ROLES OF PROTECTIVE METABOLITES AND PARALYTIC HYPOMETABOLISM

INTRODUCTION

Hypoxia/anoxia is an important component of many pathologies including stroke, heart attack, and sleep apnea. However, both the pathways by which anoxia and hypoxia negatively affect tissues and the mechanisms responsible for the wide variation in tolerance of anoxia remain poorly understood. Adult humans from different regions show large variation in tolerance to hypoxia (Beall, 2007; Gilbert-Kawai et al., 2014; Hochachka et al., 1999), and neonatal mammals are much more tolerant of anoxia than adults (Parer, 1998; Singer, 1999). Some lower vertebrates such as turtles and carp can tolerate days and even months of anoxia (Bickler and Buck, 2007). Invertebrates generally exhibit greater capacities to withstand oxygen deprivation than vertebrates (Schmitz and Harrison, 2004), and insects have been shown to have developmental stage-specific variation in hypoxia/anoxia tolerance (Brust and Hoback, 2009; Callier et al., 2015; Cavallaro and Hoback, 2014; Harrison et al., 2018). Here I examine metabolic correlates of anoxia to partially test several hypotheses to explain developmental variation in anoxia tolerance of *Drosophila melanogaster*.

Adult *D. melanogaster* live in air and would likely only experience hypoxia if drowned by rain or falling into water. *Drosophila* larvae live in rotting fruit in nature and

in near-anoxic media in the lab (Callier et al., 2015), and so likely evolved to cope with viscous, anoxic conditions. Yet surprisingly, adults can recover from hours of oxygen deprivation (up to 12 h), while larvae only survive up to 2 h in anoxia (Callier et al., 2015). In both developmental stages, anoxia causes ATP to fall rapidly to low levels as extracellular [K^+] and [H^+] strongly increase; adults survive despite accumulating very high levels of K^+ and H^+ in the hemolymph (Campbell et al., 2018). The objective of this study was to test whether adult and larval *D. melanogaster* differ with respect to patterns of carbohydrate depletion, rates and pathways of anaerobic metabolism, and/or the concentration of protective metabolites that might explain their differential tolerance of anoxia.

One possible explanation for the higher anoxia tolerance of adult relative to larval *Drosophila* is that adults might have higher carbohydrate stores, or a higher ratio of stores to anaerobic metabolic rate, enabling them to sustain anaerobic metabolism for a longer time. For all anoxia-tolerant animals, upregulation of anaerobic metabolism is critical to sustain life when aerobic metabolism is suspended by anoxia. For the highly anoxia-tolerant crucian carp and painted turtle, the limiting factor in anoxic survival is the exhaustion of glycogen stores (Nilsson, 2010). Similar to vertebrates, invertebrates rely on carbohydrate stores to fuel anaerobic metabolism, primarily glycogen (Harrison, 2015; Wegener, 1993). To test this hypothesis, I measured glycogen, trehalose and glucose concentrations during anoxia in *D. melanogaster* adults and larvae. I also assessed rates of anaerobic metabolism by measuring all of the putative anaerobic end-products in *Drosophila* (lactate, alanine, acetate, succinate, malate), as prior calorimetric studies were unable to assess metabolic rate during the first 30 min of anoxia (Callier et al. 2015). If

carbohydrate limitations contribute to the relatively poor anoxia-tolerance of larvae, I predict larvae should have lower total carbohydrate stores or lower carbohydrate stores relative to anaerobic metabolic rate, and that total carbohydrate stores should decline strongly during anoxia.

Another possibility is that adults may more strongly reduce metabolic rate during anoxia than larvae or utilize anaerobic pathways that are less disruptive to homeostasis. When measured during 30-120 minutes of anoxic exposure, larvae and adults similarly reduce metabolic rates to ~3% of normoxia (Callier et al., 2015). However, metabolic differences may be strongest during the first 30 min of exposure to anoxia. Like most adult insects, adults are paralyzed within 30 seconds by anoxia; in contrast, larvae exhibit an escape behavior for up to 30 minutes in anoxia (Callier et al., 2015). There is also suggestive data that adults and larvae may use different anaerobic pathways. Lactate accumulation fully accounts for metabolic heat production in larvae while only accounting for ~30% for adults (Callier et al. 2015). Adults have been shown to accumulate significant quantities of alanine and acetate in addition to lactate, and models suggest this may improve glucose use efficiency and ATP/H^+ ratio (Feala et al., 2007); larval anaerobic pathways other than lactate production have not yet been assessed.

A third possible contributor to the greater anoxia-tolerance of adult *D. melanogaster* could be a greater concentration of protective organic solutes that act as antioxidants or protect against protein unfolding. Organisms tolerant to environmental stressors often accumulate large quantities of organic osmolytes for cytoprotective purposes (Yancey, 2001). These fall into several chemical classes: small carbohydrates (e.g. trehalose), polyols (e.g. glycerol, sorbitol), small neutral amino acids (e.g. glycine,

proline) and derivatives (e.g. ß-alanine, taurine), and methylamines [e.g. *N*-trimethylamine oxide (TMAO), betaine]. Betaine, proline, trehalose, and polyols have been shown to be particularly protective of macromolecules during osmotic stress in marine animals (Yancey, 2005). Cold tolerant insects accumulate high levels of polyols (e.g. sorbitol, glycerol) to prevent freezing, and desiccation-resistant animals produce high levels of trehalose and proline to protect against cell membrane disruption (Clegg, 2001; Storey, 1997). Alcoholic metabolites are commonly increased in response to anoxia in insects (Chen et al., 2002; Storey and Storey, 1990; Wegener, 1993). In particular, trehalose is known to be important to anoxia tolerance in adult *D. melanogaster*. Overexpression of trehalose-6-phosphate synthase (*tps1*) increases levels of trehalose and tolerance to anoxia in adult *D. melanogaster* (Chen et al., 2002; Chen et al., 2003). Moreover, expression of the *Drosophila* trehalose synthase enzyme in human cells extends tolerance to anoxia (Chen et al., 2003). Here I tested whether the better anoxia tolerance of adult *D. melanogaster* is associated with higher levels of protective metabolites, or a greater capacity to synthesize such metabolites during anoxia.

METHODS

Insects, general conditions, and anoxic exposure

Flies were maintained and selected as previously described (Campbell et al., 2018). For anoxic exposure, groups of 30 adult females or larvae were placed into plastic vials and purged with humidified nitrogen for the duration of the treatment time, using gas flows, humidification protocols, and chambers as previously described (Campbell et al., 2018). Individuals were transferred immediately from anoxia into liquid nitrogen to

prevent reoxygenation before metabolite measurements. All experiments were conducted at 25±1˚C.

Carbohydrate Assays

I measured whole-body concentrations of glucose, glycogen, and trehalose across anoxic exposure times corresponding to 0%-100% mortality—up to 2 h for larvae and 12 h for adults. Groups of 5 adult females or 5 late 3rd instar larvae were homogenized in 100 µL of ice-cold phosphate buffered solution (PBS; 137 mmol l^{-1} NaCl, 2.7 mmol l^{-1} KCl, 10 mmol l^{-1} Na_2HPO_4, 1.8 mmol l^{-1} KH_2PO_4, pH 7.4) and a 10 µL aliquot was saved for protein assays. Carbohydrate assay methods were modified from Tennessen et al. (2014). Briefly, measurement of glucose required the use of glucose oxidase activity to catalyze the oxidation of glucose to hydrogen peroxide that was quantified at an absorbance of 540 nm. The addition of trehalase and amyloglucosidase allowed for trehalose and glycogen to be broken down into glucose units to be quantified with the same glucose oxidase assay (Sigma; GAGO-20). All samples were assayed in triplicate.

Nuclear magnetic resonance (NMR) based metabolomics

For the experiments testing whether adults and larvae differ in pathways of anaerobic metabolism, and in levels of protective metabolites, I measured both larvae and adults at four time points during 1 h of anoxia—0, 0.25 h, 0.5 h, and 1 h, as this was a time frame that could be survived by both stages. Larval and adult samples (n=4 samples per stage and treatment time) were then assayed for a variety of metabolites using proton NMR (^{1}H-NMR).

For NMR sample preparations, groups of 30 flies were homogenized in 400 µL of ice-cold acetonitrile (50% v/v) and centrifuged at 17,000 × g for 10 minutes at 4˚C. To remove large macromolecules that may interfere with the NMR signal, the supernatant was passed through a 10 kDa microfilter (Nanosep OD010C33; Pall Life Sciences, Port Washington, New York, USA) at 6,000× g for 45 minutes at 4˚C. The remaining supernatant was then lyophilized and stored at -80˚C. Immediately before analysis with NMR, samples were hydrated using 650 µL of 100% deuterium (D_2O) with 0.01 mg/ml of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS; DLM-6DB-10X0.7; Cambridge Isotope Laboratories INC, Tewksbury, MA, USA) and transferred to a 5 mm NMR tube (SVCP-5-178; Norell Inc., Morganton, NC, USA) for immediate analysis. NMR spectra were acquired using a Bruker 400 MHz Advance II equipped with a broad band probe and analyzed at 298.22 K. Proton spectra, at 400.13 MHz, were acquired using 30°-pulses, a spectral width of 9615.385 kHz, collecting 512 scans with a length of 131072 data points with a relaxation delay of 5.0 sec. All NMR measurements were conducted in the ASU Magnetic Resonance Research Center.

Representative spectra from one adult and larval sample can be seen in Figure 3.1. Spectra were processed using the Chenomx 8.1 NMR Suite (Chenomx, Inc., Edmonton, Alberta, Canada). An exponential line-broadening of 0.5 Hz was applied prior to Fourier transformation. All spectra were referenced to the DSS signal at 0 ppm, automatically phased and baseline corrected. Peaks in the spectra were identified, aligned and quantified within the Chenomx software using “targeted profiling” algorithms (Weljie et al., 2006). ^{1}H-NMR spectra signals were assigned using the built-in Chenomx database or chemical shifts of additional standards not found in the database. Spectra for standards

were collected using two-dimensional NMR methods—homonuclear correlation spectroscopy (COSY) and heteronuclear single quantum correlation spectroscopy (HSQC)—to identify chemical shifts and confirm the locations of metabolites for target metabolites. The signal intensities of DSS were consistent across runs and therefore absolute rather than relative concentrations were calculated. A total of 49 metabolites were identified (mean ± SE shown Table 3.1).

Statistics

Carbohydrate levels were compared within life stages using ANOVA to assess the effects of time in anoxia and t-tests were used to compare carbohydrates between life stages during normoxia. Main effects and post hoc tests were conducted at a family-wise alpha and Type I error rate of 0.05.

Multiple methods were used to compare metabolomic data within and between groups. Firstly, principal component analysis (PCA) was used to identify "fingerprints" specific to each stage (larvae and adult) and time in anoxia (0, 15, 30, 60 min). PCA is commonly used to analyze complex datasets where variation can be reduced to a few components that can explain a large portion of the variation. Data were log transformed and scaled using Pareto scaling (Craig et al., 2006), wherein the concentrations of each metabolite were divided by the square root of the standard deviation of that metabolite. This method helps to distribute the importance of metabolites differing in concentration and reduces the weighting of a metabolite if the variance in measurements is large (Craig et al., 2006). Secondly, partial least squares discriminant analysis (PLS-DA) was used to further identify differences in metabolite profiles between stages and duration of anoxia. PLS-DA is similar to PCA; however, the components are structured to maximize their

correlation by treatment group instead of explaining maximal variation. After PLS-DA, t-tests were used to compare the fold differences in metabolites between clusters, and volcano plots were used to visualize significant differences between groups. Because I measured metabolites at four timepoints throughout anoxia, factorial ANOVAs were carried out to test for effects of treatment (anoxic vs normoxia), stage (larval vs. adult) and stage × treatment interaction on each metabolite.

All statistics were conducted using R software (Team, 2016). Various R packages [*doBy* (Højsgaard and Halekoh, 2016), *ggplot2* (Wickham, 2009), *lsmeans* (Lenth, 2016), *multcomp* (Hothorn et al., 2008)] were used for ANOVAs, posthoc testing and figures. Multivariate analyses were conducted using various R packages—*ropls* for PCA and PLS-DA (Thevenot et al., 2015), *muma* for the complementary univariate testing (Gaude et al., 2013), *ggplot2* for creating volcano plots (Wickham, 2009).

RESULTS AND DISCUSSION

Depletion of carbohydrate stores does not explain the duration of anoxia that can be tolerated in adult and larval Drosophila

I had hypothesized that adults might have higher carbohydrate stores than larvae. However, adults and larvae did not differ in initial (normoxic) whole-body total carbohydrate stores (glycogen + trehalose + glucose) or normoxic glycogen content, Figure 3.2). Adults and larvae did differ in the form of their carbohydrate stores, as normoxic adults had higher whole-body glucose levels (F=50.58, p<0.001) and larvae had higher whole-body trehalose levels (F=136.21, p<0.001).

I had also hypothesized that a lower ratio of metabolic rate to carbohydrate stores might allow adults to better conserve carbohydrate stores. However, total whole-body carbohydrate stores (glycogen + trehalose + glucose) did not decline during anoxia in either adults or larvae (Figure 3.2). Whole-body glycogen stores did decline in anoxia, but as this occurred, glucose accumulated (Figure 3.2). Glycogen decreased significantly at the first timepoint measured for each life stage— ~50% within the first 6 hours in adults (F=12.24, p=0.008) and by ~25% within an hour in larvae (F=1.45, p=0.31). Glucose levels doubled in adults after 6 h of anoxia (F=14.05, p=0.005), and similarly, larval glucose doubled within the first h of anoxia (F=32.69, p=0.001). Trehalose, on the other hand showed opposite effects between stages; adults increased trehalose 2-fold after 6 h (F=14.45, p=0.008) and larvae decreased by 25% after 1 h of anoxia (F=4.22, p=0.072). The accumulation of whole-body glucose in both adults and larvae during anoxia suggests that depletion of carbohydrate is unlikely to explain the duration of anoxia that can be tolerated.

If there is any role for carbohydrate depletion in determining anoxic death in adult flies, it could possibly relate to glycogen stores in critical tissues. Adult flies retained 40% of their whole-body glycogen after 12 h of anoxia, the maximal duration of anoxia that can be tolerated by adult flies at this temperature. Insects have been shown to exhibit decreases in glycogen down to ~20% of normal with no ill effects (Wegener, 1993), suggesting that sufficient glycogen remains available throughout the duration of anoxia that can be survived by flies, unless levels are much lower in tissue such as the brain.

The constancy of summed glycogen, trehalose and glucose during anoxia raises the question of the source of carbohydrate for glycolysis. For larvae, serine, xylitol,

mannitol, and glycerol decline in a manner that can approximately quantitatively account for the accumulation of lactate and alanine during anoxia, suggesting that these "novel" glycolytic substrates may be the primary anaerobic substrates in larvae. For adults, none of the metabolites I measured decline sufficiently to account for the accumulation of anaerobic endproducts during anoxia. I speculate that glycoproteins or glycolipids might serve as carbohydrate sources in anaerobic adults. In support of this hypothesis, concentrations of both phosphocholine and glycerol-3-phosphocholine fall with time in anoxic adults (Figure 3.6).

Effects of developmental stage and anoxia on metabolite profiles

Pooling across all conditions adults and larvae differed strongly in their metabolite concentrations (principle component analysis). For normoxic animals, metabolic profiles also differed significantly between adults and larvae (Figure 3.3A). Normoxic adults had significantly higher concentrations of 13 metabolites relative to larvae; including beta-alanine, alanine, aspartate, betaine, fatty acids, glutamine, methionine, phosphocholine, proline, sarcosine, succinate, taurine, and tryptophan (Figure 3.3B). Normoxic larvae had higher levels of nine metabolites relative to adults, including 1,6-anhydro-beta-glucose, choline, lactate, glucose-1-phosphate, glucose-6-phosphate, histidine, tyrosine, valine, and xylitol (Figure 3.3B). Tyrosine levels were more than 25x higher in larvae, consistent with the previously demonstrated elevations of tyrosine prior to molting in insects (McDermid and Locke, 1983). Mannitol and xylitol totaled more than 25 mmol kg^{-1} in larvae, but less than 15 mmol kg^{-1} in adults; these appear to be quantitatively very important sugar alcohols in *Drosophila*, and perhaps other insects, especially in larvae. Beta-alanine concentrations are about 7x higher in

adults than larvae, with concentrations of about 10 mmol kg^{-1} in adults. Higher beta-alanine levels are associated with a higher capacity to avoid cellular damage during hypoxic bouts by preventing osmotic stress (Vairetti et al., 2002).

Normoxic samples clustered separately from the anoxic samples within each stage (PLS-DA: adults: Figure 3.4A, larvae: Figure 3.4C). Thus, there were large changes in the metabolite profile after exposure to anoxia that were mostly consistent through the first hour of anoxia. Using univariate analysis, 15 metabolites significantly decreased in adults during anoxia, including fatty acids, multiple Kreb cycle intermediates (malate, fumarate and α-ketoglutarate), while putative anaerobic endproducts increased (lactate, alanine, and succinate, Figure 3.4B). For larvae, eight metabolites were significantly higher in normoxia, including several amino acids (glutamate, leucine, cysteine, tyrosine, and histidine) and two polyols (mannitol and glycerol); seven metabolites including known end products (alanine, lactate, succinate) and two protective metabolites (taurine, proline) increased in anoxia (Figure 3.4D). Adults and larvae differed strongly in their metabolite profiles during anoxia; PLS-DA models comparing adults and larvae after 60 min of anoxia show distinct clusters between life stages (Figure 3.3C); after 60 min of anoxia, adults had significantly higher concentrations of ten metabolites including aspartate, betaine, glycine, glucose-6-phosphate, acetate, glucose, sarcosine, phosphocholine, succinate, and beta-alanine. After 60 min of anoxia, larvae had higher concentrations of eight metabolites, including asparagine, glutamine, histidine, tyrosine, lactate, malate, trehalose, and ornithine (Figure 3.3D).

Evidence that lower anaerobic metabolic rate associated with paralytic hypometabolism and alternative anaerobic end-products contributes to better anoxia tolerance in adults

Our measurements of the accumulation rates of anaerobic end-products during the first 30 min suggest that the paralysis of the adults enables them to more strongly suppress metabolism during anoxia (Figure 3.5). During the first 30 min of anoxia, anaerobic metabolic rate is estimated to be roughly 60% higher in larvae, suggesting that larvae may experience more acid-base and energetic disruptions (Figure 3.5). Larvae accumulate lactate at two to three-times the rate of adults during the first 15 min of anoxia (Figure 3.6). This is likely due to lactate production by skeletal muscle, as the larvae are locomoting strongly during this time, while adults are paralyzed. After one hour, lactate levels rose to approximately 32 mmol kg^{-1} in larvae and 19 mmol kg^{-1} in adults (Figure 3.6). After 60 min of anoxia, total anaerobic end-products were equivalent in adults and larvae, consistent with our prior calorimetric study that demonstrated that adults and larvae have similar metabolic rates during 30-120 min of anoxic exposure.

Some anaerobic pathways alternative to lactate generation are thought to have benefits for ATP production, pH regulation, and balancing of redox potential (Murphy and Steenbergen, 2007). Both adults and larvae utilized alanine as an anaerobic end-product, however the ratio of alanine to lactate was higher in adults (Figure 3.5, 3.6). As previously reported (Feala et al., 2007), acetate and succinate increase in anoxic *Drosophila* adults, but this is not observed in larvae (Figure 3.6). These are relatively small (~10%) components of anaerobic endproducts in adults; yet they account for ~10% of heat dissipation during the first 30 mins of anoxia in adults and ~1% of heat dissipation in larvae. One benefit of using alanine, succinate and acetate pathways as

anaerobic end-products is increased ATP:proton production (Feala et al., 2007). Also, hydrolysis of acetyl-CoA to acetate is ADP-linked and contributes to ATP production in a manner that utilizes rapidly accumulating mitochondrial acetyl-CoA that cannot be metabolized in the absence of oxygen (Hochachka and Somero, 2002). Together, our data are consistent with the hypothesis that use of alternative anaerobic pathways contributes to the better capacity of adults to survive anoxic exposure.

Evidence for a role for protective metabolites in the higher anoxia tolerance of adults

To assess the hypothesis that higher levels of protective metabolites in adults attributes to their superior anoxia tolerance, I used factorial ANOVA to test the effects of stage and time in anoxia on the total concentration of a subset of metabolites hypothesized to be protective—β-alanine, betaine, glycine, proline, taurine, trehalose, and three polyols (glycerol, mannitol and xylitol). There was a significant interaction between time and stage ($F_{3,20}$=6.51, p=0.003), which can be seen as total protective metabolites decrease in larvae throughout anoxia while increasing in adults (Figure 3.7). During normoxia, larvae were nearly 40% higher in total protective metabolites than adults ($F_{1,5}$=27.90, p=0.003); individually, the protective metabolites glycerol, trehalose, mannitol and xylitol were all higher in larvae, while β-alanine was the sole protective metabolite higher in adults in normoxia. In anoxia, total protective metabolites were significantly different between life stages after 15 min ($F_{1,6}$=7.44, p=0.034); no other timepoints were statistically different between life stages. However, factorial ANOVAs comparing individual protective metabolites between time in anoxia and life stage indicate that protective metabolites β-alanine, betaine, glycine, taurine, glycerol, mannitol, and xylitol were either higher or increased during anoxia in adults (Figure 3.6,

Table 3.2), supporting the idea that induction of protective metabolites may contribute to the higher tolerance in adults.

At this point, the potential mechanisms by which induction of putative protective metabolites during anoxia might enhance adult *Drosophila* survival of anoxia can only be inferred from studies of other systems. Our data showed that β-alanine was nearly 5x higher in adults, and glycine and alanine increased during anoxia in adults (Figure 3.6). Small amino acids and related derivatives, particularly β-alanine, glycine and alanine, can exhibit cytoprotective properties by preventing cell membrane permeabilization and thus reducing osmotic stress during hypoxia-derived ATP depletion (Weinberg et al., 1987); cells incubated with high levels of glycine, alanine or β-alanine were more likely to survive an oxygen deprived state (Baines et al., 1990; Frank and Rauen, 2000; Pan et al., 2005; Vairetti et al., 2002). Betaine was higher and increased during anoxia in adults (Figure 3.6). Betaine has been shown to be important in protecting against cellular stress during salinity and heat stress (Diamant et al., 2001); at least partly due to stabilizing interactions with membrane phospholipids (Rudolph et al., 1986). Another interesting metabolite is taurine, which is nearly twice as high after 60 min of anoxia in adults (Figure 3.6). Taurine increases during anoxic exposure in turtles (Lehmann et al., 1988), and has been shown to decrease neuronal excitability and prevent cellular Ca^{2+} overload during anoxic exposure (Schurr and Rigor, 1987). Together, these are suggestive data that *D. melanogaster* have multiple protective organic metabolites (betaine, β-alanine, glycine, taurine) potentially contributing to better anoxia-tolerance in adults.

I expected trehalose to be an important protective metabolite that might differentiate adult and larval anoxia tolerance based on the prior demonstration that

trehalose is important in the capacity of adults to survive anoxia. Trehalose is commonly found in high levels in adult *Drosophila* during anoxia (Chen et al., 2002; Feala et al., 2007; Storey and Storey, 1990) and in response to hot or cold stress (Malmendal et al., 2006; Overgaard et al., 2007). This can be explained at least partially by the prevention of detrimental accumulation of protein aggregates by trehalose (Chen et al., 2002), and higher levels of trehalose correlate with better survival during lengthy hypoxic bouts (Chen et al., 2003). However, larvae have trehalose levels at least twice as high as adults, so trehalose levels cannot explain the higher anoxia-tolerance of adult *Drosophila*.

CONCLUSION

I used a combination of H-NMR based metabolomics and biochemical assays to investigate four hypotheses related to the variation in anoxia tolerance between larvae and adult *D. melanogaster*. This study demonstrates that the dramatic difference in anoxia tolerance between larval and adult *Drosophila* is unlikely to be related to carbohydrate stores, or a differential depletion of carbohydrate stores needed for glycolysis. Secondly, despite the fact that metabolic rates weren't different after 30 min of anoxia, adults do suppress metabolic rates to levels 60% of larvae during the first 30 min of anoxia, likely due to the paralysis of adults vs the escape locomotion of the larvae. Third, adults utilize alternate anaerobic end products (alanine, succinate and acetate) more so than larvae, likely attaining a better ATP/H^+ ratio and redox balance. Lastly, adults have higher levels or increase concentrations of several putatively protective metabolites (i.e. polyols, ß-alanine, taurine) that likely reduce cellular damage associated with osmotic or antioxidant stress during or after anoxia.

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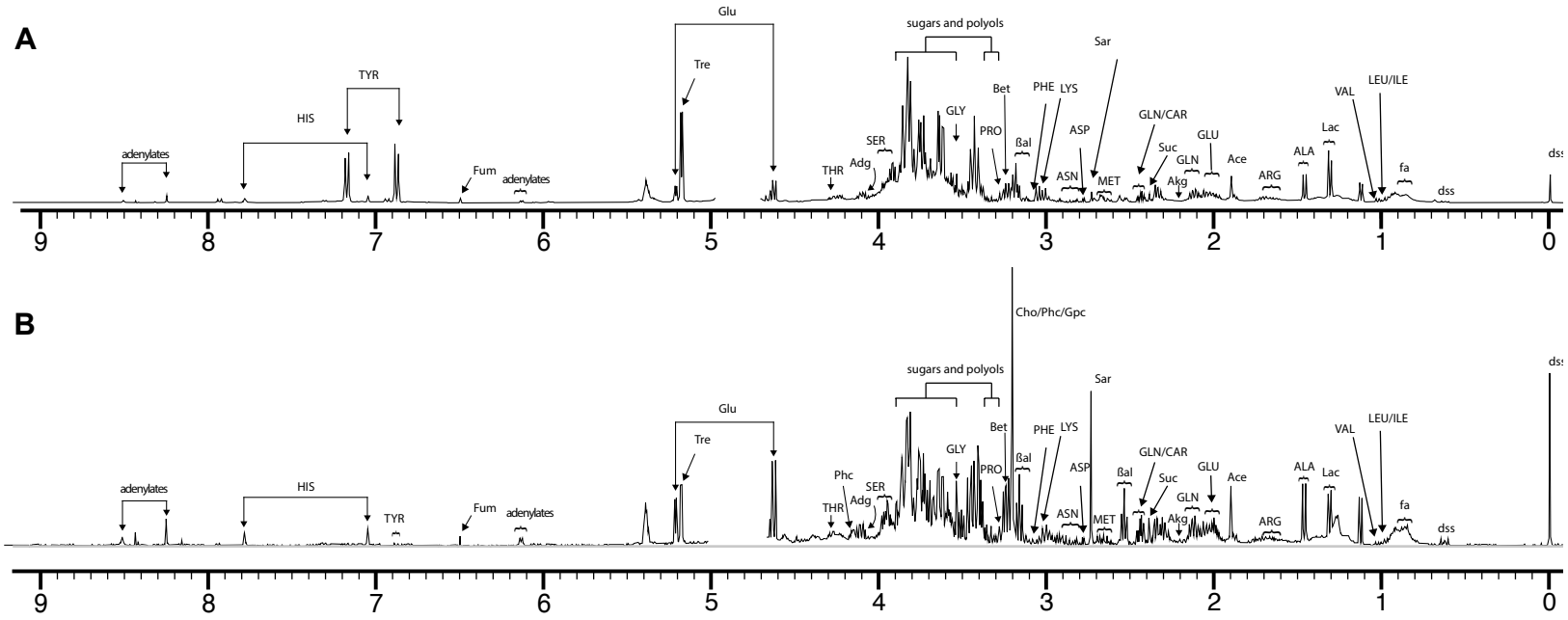

Figure 3.1: Representative ^{1}H-NMR spectra for larvae (A) and adults (B) in normoxia. Abbreviations can be found in Table 3.1.

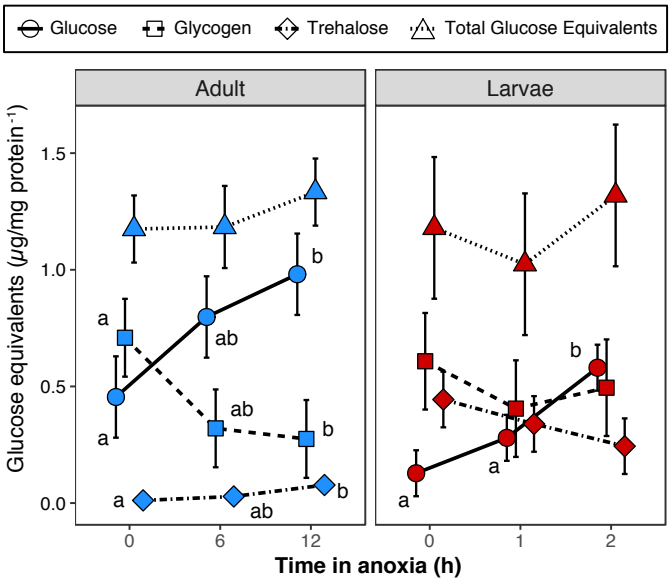

Figure 3.2: Whole-body carbohydrate levels for adult (blue) and larvae (red) during exposure to anoxia. Note the different x axes; adults survive and were tested over 12 h of anoxia, while larvae survive and were tested over 2 h of anoxia. Glucose increased and glycogen decreased over time in anoxia, while trehalose increased slightly for adults and decreased for larvae. Total glucose units (a measure of total carbohydrate stores) show no change over time in anoxia in adults or larvae. Different letters denote significantly different levels for each carbohydrate at $p<0.01$ (Tukey posthoc test).

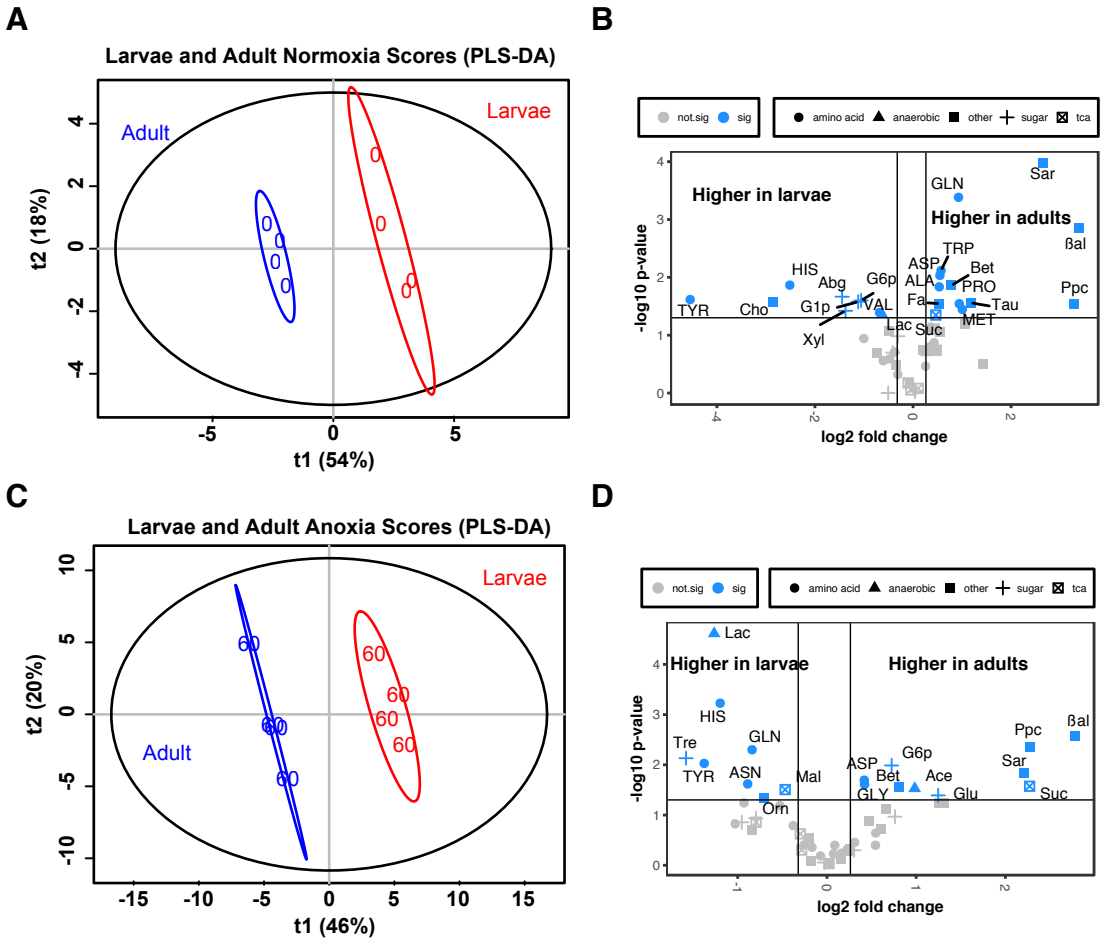

Figure 3.3: PLS-DA plots comparing metabolites between larvae and adults in normoxia (A) and after 60 min of anoxia (C), along with volcano plots for each (B&D). For volcano plots, labeled variables and blue points are significant (P value<0.05) and showed Fold Changes >1.2 or <0.8. Abbreviations are shown in Table 3.2 and PLS-DA results are shown in Table 3.3

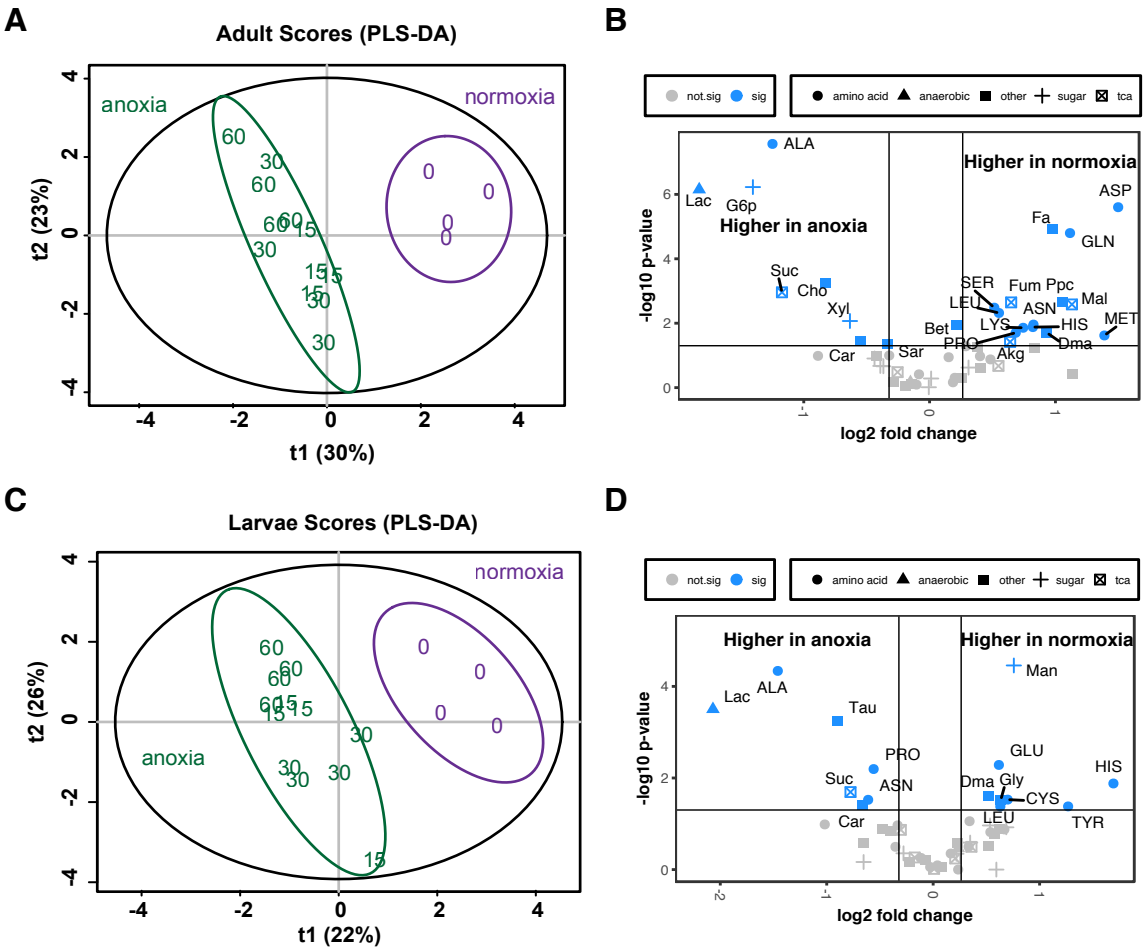

Figure 3.4: A&C) PLS-DA score plots and B&D) volcano plots comparing normoxic to anoxic metabolites for adults (A, B), and larvae (C, D). For volcano plots, labeled variables and blue points are significant (P value<0.05) and show Fold Changes >1.2 or <0.8. Abbreviations are shown in Table 3.2 and PLS-DA results are shown in Table 3.3.

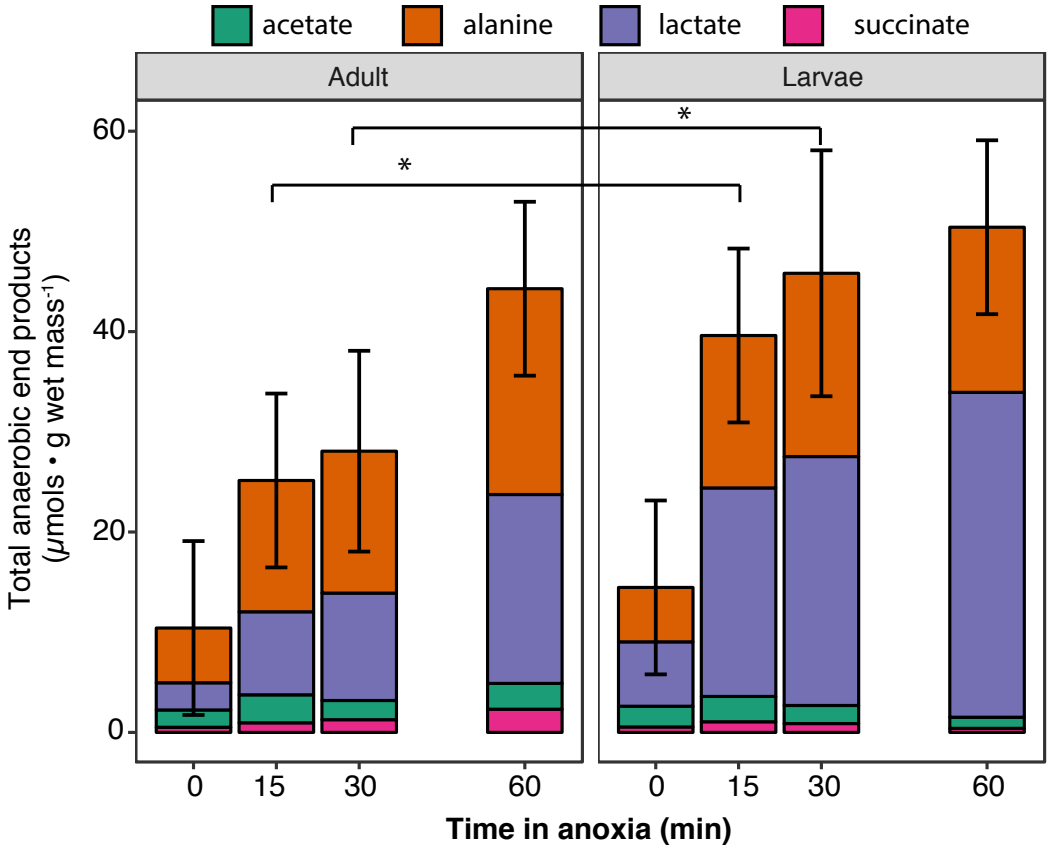

Figure 3.5: Total anaerobic end products in adults (left) and larvae (right) during 60 min of exposure to anoxia. Mean ±95% CI and * represents significance at $p<0.05$ (t-test).

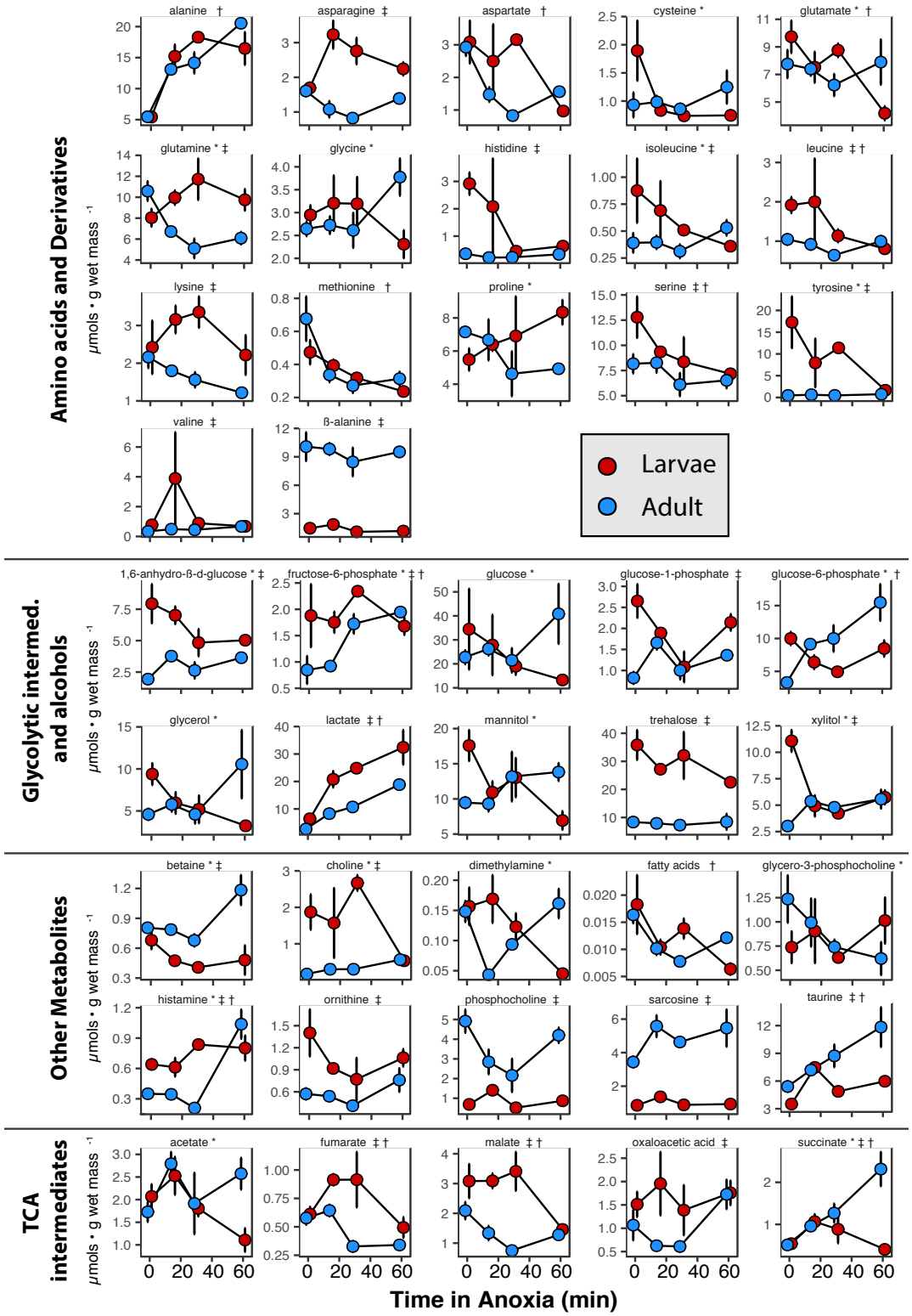

Figure 3.6: All metabolites significant for the effect of time (†), stage (‡), or the interaction (*).

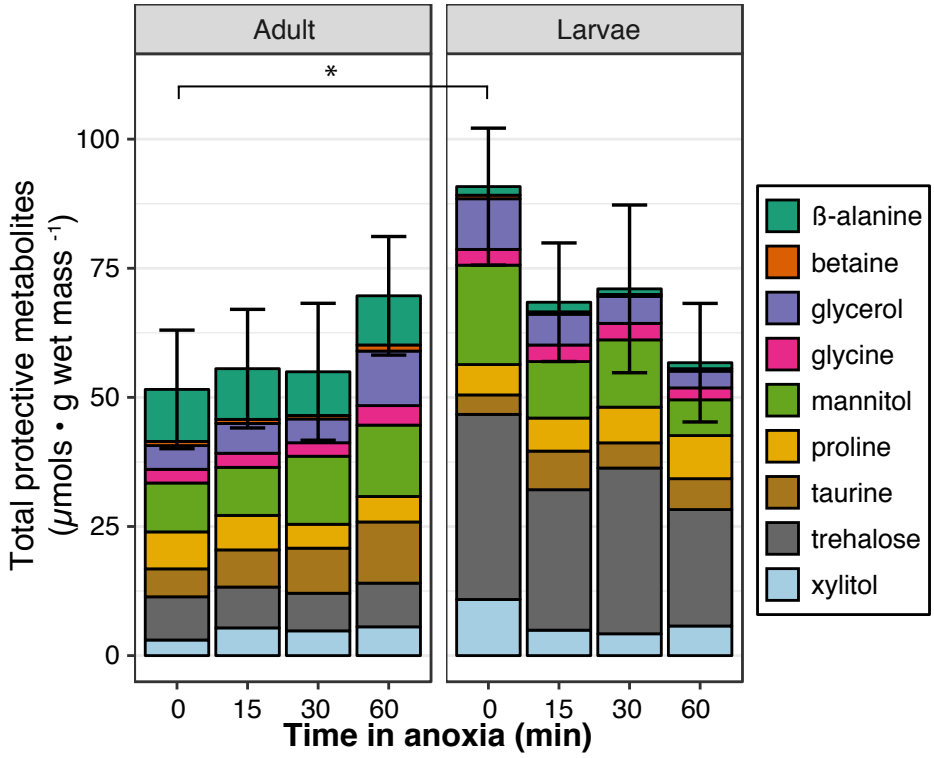

Figure 3.7: Total concentrations of putative protective metabolites in adult (left) and larval (right) *Drosophila* over 60 min of anoxia. Total protective metabolites increased in adults and decreased in larvae throughout anoxia (ANOVA interaction: $F_{1,20}$=6.51, p=0.003). Mean ±95% CI and * represents significance at p<0.05 between life stages (Tukey post-hoc test).

Table 3.1: Mean ± SE for each metabolite measured (µmol • g wet mass $^{-1}$) during normoxia and after exposure to anoxia.

stage	type	variable	abbr	Normoxia	15 min	30 min	60 min
adult	amino acid	alanine	ALA	8.413 +/- 0.672	20.16 +/- 0.67	27.397 +/- 5.904	31.598 +/- 0.64
		arginine	ARG	7.441 +/- 1.039	7.304 +/- 1.135	6.961 +/- 2.211	7.945 +/- 0.645
		asparagine	ASN	2.471 +/- 0.238	1.66 +/- 0.362	1.832 +/- 0.568	2.138 +/- 0.213
		aspartate	ASP	4.503 +/- 0.408	2.27 +/- 0.345	1.694 +/- 0.448	2.403 +/- 0.194
		cysteine	CYS	1.434 +/- 0.338	1.513 +/- 0.169	1.649 +/- 0.342	1.92 +/- 0.451
		glutamate	GLU	11.932 +/- 1.565	11.38 +/- 0.754	10.686 +/- 1.398	12.151 +/- 2.516
		glutamine	GLN	16.284 +/- 1.434	10.334 +/- 0.297	9.596 +/- 2.011	9.369 +/- 0.909
		glycine	GLY	4.071 +/- 0.244	4.194 +/- 0.297	4.629 +/- 0.737	5.808 +/- 0.624
		histidine	HIS	0.551 +/- 0.06	0.335 +/- 0.033	0.365 +/- 0.022	0.517 +/- 0.059
		isoleucine	ILE	0.601 +/- 0.139	0.609 +/- 0.094	0.604 +/- 0.141	0.816 +/- 0.11
		leucine	LEU	1.607 +/- 0.211	1.413 +/- 0.213	1.246 +/- 0.27	1.534 +/- 0.199
		lysine	LYS	3.326 +/- 0.445	2.768 +/- 0.129	2.834 +/- 0.488	1.873 +/- 0.139
		methionine	MET	1.041 +/- 0.207	0.518 +/- 0.067	0.524 +/- 0.117	0.482 +/- 0.065
		phenylalanine	PHE	0.441 +/- 0.035	0.516 +/- 0.031	0.625 +/- 0.159	0.725 +/- 0.084
		proline	PRO	11.008 +/- 0.459	10.271 +/- 1.899	9.475 +/- 2.775	7.585 +/- 0.337
		serine	SER	12.573 +/- 1.453	12.715 +/- 1.508	10.945 +/- 1.992	10.037 +/- 1.206
		threonine	THR	2.049 +/- 1.035	5.659 +/- 0.842	5.103 +/- 1.141	2.964 +/- 0.784
		tryptophan	TRP	0.494 +/- 0.01	0.491 +/- 0.062	0.551 +/- 0.117	0.576 +/- 0.061
		tyrosine	TYR	0.72 +/- 0.226	0.963 +/- 0.248	0.811 +/- 0.195	1.145 +/- 0.219
		valine	VAL	0.514 +/- 0.049	0.753 +/- 0.185	0.749 +/- 0.129	1.031 +/- 0.155
	other	acetate	Ace	2.66 +/- 0.338	4.295 +/- 0.399	3.353 +/- 0.839	3.96 +/- 0.535
		betaine	Bet	1.239 +/- 0.07	1.21 +/- 0.066	1.226 +/- 0.195	1.821 +/- 0.231
		carnitine	Car	1.067 +/- 0.101	1.523 +/- 0.346	2.711 +/- 1.207	2.382 +/- 0.48
		choline	Cho	0.263 +/- 0.029	0.464 +/- 0.058	0.571 +/- 0.129	0.867 +/- 0.078
		dimethylamine	Dma	0.228 +/- 0.027	0.067 +/- 0	0.189 +/- 0.045	0.248 +/- 0.037
		fatty acids	Fa	0.025 +/- 0.002	0.016 +/- 0.002	0.015 +/- 0.004	0.019 +/- 0.001
		glycerol	Gly	7.066 +/- 1.031	8.882 +/- 1.313	7.9 +/- 1.449	16.251 +/- 6.236
		histamine	Hst	0.539 +/- 0.05	0.53 +/- 0.068	0.494 +/- 0.173	1.597 +/- 0.22
		lactate	Lac	4.168 +/- 0.42	12.749 +/- 1.405	18.549 +/- 2.667	29.003 +/- 2.185
		ornithine	Orn	0.88 +/- 0.118	0.831 +/- 0.091	0.695 +/- 0.08	1.17 +/- 0.246
		phosphocholine	Phc	7.565 +/- 0.902	4.38 +/- 0.963	3.27 +/- 0.916	6.459 +/- 0.597
		sarcosine	Sar	5.296 +/- 0.503	8.584 +/- 0.992	8.943 +/- 1.825	8.407 +/- 1.697
		glycerophosphocholine	Gpc	1.902 +/- 0.376	1.528 +/- 0.381	1.485 +/- 0.355	0.955 +/- 0.261
		taurine	Tau	8.323 +/- 0.896	11.069 +/- 0.822	16.404 +/- 3.257	18.221 +/- 3.293
		ß-alanine	ßal	15.501 +/- 2.316	15.111 +/- 0.956	15.969 +/- 3.375	14.643 +/- 0.821
	sugar	1,6-anhydro-d-glucose	Adg	2.979 +/- 0.572	5.794 +/- 0.47	4.239 +/- 0.73	5.6 +/- 0.528
		glucose	Glu	35.028 +/- 4.446	40.375 +/- 4.286	39.601 +/- 8.574	62.813 +/- 19.191
		fructose-6-phosphate	F6p	1.304 +/- 0.405	1.417 +/- 0.109	2.625 +/- 0.202	2.997 +/- 0.101
		glucose-1-phosphate	G1p	1.266 +/- 0.217	2.553 +/- 0.253	1.773 +/- 0.327	2.095 +/- 0.069
		glucose-6-phosphate	G6p	5.096 +/- 0.32	14.065 +/- 0.568	17.628 +/- 3.127	23.813 +/- 4.268
		mannitol	Man	14.569 +/- 1.212	14.317 +/- 1.673	24.004 +/- 5.347	21.265 +/- 1.94
		trehalose	Tre	12.872 +/- 1.67	12.166 +/- 1.846	15.683 +/- 4.656	13.017 +/- 4.337
		xylitol	Xyl	4.649 +/- 0.53	8.259 +/- 1.031	7.998 +/- 0.752	8.572 +/- 1.361
	tca	alpha-ketoglutarate	Akg	1.442 +/- 0.157	1.068 +/- 0.194	1.371 +/- 0.234	1.196 +/- 0.149
		fumarate	Fum	0.889 +/- 0.092	0.99 +/- 0.078	0.659 +/- 0.163	0.523 +/- 0.066
		malate	Mal	3.215 +/- 0.444	2.058 +/- 0.367	1.705 +/- 0.551	1.968 +/- 0.167
		oxaloacetate	Oxa	1.642 +/- 0.497	0.963 +/- 0.131	1.028 +/- 0.15	2.655 +/- 0.481
		pyruvate	Pyr	0.765 +/- 0.138	1.465 +/- 0.119	1.219 +/- 0.37	0.779 +/- 0.077
		succinate	Suc	0.788 +/- 0.044	1.475 +/- 0.054	2.198 +/- 0.344	3.561 +/- 0.621

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stage	type	variable	abbr	Normoxia	15 min	30 min	60 min
larvae	amino acid	alanine	ALA	8.354 +/- 0.816	23.402 +/- 2.907	28.14 +/- 0.2	25.348 +/- 4.05
		arginine	ARG	8.304 +/- 0.55	8.095 +/- 0.362	6.159 +/- 0.298	7.781 +/- 0.8
		asparagine	ASN	2.607 +/- 0.242	4.971 +/- 0.619	4.245 +/- 0.586	3.454 +/- 0.294
		aspartate	ASP	4.746 +/- 1.008	3.846 +/- 1.736	4.859 +/- 0.149	1.489 +/- 0.143
		cysteine	CYS	2.914 +/- 0.82	1.282 +/- 0.114	1.134 +/- 0.114	1.148 +/- 0.135
		glutamate	GLU	14.973 +/- 1.793	11.576 +/- 1.719	13.461 +/- 0.757	6.441 +/- 0.786
		glutamine	GLN	12.373 +/- 1.306	15.32 +/- 1.05	18.03 +/- 3.035	14.997 +/- 1.572
		glycine	GLY	4.537 +/- 0.324	4.933 +/- 0.928	4.912 +/- 0.897	3.551 +/- 0.468
		histidine	HIS	4.489 +/- 0.628	3.201 +/- 2.699	0.692 +/- 0.058	0.978 +/- 0.081
		isoleucine	ILE	1.346 +/- 0.458	1.062 +/- 0.42	0.783 +/- 0.045	0.553 +/- 0.049
		leucine	LEU	2.95 +/- 0.311	3.077 +/- 1.692	1.747 +/- 0.251	1.24 +/- 0.057
		lysine	LYS	3.726 +/- 1.085	4.861 +/- 0.562	5.16 +/- 0.638	3.411 +/- 0.808
		methionine	MET	0.729 +/- 0.112	0.606 +/- 0.056	0.49 +/- 0.039	0.364 +/- 0.043
		phenylalanine	PHE	0.939 +/- 0.209	1.054 +/- 0.632	0.94 +/- 0.146	0.589 +/- 0.066
		proline	PRO	8.445 +/- 1.027	9.843 +/- 0.591	10.628 +/- 3.669	12.838 +/- 1.137
		serine	SER	19.679 +/- 3.157	14.385 +/- 0.524	12.867 +/- 3.762	11.034 +/- 0.743
		threonine	THR	6.556 +/- 2.721	4.838 +/- 0.968	5.255 +/- 0.578	3.311 +/- 0.243
		tryptophan	TRP	0.509 +/- 0.093	0.684 +/- 0.232	0.385 +/- 0.048	0.441 +/- 0.012
		tyrosine	TYR	26.57 +/- 9.113	12.221 +/- 8.554	17.513 +/- 0.126	2.542 +/- 0.282
		valine	VAL	1.195 +/- 0.14	5.989 +/- 4.753	1.359 +/- 0.077	1.044 +/- 0.076
	other	acetate	Ace	3.184 +/- 0.414	3.887 +/- 0.641	2.777 +/- 0.265	1.705 +/- 0.397
		betaine	Bet	1.046 +/- 0.138	0.729 +/- 0.07	0.627 +/- 0.012	0.739 +/- 0.226
		carnitine	Car	1.379 +/- 0.188	2.579 +/- 0.17	0.792 +/- 0.178	1.802 +/- 0.544
		choline	Cho	2.879 +/- 0.741	2.417 +/- 1.47	4.106 +/- 0.33	0.824 +/- 0.046
		dimethylamine	Dma	0.241 +/- 0.048	0.26 +/- 0.061	0.189 +/- 0.034	0.069 +/- 0.003
		fatty acids	Fa	0.028 +/- 0.008	0.016 +/- 0.002	0.021 +/- 0.003	0.01 +/- 0.002
		glycerol	Gly	14.432 +/- 1.981	9.16 +/- 1.97	7.987 +/- 2.519	4.975 +/- 0.718
		histamine	Hst	0.985 +/- 0.076	0.942 +/- 0.141	1.287 +/- 0.045	1.233 +/- 0.19
		lactate	Lac	9.886 +/- 1.82	32.014 +/- 4.567	38.215 +/- 2.435	49.884 +/- 9.64
		ornithine	Orn	2.157 +/- 0.491	1.413 +/- 0.089	1.185 +/- 0.447	1.636 +/- 0.182
		phosphocholine	Phc	1.06 +/- 0.373	2.167 +/- 0.197	0.797 +/- 0.028	1.342 +/- 0.262
		sarcosine	Sar	1.323 +/- 0.356	2.097 +/- 0.174	1.363 +/- 0.184	1.435 +/- 0.125
		glycerophosphocholine	Gpc	1.135 +/- 0.251	1.391 +/- 0.508	0.971 +/- 0.05	1.559 +/- 0.366
		taurine	Tau	5.401 +/- 0.48	11.503 +/- 0.314	7.508 +/- 0.606	9.196 +/- 0.602
		ß-alanine	ßal	2.248 +/- 0.471	2.855 +/- 0.419	1.665 +/- 0.023	1.788 +/- 0.136
	sugar	1,6-anhydro-d-glucose	Adg	12.215 +/- 2.382	10.799 +/- 1.074	7.444 +/- 1.651	7.743 +/- 0.421
		glucose	Glu	52.996 +/- 25.856	42.831 +/- 19.321	29.241 +/- 5.504	20.437 +/- 2.304
		fructose-6-phosphate	F6p	2.892 +/- 0.918	2.699 +/- 0.307	3.606 +/- 0.016	2.585 +/- 0.257
		glucose-1-phosphate	G1p	4.08 +/- 0.614	2.907 +/- 0.158	1.669 +/- 0.559	3.297 +/- 0.302
		glucose-6-phosphate	G6p	15.388 +/- 1.543	9.844 +/- 1.614	7.596 +/- 0.093	13.031 +/- 1.907
		mannitol	Man	27.066 +/- 3.369	16.829 +/- 2.407	20.052 +/- 4.272	10.663 +/- 2.017
		trehalose	Tre	28.102 +/- 15.959	41.838 +/- 2.615	49.368 +/- 12.851	34.679 +/- 2.11
		xylitol	Xyl	17.032 +/- 1.576	7.579 +/- 1.534	6.506 +/- 0.058	8.828 +/- 1.014
	tca	alpha-ketoglutarate	Akg	1.537 +/- 0.309	1.428 +/- 0.277	0.826 +/- 0.088	0.963 +/- 0.076
		fumarate	Fum	0.941 +/- 0.098	1.406 +/- 0.076	1.407 +/- 0.374	0.76 +/- 0.138
		malate	Mal	4.743 +/- 0.878	4.754 +/- 0.383	5.245 +/- 0.997	2.244 +/- 0.122
		oxaloacetate	Oxa	2.327 +/- 0.412	3.007 +/- 1.041	2.134 +/- 0.823	2.704 +/- 0.399
		pyruvate	Pyr	1.175 +/- 0.142	1.61 +/- 0.031	1.003 +/- 0.142	0.891 +/- 0.138
		succinate	Suc	0.834 +/- 0.075	1.636 +/- 0.275	1.36 +/- 0.506	0.625 +/- 0.083

Table 3.2: Results of factorial ANOVAs for each metabolite comparing metabolite concentration by time in anoxia and life stage.

			Time			**Stage**			**Time:Stage**		
	Metabolite	Abbreviation	F-stat	*p*		F-stat	*p*		F-stat	*p*	
amino acid	alanine	ALA	35.247	<0.001	***	0.004	0.949		0.696	0.412	
	arginine	ARG	0.014	0.906		2.087	0.161		0.411	0.527	
	asparagine	ASN	0.022	0.883		26.502	<0.001	***	0.275	0.605	
	aspartate	ASP	10.666	0.003	**	1.073	0.31		1.071	0.311	
	cysteine	CYS	1.374	0.252		0.005	0.944		6.219	0.02	*
	glutamate	GLU	10.652	0.003	**	0.202	0.657		9.157	0.006	**
	glutamine	GLN	2.127	0.157		11.173	0.003	**	7.422	0.012	*
	glycine	GLY	0.090	0.767		0.236	0.632		9.329	0.005	**
	histidine	HIS	3.058	0.093		21.226	<0.001	***	3.560	0.071	
	isoleucine	ILE	1.080	0.309		4.333	0.048	*	7.472	0.011	*
	leucine	LEU	5.325	0.03	*	5.652	0.025	*	4.135	0.053	
	lysine	LYS	3.886	0.06		9.906	0.004	**	1.667	0.208	
	methionine	MET	18.650	<0.001	***	0.426	0.52		0.043	0.838	
	phenylalanine	PHE	0.108	0.745		3.339	0.08		2.951	0.098	
	proline	PRO	0.003	0.957		2.088	0.161		8.383	0.008	**
	serine	SER	10.042	0.004	**	6.796	0.015	*	0.936	0.343	
	threonine	THR	0.029	0.867		2.000	0.17		0.848	0.366	
	tryptophan	TRP	0.016	0.901		0.044	0.836		1.243	0.276	
	tyrosine	TYR	4.185	0.051		73.398	<0.001	***	11.557	0.002	**
	valine	VAL	0.188	0.668		10.423	0.003	**	2.888	0.102	
anaerobic	acetate	Ace	2.322	0.14		2.638	0.117		8.460	0.008	**
	lactate	Lac	55.767	<0.001	***	20.186	<0.001	***	0.773	0.388	
other	b.alanine	Bal	1.348	0.257		372.828	<0.001	***	0.846	0.366	
	betaine	Bet	0.003	0.953		23.903	<0.001	***	7.089	0.013	*
	carnitine	Car	1.817	0.19		0.138	0.713		2.790	0.107	
	choline	Cho	0.033	0.857		42.868	<0.001	***	16.522	<0.001	***
	dimethylamine	Dma	3.340	0.08		0.094	0.761		14.100	0.001	***
	fatty acids	Fa	5.059	0.034	*	0.533	0.472		1.751	0.198	
	glycerol	Gly	0.971	0.334		0.156	0.696		20.056	<0.001	***
	histamine	Hst	12.040	0.002	**	12.123	0.002	**	4.703	0.04	*
	ornithine	Orn	0.073	0.789		20.788	<0.001	***	1.294	0.266	
	phosphocholine	Ppc	0.005	0.945		45.018	<0.001	***	0.040	0.843	
	sarcosine	Sar	1.029	0.32		138.834	<0.001	***	0.642	0.431	
	glycero-3-phosphocholine	Gpc	1.070	0.311		0.096	0.759		5.244	0.031	*
	taurine	Tau	15.793	0.001	***	13.104	0.001	**	2.110	0.159	
sugar	fructose-6-phosphate	F6p	9.507	0.005	**	8.761	0.007	**	8.700	0.007	**
	glucose	Glu	0.171	0.683		2.716	0.112		5.413	0.028	*
	glucose-1-phosphate	G1p	0.246	0.624		13.409	0.001	**	1.831	0.188	
	glucose-6-phosphate	G6p	12.733	0.001	**	0.321	0.576		16.449	<0.001	***
	mannitol	Man	2.684	0.114		0.000	0.995		22.414	<0.001	***
	trehalose	Tre	0.503	0.485		70.114	<0.001	***	0.103	0.751	
	1,6-anhydro-ß-d-glucose	Abg	0.046	0.832		37.185	<0.001	***	7.675	0.01	*
	xylitol	Xyl	0.006	0.941		5.931	0.022	*	7.076	0.013	*
tca	a-ketoglutarate	Akg	3.035	0.094		0.037	0.85		1.076	0.31	
	fumarate	Fum	10.993	0.003	**	9.893	0.004	**	0.730	0.401	
	malate	Mal	12.238	0.002	**	24.334	<0.001	***	0.862	0.362	
	oxaloacetic acid	Oxa	2.821	0.105		10.004	0.004	**	1.493	0.233	
	pyruvate	Pyr	3.224	0.085		2.922	0.1		0.679	0.418	
	succinate	Suc	6.704	0.016	*	15.159	0.001	***	29.660	<0.001	***

Significance at p<0.001***, p<0.01**, p<0.05*

Table 3.3: Results from multivariate PLS-DA comparing adults and larvae during normoxia and anoxia. R2X represents the proportion of the variation in anoxia explained by the X variables, R2Y is the total proportion of the variation in anoxia explained by the full PLS-DA model, and Q2 is the cross validated R^2 or the proportion of anoxia predicted by the PLS-DA model. RMSEE is the root mean square error of estimation, or the square root of the mean error between the actual and the predicted responses.

Group	**Comparison**	**R2X**	**R2Y**	**Q2**	**RMSEE**
Normoxia	adult vs larvae	0.717	0.994	0.967	0.050
Anoxia	adult vs larvae	0.663	0.994	0.965	0.050
Larvae	anoxia vs normoxia	0.621	0.980	0.890	0.071
Adult	anoxia vs normoxia	0.672	0.975	0.843	0.080

CHAPTER 4

GENOME-WIDE ASSOCIATION ANALYSIS OF ANOXIA TOLERANCE IN *DROSOPHILA MELANOGASTER*

INTRODUCTION

Oxygen limitation plays a key role in many pathologies, some as a result of high tissue metabolism (i.e. cancer) and others from deprivation of O_2 supply including stroke, heart attack, and sleep apnea (Eltzschig and Eckle, 2011). However, as yet, there remain many controversies concerning the basic mechanisms by which tissue anoxia and hypoxia negatively impact organisms. In nature, animals occasionally run the risk of being exposed to low oxygen environments and must respond in a manner to allow them to survive until oxygen levels are restored. Some lower vertebrates can survive long bouts of anoxia (i.e. carp and turtles; Bickler and Buck, 2007). However, within mammals, humans can tolerate only a few minutes of anoxia before severe brain damage occurs while the most tolerant mammal (naked mole rat) can survive 18 min of anoxic exposure (Park et al., 2017). While there have been many studies examining interspecific variation in anoxia-tolerance, we have little understanding of the genetic bases to intraspecific variation in anoxia-tolerance.

Differences between anoxia-sensitive and anoxia-tolerant species are believed to primarily involve differences in abilities to downregulate energy turnover and/or abilities to upregulate anaerobic ATP production (Hochachka et al., 1996). Matching ATP

demand and supply allows some anoxia-tolerant species to maintain ATP levels during anoxia, preventing deleterious effects such as decrease in pH, altered calcium homeostasis, increased intracellular osmotic pressure and/or mitochondrial damage (Hochachka and Somero, 2002). Plausibly this may also be true for intraspecific variation, suggesting that genetic variation in the capacities to down-regulate metabolically demanding activities (e.g. protein synthesis, ion channel activity), or variation in anaerobic capacities might underlie variation in anoxia tolerance. Additionally, variation in the capacity to survive anoxia may be related to an organism's ability to delay or repair damage that occurs during anoxia or reoxygenation. Previous studies have indicated that mechanisms that protect against protein unfolding can be important in anoxia-tolerance, including induction of heat shock proteins (King and MacRae, 2015), and trehalose (Chen et al., 2002), suggesting that genes involved in coping with protein unfolding might be important in intraspecific variation in anoxia tolerance. Anoxic exposure is also associated with oxidative damage after reperfusion (Granger and Kvietys, 2015) and inflammation (Eltzschig and Carmeliet, 2011), suggesting that genes that affect ROS production, removal, or damage repair, or genes that influence the inflammatory response might be important in mediating intraspecific variation in anoxia tolerance.

One strategy for understanding the genetic basis of tolerance to hypoxia is through the use of model organisms and their translational power to understand oxygen-mediated pathways in human disease (Farahani and Haddad, 2003). *Drosophila melanogaster* is a well-suited model for multiple reasons—it is hypoxia-tolerant, the

majority of major metabolic pathways are conserved between flies and humans, and >60% of the fly genome is conserved humans. While anoxia tolerance of larval *Drosophila* has yet to be thoroughly investigated, there have been extensive research on anoxia tolerance in adults. Adults do not survive more than 12 h of anoxia and the rapid decrease in survival after 4 h coincides with large accumulations of protein aggregations (Chen et al., 2002). Heat shock proteins (HSP) and trehalose are two mechanisms associated with the prevention of protein unfolding (Azad et al., 2009; Chen et al., 2002). Flies exposed to severe hypoxia upregulate HSP expression that corresponds with increased survival (Azad et al., 2009), and overexpression of trehalose-6-phosphate synthase (*tps1*) increased survival in anoxia (Chen et al., 2003). Another important gene found to benefit anoxia tolerance is involved with RNA editing of ion channels (Ma et al., 2001; Palladino et al., 2000) and plays a role in ROS metabolism by regulating expression of ROS scavengers (Chen et al., 2004). Additionally, genes involved in metabolism, immune response, oxidative stress, and protein unfolding are overepresented in flies exposed to severe hypoxia (Azad et al., 2009; Gleadle and Ratcliffe, 1998; Liu et al., 2006; Zhou et al., 2007). However, the causal mechanisms are not yet clear, and the relative importance of these various responses remain elusive.

In the previous two chapters, I examined multiple hypotheses based on the current paradigm regarding anoxia tolerance. Vertebrate studies demonstrate that the matching of ATP supply and demand in a manner that preserves cellular ionic homeostasis is responsible for surviving anoxic exposure. In response to anoxia, adult *Drosophila* are ~8x more tolerant than larvae (Callier et al., 2015). Yet, adults are able to tolerate long

durations of anoxia with minimal ATP and tolerate large disturbances in ionic status (Campbell et al., 2018). On the contrary, larval ATP falls to near zero more quickly and the degree of extracellular ionic disturbance is less pronounced in larvae (Campbell et al., 2018). Secondly, metabolomics experiments demonstrate that adults have lower metabolic rates during the first 30 min of anoxia and upregulated protective metabolites during the first h of anoxia while larvae did not (see Chapter 3). As of yet, no studies have examined the genetic basis to variation in anoxia tolerance among individual *D. melanogaster*.

Here, I use the *Drosophila* Genetic Reference Panel (DGRP) to conduct a genome-wide association study to identify genetic variation and target genes related to anoxia tolerance in adult and larval *D. melanogaster*. The DGRP consists of more than 200 fully sequenced inbred strains derived from a single outbred population of flies developed as a resource for whole genome association mapping (Mackay et al., 2012). This resource allows for a phenotypic trait to be directly mapped to the genetic variation between lines to identify target genes that contribute to phenotypic variation. In addition to identifying target genes associated with variation in anoxia tolerance among individual larvae and adults, I asked whether anoxia tolerance in the DGRP is correlated with other phenotypes previously investigated for the DGRP. In particular, I addressed whether the variation in anoxia tolerance is associated with carbohydrate stores, resistance to oxidative stress, or the strength of immune defenses.

METHODS

Fly stocks and anoxia survival phenotypes

DGRP lines were obtained from the Bloomington Stock Center. All flies were maintained on the standard cornmeal molasses diet and reared at 25˚C. Adults 3-4 days old and late 3rd instar larvae (LL3) were collected and separated into groups of 10-20 individuals per replicate, with a minimum of 3 replicate vials per line—adults were also separated by sex and assayed separately. Anoxia treatments were conducted as previously described (Campbell et al., 2018). Behavioral responses were rapid—demonstrated by larvae climbing out of the media and adults becoming paralyzed in less than one minute—indicating that the perfusion of nitrogen into vials was rapid. The duration of anoxic exposure was based on previous experiments; adults were exposed to 6 h of anoxia and larvae were exposed to 1 h of anoxia; exposures that cause approximately 50% mortality in outbred lines (Callier et al. 2015; Campbell et al. 2018). After exposure to anoxia, vials were removed from the chamber and placed on their sides in an incubator to allow for recovery under normal conditions. After 24 hours, the number of survivors were counted for each vial; adult survivors were counted as the number with the ability to move. Previous data (Callier et al., 2015) show that LL3 pupate within 24 hours after anoxia, and therefore the number of pupae present after 24 hours of recovery was considered the number of surviving larvae. The survival phenotype was presented as the proportion surviving for each replicate.

Quantitative Genetic Analyses

A linear mixed model was used to determine variance components in anoxia survival across DGRP lines and between sexes for adults. Variances were portioned for each phenotype using the model: $Y = \mu + Sex + Line + Sex * Line + \varepsilon$, where *Sex* is

fixed, *Line* is random, and ε is the error variance. Reduced models were also fit using: $Y = \mu + Line + \varepsilon$ for each sex separately and for larvae. Variance components were estimated using the restricted maximum likelihood (REML) method. A minimum of three replicates and ten flies per replicate were analyzed per DGRP line and sex, which allowed the calculations of broad-sense heritability (H^2) for each of five analyses: adults pooled by sex, male and female analyzed separately, larvae, and a pooled analysis of adults and larvae. Broad sense heritability (H^2) was calculated as $H^2 = (\sigma_L^2 + \sigma_{SL}^2)/(\sigma_L^2 + \sigma_{SL}^2 + \sigma_E^2)$, where σ_L^2 is the variance component among lines, σ_{SL}^2 is the line-by-sex or line-by-stage variance component, and σ_E^2 is the sum of all other sources of variation. H^2 was calculated for larvae and each sex separately using $H^2 = \sigma_L^2/(\sigma_L^2 + \sigma_E^2)$. Cross-sex ($r_{gsex}$) and cross-stage genetic correlation (r_{gstage}) was calculated as $r_{gsex} = \sigma_L^2/\sqrt{(\sigma_{LM}^2 \times \sigma_{LF}^2)}$ for adults and $r_{gstage} = \sigma_L^2/\sqrt{(\sigma_{LL}^2 \times \sigma_{LA}^2)}$ for life stage, where σ_L^2 is the variance component among lines for sexes or life stages combined, σ_{LM}^2 and σ_{LF}^2 are variance components among lines for males and females, σ_{LL}^2 is the variance for component for larvae, and σ_{LA}^2 is the variance component for adults pooled by sex.

Genome-wide Association Study

Associations for anoxia survival were computed using line means for survival phenotypes using the DGRP pipeline (dgrp2.gnets.ncsu.edu); the DGRP pipeline consists of >2.4 million SNPs with minor allele frequencies (MAF) present in at least four lines (Huang et al., 2014), and adjusts means for the effects of *Wolbachia* infection and 5 major chromosomal inversions. Associations were performed for adults and larvae using a linear mixed model: $Y = \mu + SNP + Sex + SNP(Sex) + Line(SNP) + \epsilon$, at a

nominal P-value threshold of $p<10^{-5}$ to be significantly associated with trait variation. A reduced model omitting the fixed effect of sex was used for associations of larvae and separate sexes of adults. Next, gene ontology analyses were performed using the DAVID Functional Annotation Tool and GOFinder (Huang et al., 2009a; Huang et al., 2009b), with Bonferroni correction for multiple tests. All stats were conducted using R software and various packages [doBy (Højsgaard and Halekoh, 2016), ggplot2 (Wickham, 2009), dplyr (Wickham et al., 2017), nlme (Pinheiro et al., 2017)].

RESULTS

Quantitative genetics of anoxia tolerance

Survival phenotypes were collected for adult males and females in 171 lines after exposure to 6 hours of anoxia (Fig 4.1A, Appendix A). Survival ranged from 0-100% in females and 0-98% in males. There was a significant amount of genetic variation in anoxia tolerance, with a broad inheritance (H^2) of 0.552 (Table 4.1). Adults exhibited a sexually dimorphic response to anoxic exposure with females being generally more tolerant than males (main effect of sex, P<0.0001, Table 4.1). Additionally, a strong phenotypic correlation between male and female anoxia tolerance (Figure 4.1B), a cross genetic correlation of r_{gsex}=0.999, and a nonsignificant interaction term (Table 4.1) suggest that although females are more tolerant, the genotype did not affect the difference between males and females. Thus, some polymorphisms identified by the GWA may be sex-specific, while the majority may affect both sexes.

For larvae, survival was measured for 169 lines after exposure to 1 hour of anoxia (Fig. 4.2A, Appendix A). Larval survival varied tremendously between lines and spanned

a range of 20-98% survival with a broad sense heritability of H^2= 0.695 (Table 4.1). Anoxia tolerance in larvae was correlated with combined adult anoxia tolerance (Figure 4.2B). However, the low correlation between adult and larval anoxia tolerance (r=0.18, p=0.02), along with a low cross-genetic correlation (Table 4.1, r_{gs}=0.249) suggests that, in general, different genes affected anoxia tolerance larvae and adults.

Additionally, I compared my anoxia phenotypes to numerous other physiological and life history traits previously measured for the DGRP; this allows for the approximation of genetic correlation between these phenotypes and anoxia tolerance (Table 4.2). Correlations were conducted on 16 traits in adults combined by sex, 48 in males, 40 in females, and 31 traits in larvae. Anoxia tolerances in both males and females were positively correlated with three identical traits [chill coma recovery, starvation resistance (Mackay et al., 2012), and alcohol tolerance (Morozova et al., 2015)] and negatively correlated with one trait [sensitivity to oxidative stress (Jordan et al., 2012). Of the 8 traits found to be significantly correlated with anoxia-tolerance in only males (Table 4.2), anoxia tolerance was most strongly correlated with decreasing susceptibility to unfolded proteins (r=-0.317, p=0.001; Chow et al., 2013). Of the 11 traits found to be significantly correlated with anoxia tolerance in only females, anoxia was most correlated with resistance to fungal infection (r=0.424, p=0.001; Wang et al., 2017) and decreasing lipid content (r=-0.343, p=0.001; Nelson et al., 2016). For larvae, 6 traits were correlated with anoxia (Table 4.2). The most correlated trait was State 3 mitochondrial respiration rate (r=0.415, p=0.011; Jumbo-Lucioni et al., 2012). Additionally, larval anoxia tolerance

was positively correlated with two traits for resistance to oxidative stress (Jordan et al., 2012; Weber et al., 2012).

Genome-wide association analyses of anoxia tolerance

GWA analyses were performed for mean anoxia tolerance in adults and larvae exposed to 6 h and 1 h of anoxia, respectively. Analyses were performed on five traits: the anoxia-tolerance of pooled males and females, the difference in anoxia-tolerance between males and females, the anoxia-tolerance of males and females separately, and the anoxia-tolerance of larvae. Prior to analyses, data were tested for the effect of *Wolbachia* infection, polymorphic inversions, and the amount of polymorphic relatedness (Huang et al., 2014). There was a significant effect of the inversion *In_2R_NS* on anoxia-tolerance in males, females, and the difference between sexes, and an effect of the inversion *In_2L_t* on the difference between sexes in anoxia-tolerance (Appendix B). There was no effect of Wolbachia infection on anoxia tolerance.

The GWA for adults found 159 significant SNPs in or near 117 genes (Figure 4.3A, Appendix C); 56% of SNPs were found in intronic regions, while the remaining SNPs fell within coding regions (~8%), untranslated regions (UTR, ~5%) and intergenic regions (~29%). For larvae, the GWA found 32 SNPs in or near 22 genes to be significant (Fig.4.3B, Appendix D); the majority of SNPs were found in intronic regions (67%), 16% fell within coding regions, while the remainder fell within UTR (6%) and intergenic (10%) regions. Only three genes overlap between all of the four adult anoxia phenotypes (male, female, pooled sex, difference between sexes) and the larval phenotype (Figure 4.4).

Gene ontology enrichment analyses were conducted to group genes with related function to investigate possible physiological mechanisms responsible for anoxia tolerance. Adult genes showed significant enrichment in 12 functional clusters; of the top four clusters, one was related to immunoglobulin structure and three were related to ion transport (Table 4.3). Gene ontology enrichment for larval genes returned three enriched groups that involve alternative splicing, the SH3 domain, and transmembrane integrity (Table 4.4).

DISCUSSION

Natural variation and heritability of anoxia tolerance

Anoxia tolerance is highly variable across DGRP lines, sexes and between life stages. Although anoxia tolerance between sexes was strongly correlated (Fig. 4.1B), differences between sexes varied substantially in different lines; the largest difference was within DGRP_373 where 98% of females and 32% males survived 6 h of anoxia and the smallest difference was within multiple lines (DGRP_85, DGRP_100, DGRP_105, DGRP_287, DGRP_531), wherein no females or males survived anoxic exposure. Comparing between life stages, anoxia tolerance was not strongly correlated (Fig. 4.2B) and variation within lines was highly variable; for example, 98% of larvae from DGRP_530 survived 1 h of anoxia while only 3% of adults from the same line survived 6 h of anoxia. Together these results suggest that tolerance of anoxia depends on different genetic architecture in larvae and adults.

The heritability of anoxia tolerance was quite high, ranging from 0.504-0.695. Compared to other DGRP studies, tolerance to toxins (lead tolerance, H^2 = 0.76-0.80,

methylmercury tolerance, H^2 = 0.8, (Montgomery et al., 2014; Zhou et al., 2016)) tend to have very high heritability. Tolerance to radiation, which is linked to oxidative stress, also has a very high heritability (H^2 = 0.8; Vaisnav et al., 2014). However, two other studies of oxidative stress resistance found lower heritability [acute oxidative stress H^2=0.36-0.48 (Weber et al., 2012); chronic oxidative stress H^2=0.14-0.41 (Jordan et al., 2012)]. The H^2 for anoxia-tolerance was in the range or higher than reported for other parameters related to physiological stress responses [chill coma recovery H^2 = 0.36 (Mackay et al., 2012), alcohol sensitivity H^2 = 0.38-0.42 (Morozova et al., 2015), food intake H^2 = 0.45 (Garlapow and Mackay, 2015), mitochondrial function H^2 = 0.15-0.20 (Jumbo-Lucioni et al., 2012), resistance to fungal infection H^2 = 0.23-0.47 (Wang et al., 2017), and starvation resistance H^2 = 0.54 (Mackay et al., 2012)].

Correlations with immune-related responses of DGRP suggest a possible key role of immune function in anoxia tolerance

Hypoxic/anoxic bouts have been shown to illicit an inflammatory response that is key to many oxygen-mediated pathologies and can lead to organ dysfunction (Eltzschig and Carmeliet, 2011; Eltzschig and Eckle, 2011), suggesting that variation in the inflammatory response might be important in explaining variation in anoxia-tolerance. Anoxia-tolerance was also correlated with several indices of immune function in other GWA studies of DGRP phenotypes. Variation in anoxia tolerance across the DGRP lines was strongly positively correlated with a resistance to fungal infection (Table 4.2); this was the strongest correlation of all adult comparisons (r=0.424, p=0.001). Anoxia-tolerance was also positively correlated with resistance to enteric infection (r=-0.291,

p=0.001; note the correlation here is negative because this study quantified susceptibility to infection, the inverse of resistance). These results indicate that anoxia induces an inflammatory response, either directly or indirectly related to a response to cellular damage. Other studies confirm this notion by demonstrating the genes upregulated in severe hypoxia/anoxia are associated with an inflammatory response (Zhou et al., 2007). Compared to other inflammation-inducing phenotypes (i.e. oxidative stress, cold exposure), a number of genes associated with inflammation overlap between these phenotypes and the anoxia tolerance phenotype. For example, immune response genes were identified as contributing to variation in oxidative stress resistance phenotypes (Durham et al., 2014; Jordan et al., 2012; Weber et al., 2012) and are shown to be upregulated in response to cold (MacMillan et al., 2016).

In our adult GWA, immunoglobulin-like domain genes were the mostly highly significant annotation gene cluster group, with nine different genes being significantly related to anoxia-tolerance. The genes within this cluster can function in immune responses and might provide a genetic mechanism for the associations between anoxia-tolerance and immune function in the DGRP lines. However, these immunoglobulin-domain proteins are very diverse in their functions, including cell-cell recognition, muscle function, growth factor receptors and cell adhesion molecules. Four of the individual genes identified within this cluster have unknown functions, however, five of the genes in this cluster have been annotated.

Many of the specific genes within the cluster representing immune response (Table 4.3, Cluster 1) that are highly significant with adult anoxia-tolerance have been

shown to have roles as regulators of neural development. *Lar (leukocyte antigen-related)* is a receptor protein tyrosine phosphatase that has also been shown to regulate synaptic development and the circadian clock in *Drosophila* (Agrawal and Hardin, 2016). Tyrosine phosphatases are key regulators of cell function, and it is plausible that *Lar* is involved in regulating responses to anoxia or recovery. The gene *babos* has been studied as a cell adhesion protein involved in learning and memory, and the developmental plasticity of neurons (Farca-Luna and Sprecher, 2013). *Vein (vn)* is a secreted neuregulin-like EGFR ligand that has been shown to function in the regulation of growth and patterning of tissues. *Kekkon5* (*kek5)* has been identified as an extracellular BMP pathway regulator, involved in neuronal development (Derheimer et al., 2004; Evans et al., 2009). Thus, these genes might plausibly affect anoxia-tolerance by affecting brain structure prior to anoxia-exposure, or possibly be involved in the recovery and repair of anoxic-exposed brains and other tissues.

Evidence for genes mediating relationships between metabolism and anoxia tolerance

In response to anoxia, metabolism is suppressed to nearly 3% of normal in adults (Callier et al., 2015). Therefore, I predicted that genes associated with metabolic processes might be important in mediating the inter-populational differences. However, the GWA study of adults did not identify any significant genes related to metabolism, suggesting that variation in metabolic genes was not particularly important in explaining variation in anoxia-tolerance across the lines. Conversely, larval anoxia tolerance is positively correlated with higher State 3 mitochondrial respiration rate across the DGRP lines (Table 4.2). Given that mitochondrial oxidative phosphorylation is absent in anoxia,

the positive correlation between State 3 respiration rate and anoxia tolerance of larvae might arise from benefits of ATP generation during early exposure to anoxia, before the mitochondria are completely anoxic. Alternatively, mitochondria with high State 3 respiration rates might be strongly coupled, and generate less ROS during reoxygenation, or be better able to oxidatively produce ATP after sustaining damage during anoxia and reoxygenation.

Adult anoxia-tolerance was negatively correlated with glycerol and lipid content across the DGRP lines (Table 4.2). Lines that tend to accumulate glycerol and lipid might be less poised for carbohydrate metabolism, which is predominant during anaerobiosis. Alternatively, high lipid contents might cause tissues to be more susceptible to damage induced by protein aggregates formed during anoxia, or ROS. Elevated cellular lipids have often been shown to repress inflammation via the PPAR pathway (Bensinger and Tontonoz, 2008).

According to classical theories of anoxia-tolerance in vertebrates (Hochachka et al., 1996), we might have expected anoxia-tolerance to be higher in lines that accumulate higher carbohydrate stores. However, this was not the case (Table 4.2). This result supports the prior finding that carbohydrate stores are not significantly depleted during anoxic exposure in *D. melanogaster* (see Chapter 3).

Evidence for genes that may affect anoxia-tolerance by influencing ionic homeostasis

When exposed to anoxia, animals must cope with reduced ATP production and as ATP becomes depleted, ion homeostasis can be disrupted, eventually leading to cell death (Galli and Richards, 2014; Storey and Storey, 2007). Adult *Drosophila* undergo a 3-fold

increase in extracellular K^+ after 6 h of anoxic exposure (Campbell et al., 2018) and anoxia induces the loss of membrane potential in muscle (Krishnan et al., 1997), but we do not know how this disruption varies among these DGRP lines. One possibility is that these ion transport genes identified by the GWA function to maintain ionic homeostasis during anoxia. A gene responsible for mRNA editing of ion channel transcripts (*Drosophila* pre-mRNA adenosine deaminase, dADAR) has been shown to be important in regulating ion channel function (Palladino et al., 2000); this gene is expressed most abundantly in the brain, and RNAi-mediated knockdown renders mutants sensitive to anoxia and ionic stress (Ma et al., 2001). Gene ontology enrichment of GWA analyses for adult anoxia tolerance found three of the four top clusters of genes were related to ion transport/balance (Table 4.2, annotation clusters 2-4). For example, allelic variation in the pickpocket genes (*ppk19*, *ppk30*) are associated with anoxia tolerance; these are non-voltage gated amiloride-sensitive sodium channels. Also associated with anoxia-tolerance were the voltage-gated potassium channels encoded by *Elk* and *Shawl*. Plausibly allelic variation in these genes may affect ionic disruption during anoxia.

Two genes identified as linked to anoxia-tolerance are related to calcium regulation. *Dpr3* is an exciting gene to be identified by our GWA, as it is a component of the calcium release-activated channels (CRAC channel; Vig et al., 2006). As calcium entry after ionic disruption is considered a key step in cell death resulting from anoxia, it is plausible that allelic variation in *Dpr3* is affecting the magnitude of the calcium response to anoxia. *PMCA* was identified as a gene correlated to anoxia-tolerance in adults, within the ion transport cluster. *PMCA* is a plasma membrane calcium ATPase

that plays an important role in restoring intracellular calcium levels after a calcium spike (Desai and Lnenicka, 2011). The different alleles of *PMCA* may vary in their capacity to sustain calcium extrusion during anoxia.

Oatp58Da, *Oatp58Db* and *Oatp58Dc* are organic anion transport proteins, identified by GWA to be associated with adult anoxia-tolerance. *Oatp58Da* has been shown to be involved in the transport of a variety of organic anions, including toxins in the Malpighian tubules (Torrie et al., 2004). This could be a novel, previously unrecognized important function during anoxia, especially as organismal maintenance of hemolymph ions has been identified as a key component of survival of chill coma (Overgaard and MacMillan, 2017). Anoxia-tolerance of males and females among the DGRP lines was significantly correlated with chill coma recovery (Table 4.2). Chill coma damage in insects has been well demonstrated to be a result of ionic stress resulting from a dysregulation of ionic homeostasis (Overgaard and MacMillan, 2017). Furthermore, three genes identified by the GWA (*Oatp58Db*, *Oatp58Dc, CG43066*) are substantially downregulated and one was upregulated (*CG3822*) during cold exposure (MacMillan et al., 2016). Plausibly, alleles of the nine genes identified in Table 4.3, annotation cluster 2 (ion transport) that occur in the anoxia-tolerant lines may reduce ionic disruption in both anoxia and chill coma.

Possible evidence for genes that affect anoxia-tolerance by affecting the resistance to oxidative stress or unfolded proteins

In order to recover from anoxia, animals must return to oxygenated air, and this reperfusion of oxygen can elicit substantial oxidative stress, which is believed by some to

be the major mechanism of cell damage associated with anoxia (Ambrosio et al., 1987; Hashimoto et al., 2003; Milton et al., 2007). An excessive production of ROS that overcomes the buffering capacity of antioxidant defenses can lead to multiple deleterious effects on cells and organelles (Galli and Richards, 2014; Pamenter, 2014). For example, elevated ROS levels can react with unsaturated fatty acids in membranes (Behn et al., 2007), side chains of proteins to produce carbonyls, and oxidize nucleic acids, particularly guanine (Cooke et al., 2003; Dickinson and Chang, 2011). Flies exposed to severe hypoxia/anoxia upregulate genes linked to antioxidant production, supporting an important role for resisting oxidative stress in anoxia-survival (Azad et al., 2009; Liu et al., 2006). If variation in anoxia tolerance involves the ability to tolerate oxidative stress, we would predict that DGRP lines more tolerant to oxidative stress would also be more tolerant of anoxia. Indeed, anoxia tolerance was significantly correlated with multiple oxidative stress-resistance phenotypes (Table 4.2). DGRP lines with higher anoxia tolerance were more tolerant to chronic and acute oxidative stress in both larvae and adults (Table 4.2). The negative correlation between the startle response to chronic oxidative stress and anoxia tolerance in adults may result from lines that are anoxia-tolerant having higher antioxidant capacities, potentially explaining why they do not respond behaviorally until exposed to higher concentrations of oxidants. Further supporting the oxidative stress/anoxia-tolerance linkage, female anoxia tolerance was significantly correlated with virgin female lifespan (Table 4.2). Animals with longer lifespan tend to be more tolerant to stressors including oxidative stress (Martin et al., 1996). Together, these data cautiously support the hypothesis that variation in oxidative

stress resistance is a component of the variation in anoxia-tolerance across the DGRP lines. Yet, the genetic mechanisms driving differences in oxidative stress responses during anoxia are unclear. The GWA did not identify any genes traditionally linked to oxidative stress resistance, such as genes involved in detoxifying or buffering ROS. Plausibly, the identified genes are downstream of regulatory genes, such as the alternative splicing genes that were identified by our GWA analysis of larval anoxia tolerance. Another possibility is that the correlations between anoxia-tolerance and tolerance to oxidative stress arise secondarily from the effects of immune or ionic homeostatic functions that help flies cope with multiple types of stress.

Anoxia is characterized by a decrease in intracellular pH and elevations in ionic concentrations and osmotic pressures that can induce protein unfolding (Krivoruchko and Storey, 2013). As a result, anoxia can cause the formation of unfolded and misfolded proteins that have exposed hydrophobic segments, rendering them prone to aggregation and degradation. Protein aggregates can be toxic to the cell, and to prevent such aggregations, cells induce molecular chaperones in response to their formation (Giffard et al., 2004). Large increases in protein-aggregations coincide with the increase in mortality in anoxia (Chen et al., 2002). Heat shock proteins (HSPs) are a group of important molecular chaperones that repair and/or protect unfolded proteins in response to numerous environmental stressors (Feder and Hofmann, 1999). Flies exposed to hypoxia exhibit increased levels of Hsp70 and Hsp23, and flies with overexpressed HSPs had substantial increases in survival (Azad et al., 2009). The anoxia tolerance phenotype was strongly correlated with endoplasmic reticulum stress (Table 4.2), in which flies were

exposed to a commonly used chemical (tunicamycin) to induce an unfolded protein response; the negative correlation reflects a measure of the inverse of survival and can be interpreted as those lines less susceptible to protein unfolding were more tolerant to anoxia. However, as for the resistance to oxidative stress, the genetic bases to a role for variation in the response to unfolded proteins in causing variation in anoxia tolerance is unclear. No HSPs or other chaperones were identified by the GWA. Plausibly, upstream transcriptional regulators of HSPs might be responsible for this correlation, or the correlation between anoxia tolerance and the endoplasmic stress phenotype might be downstream of other genetic effects such as those influencing immune function or ion channels that mediate variation in stress-resistance of the DGRP lines.

Genes related to larval anoxia-tolerance

Of the genes with known functions shown to be associated with larval anoxia-tolerance by GWA, two were DNA regulatory genes, and two were genes involved in contractile processes. *Chiffon (chif)* is in the zinc-finger superfamily and is believed to be involved in the regulation of transcription and DNA replication (Landis and Tower, 1999). The gene *dre4* is a component of the FACT complex, which is involved in DNA replication and repair, and nucleosome organization (Tsunaka et al., 2009). Conceivably, these genes could influence DNA transcriptional or repair processes during or after anoxia. *Lasp* is a member of the nebulin family and is involved in physiological processes requiring the cytoskeleton such as spermatogenesis (Lee et al., 2008). *Paramyosin* (*Prm*) is a muscle protein that can modulate flight muscle stiffness, but also

is expressed in larvae. Possibly *Lasp* and *Prm* affects locomotory behavior in the larvae, or plausibly repair processes requiring the cytoskeleton.

Another gene identified by the GWA is *Pde11*, which likely affects larval locomotory behavior. Adults and larvae respond differently when exposed to anoxia (Callier et al., 2015); adults are paralyzed within a minute and larvae locomote for up to nearly 30 min. Conceivably, the differences in anoxia tolerance can be attributed to locomotory behavior, with animals exhibiting less locomotion having reduced anaerobic metabolism and damage. cGMP has been shown to modulate larval escape behavior in hypoxia (Vermehren-Schmaedick et al., 2010), and increased cGMP leads to a fast onset of an anoxic coma through a cGMP-activated protein kinase (Dawson-Scully et al., 2010). Furthermore, members of the phosphodiesterase (*PDE*) gene family mediate fast recovery from anoxic exposure (Xiao and Robertson, 2017). The GWA analysis for larval anoxia tolerance identified *Pde11* as one of the top genes associated with anoxia tolerance (Appendix D). If *Pde11* affects anoxia tolerance by influencing locomotor behavior, we would predict that DGRP lines with larvae that exhibit less movement during anoxia would be more tolerant. However, the proportion of larvae climbing out of the media when exposed to anoxia was not correlated with survival (r=0.14, p=0.082; Figure 4.5). However, it is plausible that anoxia tolerance is related to the magnitude of locomotory activity during the initial exposure to anoxia, with consequent effects on lactate production, pH, and ionic homeostasis. Alternatively, *Pde11*/cGMP phosphodiesterase activity may be regulating other processes that affect anoxia-tolerance.

Conclusions

This study demonstrates that anoxia tolerance is a highly variable trait, and that much of this variation is determined genetically. Male and female anoxia tolerance is tightly correlated, yet there is still a substantial amount of variation within some lines. GWA analyses for adult anoxia tolerance identified many genes with functions closely related to immune/inflammatory response, consistent with the strong up-regulation of immune genes after anoxic/hypoxic exposure. These data strongly suggest that genetically-based differences in immune function are key differentiators of anoxia-tolerance, but the mechanisms responsible remain unclear. GWA also identified multiple ion transport function genes whose allelic variation affected anoxia-tolerance in adults; examination of how these alleles affect ionic disruption or tolerance of ionic disruption may provide important insights into the maintenance of genetic variation in these ion transporters.

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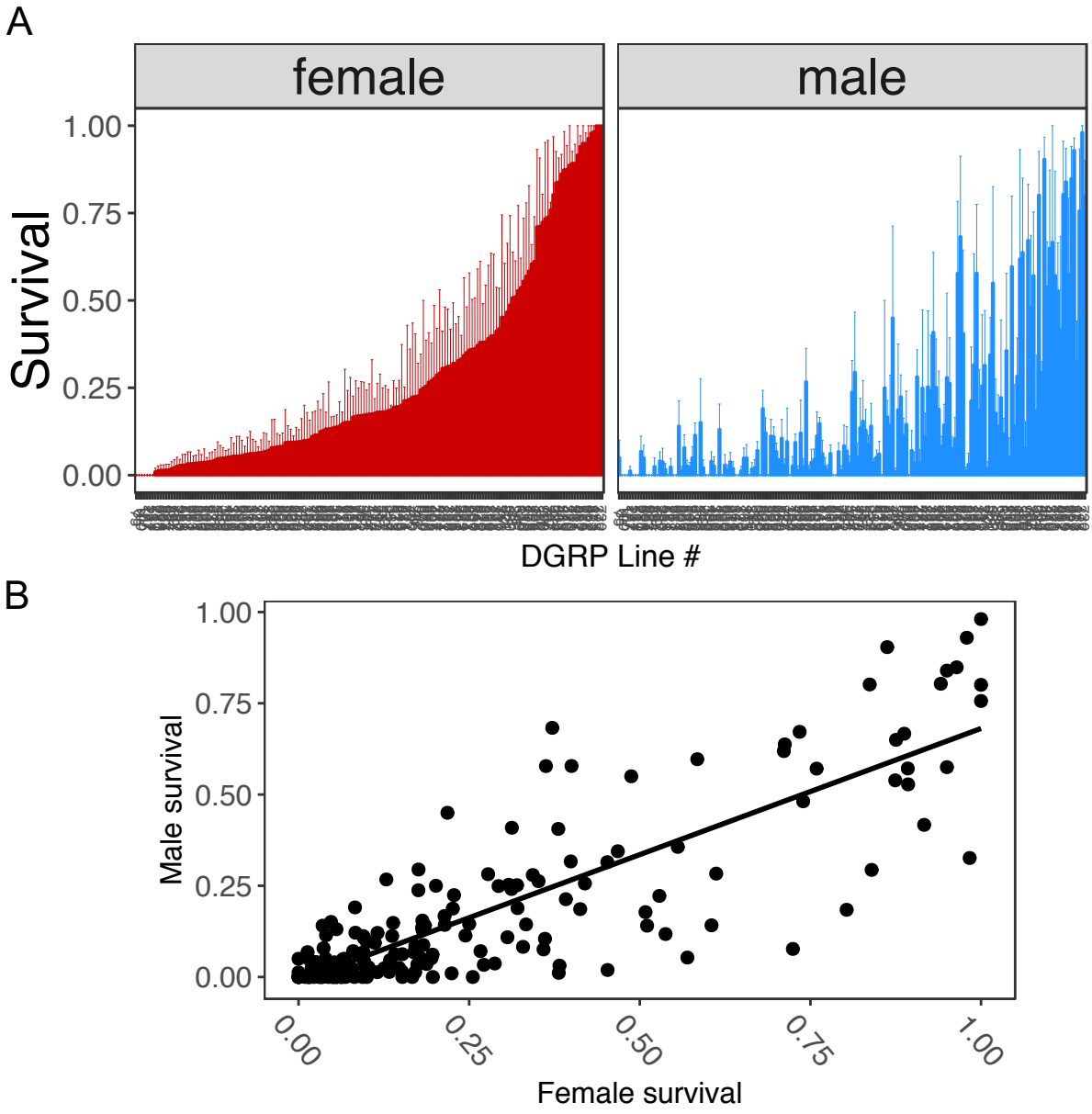

Figure 4.1: A.) Mean adult survival ± SE for each line after 6 h of anoxic exposure. B) Within a line, male and female adult survival were strongly correlated ($r = 0.839$, $p < 0.0001$).

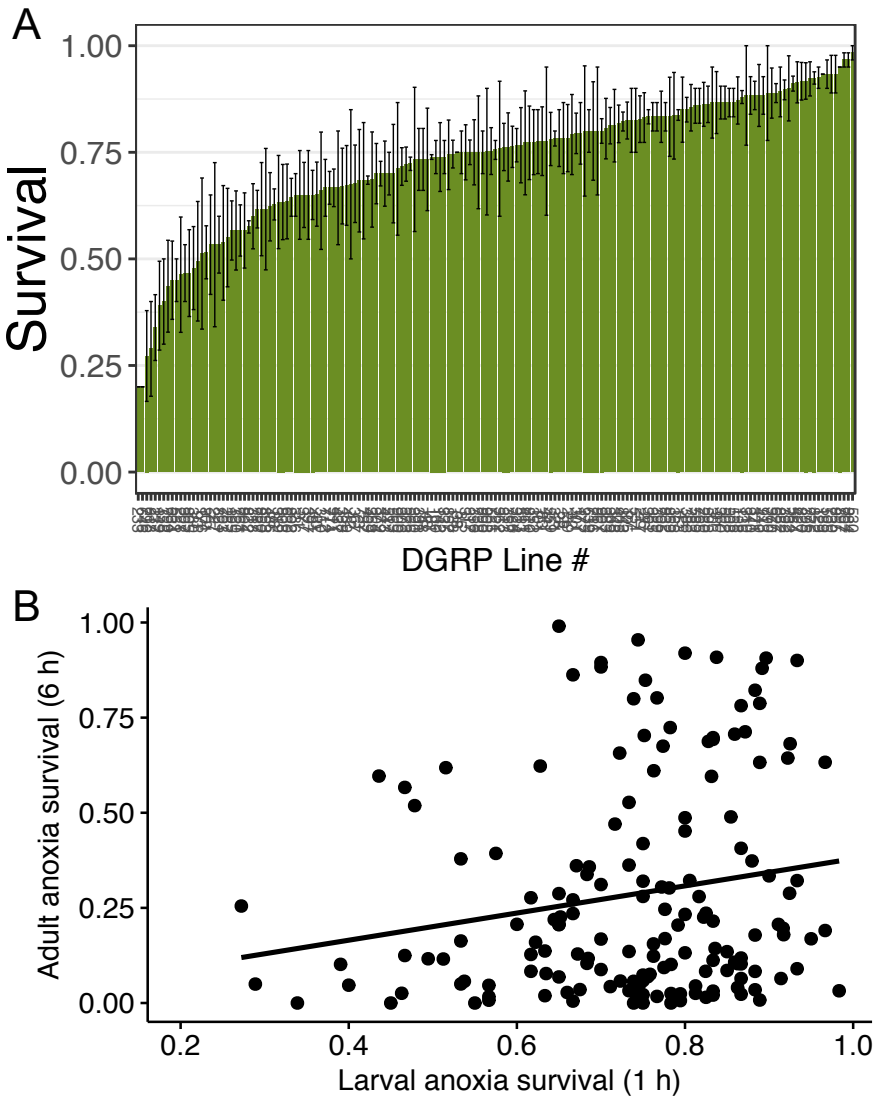

Figure 4.2: A.) Mean larval survival ± SE for each line after 1 h of anoxic exposure. B) Larval survival was not strongly correlated to sex-pooled adult survival ($r = 0.18$, $p = 0.02$).

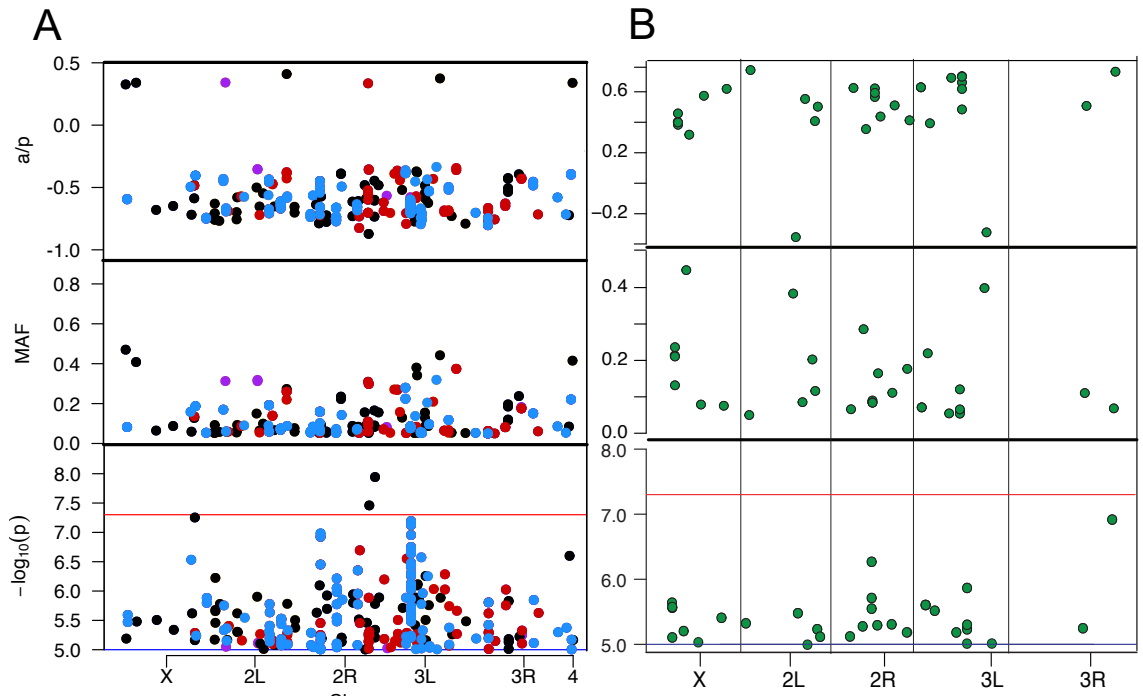

Figure 4.3: Significant SNPs identified in the GWAS are plotted for adults (A) and larvae (B). Effect sizes normalized by the phenotypic standard variation (α/p) are plotted in the top panel. The middle panel shows the minor allele frequencies (MAF) for each significant SNP. P-values plotted as -log10(p) shown in the bottom panel for only SNPs significant at $p<10^{-5}$. For adults, colors represent significant SNPs for each of the four adult traits analyzed (Female: red, male: blue, pooled sex average: purple, difference between sexes: black).

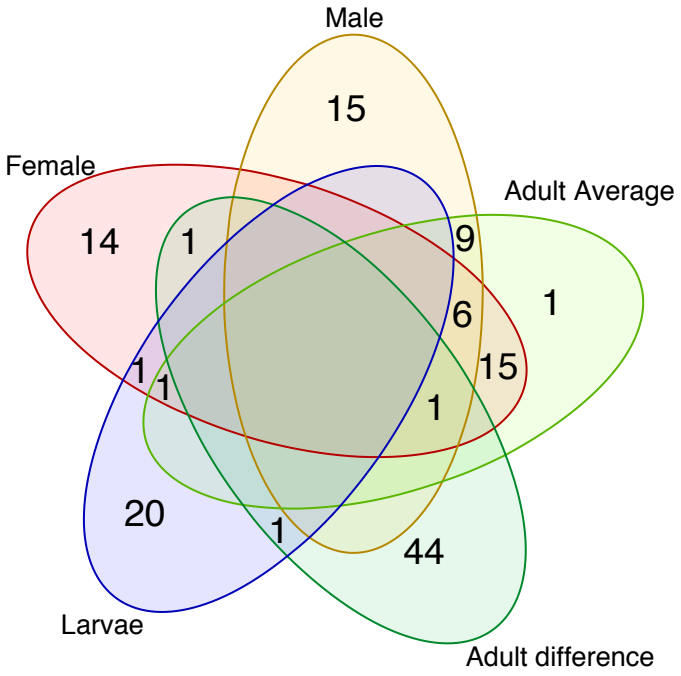

Figure 4.4: Venn diagram showing the overlap of target genes between all adult and larval phenotypes. Adult average represents male and female survival pooled together by line and adult difference represents the difference between female and male survival by line.

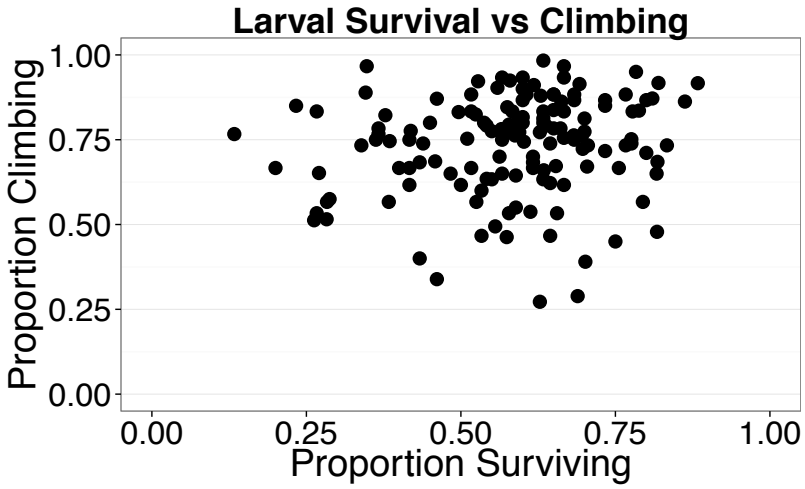

Figure 4.5: The correlation between the proportion of larvae that exhibit escape behavior and the proportion of larvae surviving 1 h of anoxia. R=0.14, p=0.082

Table 4.1: Analysis of variance (ANOVA) for anoxia tolerance in adults and larvae. H^2 is the broad-sense heritability, r_{gsex} is the cross sex, and r_{gstage} is the cross stage genetic correlation.

Analysis	**Source of Variation**	**DF**	**MSE**	*F*	*p*	*Variance (SE)*	H^2	r_{gs}
Sexes	Sex	1	2.8752	28.799	<0.0001	fixed	0.552	0.999
Pooled	Line	169	0.4572	9.962	<0.0001	0.0545 (0.018)		
	Sex:Line	170	0.0449	0.979	0.561	0.0039 (0.0048)		
	Error	953	0.0459			0.0461 (0.007)		
Female	Line	170	0.2980	6.102	<0.0001	0.0681 (0.02)	0.582	
	Error	479	0.0488			0.0490 (0.0101)		
Male	Line	170	0.2041	4.756	<0.0001	0.0437 (0.016)	0.504	
	Error	474	0.0429			0.0430 (0.0095)		
Larvae	Line	178	0.0686	3.299	<0.0001	0.0165 (0.0096)	0.695	
	Error	353	0.0208			0.0376 (0.0077)		
Adult and	Stage	1	92.860	2225.41	<0.0001	fixed	0.475	0.249
larvae pooled	Line	192	0.341	8.170	<0.0001	0.0074 (0.0062)		
	Stage:Line	146	0.162	3.884	<0.0001	0.0299 (0.0143)		
	Error	1468				0.0413 (0.0053)		

Table 4.2: Correlations between anoxia tolerance and other phenotypes measured in the DGRP.

Trait	Male			Female			Pooled Sex		Larvae			Citation
	cor	*p*		*cor*	*p*		*cor*	*p*	*cor*	*p*		
sensitivity to oxidative stress (negative geotaxis)	0.057	0.443		0.126	0.051				0.317	0.000	***	Jordan et al. (2012)
sensitivity to oxidative stress (startle response)	-0.157	0.035	*	-0.135	0.037	*			0.020	0.799		
mito function (P:O ratio)	-0.131	0.426		0.099	0.510				-0.031	0.856		Jumbo-Lucioni et al. (2012)
mito function (State 3)	0.022	0.893		-0.047	0.753				0.415	0.011	*	
mito function (State 4)	0.190	0.246		-0.198	0.182				-0.141	0.406		
chill coma recovery	0.207	0.006	**	0.160	0.016	*			0.047	0.561		Mackay et al (2012)
startle response	-0.016	0.827		0.020	0.754				-0.006	0.941		
starvation resistance	0.156	0.029	*	0.143	0.022	*			-0.057	0.453		
alcohol tolerance	0.186	0.009	**	0.159	0.011	*			-0.002	0.978		Morozova et al (2015)
resistance to fungal infection (MA549)	0.058	0.431		0.061	0.348				0.087	0.266		Wang et al (2017)
resistance to fungal infection (PA14)	0.184	0.089		0.424	0.001	***			0.201	0.080		
oxidative stress paraquat	0.125	0.110		0.021	0.761				0.012	0.884		Weber et al (2012)
oxidative stress msb	0.109	0.166		0.281	0.001	***			0.184	0.026	*	
food intake	-0.083	0.273		-0.194	0.003	**			0.124	0.119		Garlapow et al 2015
boric acid toxicity				0.098	0.167							Najarro et al (2017)
mated lifespan				-0.051	0.439							Durham et al (2014)
fecundity				0.037	0.573							
traumatic brain injury				0.052	0.446							Katzenberger et al (2015)
susceptibility to enteric infection				-0.291	0.001	***						Bou Sleiman et al. (2015)
tolerance to bacterial infection				0.117	0.093							Howick and Lazzaro (2017)
bacterial load				-0.089	0.201							
genotypic deviation of tolerance from bacterial infection				-0.023	0.742							
virgin female lifespan				0.284	0.001	***						Ivanov et al. (2015)
endoplasmic ret stress (hazard ratio)	-0.177	0.060										Chow et al (2013)
endoplasmic ret stress (LT50)	-0.317	0.001	***									
male aggression	0.027	0.707										Shorter et al (2015)
radiation resistance	-0.076	0.345										Vaisnav et al. (2014)
glucose pooled	-0.119	0.162										Unckless et al (2015)
glucose high glucose	-0.078	0.358										
glucose low glucose	-0.088	0.294										
glycerol pooled	-0.194	0.022	*									
glycerol high glucose	-0.224	0.007	**									
glycerol low glucose	-0.104	0.216										
glycogen pooled	-0.040	0.640										
glycogen high glucose	-0.084	0.320										
glycogen low glucose	0.017	0.838										
triglyceride pooled	-0.163	0.055										
triglyceride high glucose	-0.128	0.129										
triglyceride low glucose	-0.128	0.128										
protein pooled	0.009	0.919										
protein high glucose	-0.023	0.791										
protein low glucose	0.020	0.811										
meanweight (ug) pooled	-0.026	0.758										
meanweight (ug) high glucose	-0.052	0.540										
meanweight (ug) low glucose	-0.004	0.966										
low glucose PC1	0.046	0.583										
low glucose PC2	-0.039	0.645										
low glucose PC3	0.063	0.455										
low glucose PC4	-0.204	0.014	*									
low glucose PC5	-0.017	0.840										
high glucose PC1	-0.163	0.053										
high glucose PC2	-0.139	0.101										
high glucose PC3	0.046	0.588										

Significance at p<0.001***, p<0.01**, p<0.05*

Trait	Male		Female			Pooled Sex			Larvae			Citation
	cor	*p*	*cor*	*p*		*cor*	*p*		*cor*	*p*		
high glucose PC4	-0.151	0.075										Unckless et al (2015)
high glucose PC5	-0.004	0.961										
starvation resistance mean (nutrient restrictive)			0.010	0.885								Nelson et al (2016)
starvation resistance mean (nutrient rich)			0.065	0.324								
starvation resistance median (nutrient restrictive)			0.037	0.575								
starvation resistance median (nutrient rich)			0.045	0.492								
starvation resistance max (nutrient restrictive)			0.033	0.616								
starvation resistance max (nutrient rich)			0.028	0.668								
body mass (nutrient restrictive)			0.073	0.266								
body mass (nutrient rich)			-0.012	0.855								
lipid (nutrient restrictive)			-0.343	0.001	***							
lipid (nutrient rich)			-0.234	0.001	***							
protein (nutrient restrictive)			-0.057	0.388								
protein (nutrient rich)			-0.011	0.872								
glucose (nutrient restrictive)			-0.106	0.105								
glucose (nutrient rich)			-0.121	0.065								
lead tolerance	0.081	0.264	0.120	0.058								Zhou et al (2016)
lead development time	-0.018	0.811	-0.076	0.244					0.020	0.793		
DDT mortality (LD50)			0.036	0.582								Schmidt et al (2017)
embryo development time						0.027	0.867		0.139	0.393		Horvath et al (2016)
egg viability						-0.025	0.875		-0.118	0.467		
Larval amanitin resistance LC50						-0.086	0.619		-0.345	0.046	*	Mitchell et al (2017)
Larval amanitin resistance (0.2 ppm)						-0.004	0.965		0.117	0.194		
Larval amanitin resistance (2 ppm)						-0.006	0.947		0.207	0.020	*	
larval insecticide resistance (0.25 ppm)						0.175	0.022	*	0.003	0.973		Battlay et al (2016)
larval insecticide resistance (0.5 ppm)						0.064	0.402		-0.011	0.889		
larval insecticide resistance (1 ppm)						-0.047	0.541		-0.073	0.364		
larval insecticide resistance (2 ppm)						-0.041	0.595		-0.059	0.459		
larval insecticide resistance (LD50)						-0.028	0.712		0.050	0.535		
larval methylmercury survival (0 M)						-0.074	0.344		0.028	0.731		Montegomery et al (2014)
larval methylmercury survival (5 M)						-0.049	0.529		0.029	0.719		
larval methylmercury survival (10 M)						-0.068	0.381		0.006	0.946		
larval methylmercury survival (15 M)						0.044	0.575		0.117	0.148		
larval methylmercury + caffiene survival (0 M + 2 mM)						0.143	0.064		0.160	0.049	*	
larval methylmercury + caffiene survival (10 M + 2 mM)						-0.014	0.854		0.027	0.742		

Significance at p<0.001***, p<0.01**, p<0.05*

Table 4.3: Results from the gene ontology enrichment analysis for adult anoxia tolerance. Only clusters with enrichment scores > 2 were shown.

Annotation Cluster 1	**Enrichment Score: 3.135621657841971**									
Category	Term	Count	%	PValue	Genes	List Total	Pop Hits	Pop Total	Fold Enrichment	Bonferroni corrected FDR
INTERPRO	IPR013783:Immunoglobulin-like fold	9	7.8261	0.0001	LAR, CG13506, BABOS, VN, CG12484, KEK5, CG42389, CG15630, DPR3	87	177	10984	6.4196	0.0154
INTERPRO	IPR007110:Immunoglobulin-like domain	8	6.9565	0.0001	LAR, CG13506, BABOS, VN, CG12484, KEK5, CG15630, DPR3	87	135	10984	7.4817	0.0189
INTERPRO	IPR003598:Immunoglobulin subtype 2	7	6.0870	0.0002	LAR, CG13506, VN, CG12484, KEK5, CG15630, DPR3	87	109	10984	8.1080	0.0442
INTERPRO	IPR003599:Immunoglobulin subtype	7	6.0870	0.0002	LAR, CG13506, BABOS, CG12484, KEK5, CG15630, DPR3	87	111	10984	7.9619	0.0487
SMART	SM00408:IGc2	7	6.0870	0.0012	LAR, CG13506, VN, CG12484, KEK5, CG15630, DPR3	59	109	5218	5.6797	0.0803
SMART	SM00409:IG	7	6.0870	0.0013	LAR, CG13506, BABOS, CG12484, KEK5, CG15630, DPR3	59	111	5218	5.5773	0.0879
INTERPRO	IPR013098:Immunoglobulin I-set	5	4.3478	0.0017	LAR, CG13506, VN, KEK5, CG15630	87	66	10984	9.5646	0.3190
UP_KEYWORDS	Immunoglobulin domain	4	3.4783	0.0055	LAR, BABOS, VN, CG12484	107	47	13921	11.0726	0.4182
INTERPRO	IPR003961:Fibronectin, type III	4	3.4783	0.0137	LAR, CG12484, CG42389, CG15630	87	64	10984	7.8908	0.9519
Annotation Cluster 2	**Enrichment Score: 2.4931587250074165**									
Category	Term	Count	%	PValue	Genes	List Total	Pop Hits	Pop Total	Fold Enrichment	Bonferroni corrected FDR
UP_KEYWORDS	Ion transport	9	7.8261	0.0002	OATP58DA, OATP58DB, PPK30, ELK, SHAWL, OATP58DC, PMCA, PPK19, CG3822	107	208	13921	5.6294	0.0183
UP_KEYWORDS	Transport	11	9.5652	0.0108	OATP58DA, OATP58DB, PPK30, OBP99A, ELK, SHAWL, CG43066, OATP58DC, PMCA, PPK19, CG3822	107	564	13921	2.5375	0.6570
UP_KEYWORDS	Ion channel	5	4.3478	0.0165	PPK30, ELK, SHAWL, PPK19, CG3822	107	128	13921	5.0821	0.8072
Annotation Cluster 3	**Enrichment Score: 2.3314431260822235**									
Category	Term	Count	%	PValue	Genes	List Total	Pop Hits	Pop Total	Fold Enrichment	Bonferroni corrected FDR
GOTERM_BP_DIRECT	GO:0043252~sodium-independent organic anion transport	3	2.6087	0.0016	OATP58DA, OATP58DB, OATP58DC	85	8	10996	48.5118	0.3879
GOTERM_BP_DIRECT	GO:0071702~organic substance transport	3	2.6087	0.0016	OATP58DA, OATP58DB, OATP58DC	85	8	10996	48.5118	0.3879
GOTERM_MF_DIRECT	GO:0022804~active transmembrane transporter activity	3	2.6087	0.0016	OATP58DA, OATP58DB, OATP58DC	73	8	9284	47.6918	0.1639
GOTERM_MF_DIRECT	GO:0015347~sodium-independent organic anion transmembrane transporter activity	3	2.6087	0.0016	OATP58DA, OATP58DB, OATP58DC	73	8	9284	47.6918	0.1639
INTERPRO	IPR004156:Organic anion transporter polypeptide OATP	3	2.6087	0.0016	OATP58DA, OATP58DB, OATP58DC	87	8	10984	47.3448	0.3039
GOTERM_MF_DIRECT	GO:0022857~transmembrane transporter activity	4	3.4783	0.0289	OATP58DA, OATP58DB, CG3036, OATP58DC	73	86	9284	5.9153	0.9616
INTERPRO	IPR020846:Major facilitator superfamily domain	4	3.4783	0.1575	OATP58DA, OATP58DB, CG3036, OATP58DC	87	175	10984	2.8858	1.0000
Annotation Cluster 4	**Enrichment Score: 2.056464216286357**									
Category	Term	Count	%	PValue	Genes	List Total	Pop Hits	Pop Total	Fold Enrichment	Bonferroni
GOTERM_CC_DIRECT	GO:0008076~voltage-gated potassium channel complex	3	2.6087	0.0045	ELK, SHAWL, CG42732	80	13	10026	28.9212	0.2718
GOTERM_MF_DIRECT	GO:0005249~voltage-gated potassium channel activity	3	2.6087	0.0084	ELK, SHAWL, CG42732	73	18	9284	21.1963	0.6068
GOTERM_BP_DIRECT	GO:0006813~potassium ion transport	3	2.6087	0.0179	ELK, SHAWL, CG42732	85	27	10996	14.3739	0.9965

Table 4.4: Results from the gene ontology enrichment analysis for larval anoxia tolerance. Only clusters with enrichment scores > 2 were shown.

Annotation Cluster 1	**Enrichment Score: 2.5063184924448074**									
Category	Term	Count	%	PValue	Genes	List Total	Pop Hits	Pop Total	Fold Enrichment	Bonferroni corrected FDR
UP_KEYWORDS	Alternative splicing	7	24.1379	0.0003	PDE11, LASP, FS(1)H, CHIF, DRE4, PRM,	22	631	13921	7.0197	0.0115
UP_KEYWORDS	Coiled coil	10	34.4828	0.0003	PDE11, CG42231, LASP, CG8910, FS(1)H, CHIF, DRE4, PRM, CG2258, X11LBETA	22	1594	13921	3.9697	0.0120
UP_SEQ_FEATURE	splice variant	5	17.2414	0.0309	PDE11, LASP, FS(1)H, CHIF, DRE4	8	610	3113	3.1895	0.7641
UP_KEYWORDS	Phosphoprotein	5	17.2414	0.0444	LASP, FS(1)H, CHIF, DRE4, PRM	22	909	13921	3.4806	0.8703
Annotation Cluster 2	**Enrichment Score: 2.3720705430248015**									
Category	Term	Count	%	PValue	Genes	List Total	Pop Hits	Pop Total	Fold Enrichm	Bonferroni corrected FDR
UP_KEYWORDS	SH3 domain	3	10.3448	0.0024	LASP, CG2258, CG32082	22	49	13921	38.7412	0.1042
INTERPRO	IPR001452:Src homology-3 domain	3	10.3448	0.0045	LASP, CG2258, CG32082	17	70	10984	27.6908	0.1813
SMART	SM00326:SH3	3	10.3448	0.0069	LASP, CG2258, CG32082	12	61	5218	21.3852	0.1173

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APPENDIX A

MEAN SURVIVAL FOR ADULT MALES AND ADULT FEMALES EXPOSED TO 6 H OF ANOXIA, AND MEAN SURVIVAL FOR LARVAE EXPOSED TO 1 H OF ANOXIA.

	Female			Male			Larvae		
Line no.	N	Mean +/- SE	Variance	N	Mean +/- SE	Variance	N	Mean +/- SE	Variance
21	4	0.724 +/- 0.08	0.025	3	0.077 +/- 0.077	0.018	-	-	-
26	4	0.803 +/- 0.165	0.109	3	0.184 +/- 0.159	0.075	3	0.831 +/- 0.058	0.01
28	-	-	-	-	-	-	3	0.783 +/- 0.067	0.013
31	4	0.226 +/- 0.178	0.127	4	0.188 +/- 0.188	0.141	3	0.65 +/- 0.104	0.032
32	3	0.079 +/- 0.079	0.019	3	0.022 +/- 0.022	0.001	3	0.792 +/- 0.094	0.026
38	-	-	-	-	-	-	2	0.75 +/- 0	0
40	3	0.228 +/- 0.092	0.026	3	0.224 +/- 0.062	0.012	3	0.652 +/- 0.075	0.017
42	4	0.049 +/- 0.036	0.005	4	0.022 +/- 0.022	0.002	2	0.675 +/- 0.175	0.061
45	3	0.1 +/- 0.1	0.03	4	0.096 +/- 0.096	0.037	3	0.85 +/- 0.076	0.018
48	4	0.508 +/- 0.13	0.068	3	0.178 +/- 0.097	0.028	3	0.733 +/- 0.12	0.043
57	4	0.453 +/- 0.153	0.094	4	0.019 +/- 0.019	0.001	2	0.825 +/- 0.075	0.011
59	3	0.183 +/- 0.074	0.016	3	0.088 +/- 0.027	0.002	3	0.783 +/- 0.083	0.021
69	3	0.022 +/- 0.022	0.001	3	0 +/- 0	0	-	-	-
73	4	0.174 +/- 0.07	0.019	4	0.034 +/- 0.021	0.002	3	0.683 +/- 0.12	0.043
75	3	0 +/-0	0	4	0.05 +/- 0.05	0.01	3	0.533 +/- 0.117	0.041
83	-	-	-	-	-	-	3	0.776 +/- 0.081	0.019
85	4	0 +/- 0	0	3	0 +/- 0	0	2	0.75 +/- 0.05	0.005
88	3	0.756 +/- 0.184	0.101	-		-	3	0.628 +/- 0.036	0.004
91	3	0.529 +/- 0.243	0.177	3	0.222 +/- 0.222	0.148	3	0.867 +/- 0.073	0.016
93	-	-	-	-	-	-	3	0.494 +/- 0.14	0.059
100	3	0 +/- 0	0	3	0 +/- 0	0	3	0.739 +/- 0.039	0.005
101	3	0.056 +/- 0.056	-	3	0.131 +/- 0.072	-	2	0.775 +/- 0.075	0.011
105	3	0 +/- 0	0	4		0	3	0.825 +/- 0.043	0.006
109	4	0.038 +/- 0.013	0.001	4	0.023 +/- 0.013	0.001	-	-	-
129	4	0.854 +/- 0.056	0.013	-	-	-	2	0.925 +/- 0.025	0.001
136	3	0.556 +/- 0.179	0.097	3	0.357 +/- 0.221	0.147	3	0.88 +/- 0.015	0.001
138	3	0.102 +/- 0.055	0.009	3	0.027 +/- 0.027	0.002	3	0.746 +/- 0.033	0.003
142	4	0.063 +/- 0.034	0.005	4	0 +/- 0	0	3	0.739 +/- 0.081	0.02
149	3	0.141 +/- 0.069	0.014	3	0.062 +/- 0.038	0.004	3	0.39 +/- 0.104	0.033
153	4	1.00 +/-0	0	3	0.756 +/- 0.177	0.094	4	0.838 +/- 0.012	0.001
158	4	0.711 +/- 0.221	0.196	3	0.619 +/- 0.312	0.293	3	0.872 +/- 0.055	0.009
161	4	0.823 +/- 0.04	0.006	-		-	-	-	-
176	4	0.178 +/- 0.041	0.007	4	0.053 +/- 0.037	0.006	3	0.512 +/- 0.177	0.094
177	4	0 +/- 0	0	4	0.012 +/- 0.012	0.001	3	0.794 +/- 0.072	0.016
181	3	0.069 +/- 0.05	0.008	3	0 +/- 0	0	3	0.739 +/- 0.006	0
189	4	0.134 +/- 0.033	0.004	4	0.046 +/- 0.029	0.003	2	0.933 +/- 0	0
195	4	0.053 +/- 0.021	0.002	4	0.04 +/- 0.026	0.003	3	0.567 +/- 0.069	0.014
208	3	0.612 +/- 0.128	0.049	3	0.283 +/- 0.109	0.036	-	-	-
217	3	0.917 +/- 0.083	0.021	3	0.417 +/- 0.267	0.213	3	0.828 +/- 0.055	0.009
227	4	0.185 +/- 0.06	0.014	4	0.141 +/- 0.065	0.017	3	0.533 +/- 0.192	0.111
228	4	0.033 +/- 0.033	0.004	3	0.024 +/- 0.024	0.002	3	0.463 +/- 0.135	0.055
233	-	-	-	-	-	-	2	0.2 +/- 0	0
235	4	0.511 +/- 0.102	0.042	4	0.141 +/- 0.089	0.032	-	-	-
237	-	-	-	-	-	-	3	0.339 +/- 0.077	0.018
239	4	0.05 +/- 0.029	0.003	4	0 +/- 0	0	3	0.783 +/- 0.079	0.019
256	4	0.029 +/- 0.017	0.001	4	0.038 +/- 0.038	0.006	-	-	-
280	4	0.013 +/- 0.013	0.001	4	0.068 +/- 0.044	0.008	4	0.862 +/- 0.052	0.011

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287	4	0 +/- 0	0	4	0 +/- 0	0	3	0.55 +/- 0.115	0.04
301	4	0.036 +/- 0.036	0.005	4	0.012 +/- 0.012	0.001	3	0.794 +/- 0.047	0.007
303	-	-	-	-	-	-	3	0.867 +/- 0.083	0.021
304	4	0.035 +/- 0.022	0.002	4	0.141 +/- 0.072	0.021	4	0.7 +/- 0.071	0.02
306	4	0.362 +/- 0.145	0.084	4	0.578 +/- 0.205	0.169	3	0.717 +/- 0.044	0.006
307	4	0.137 +/- 0.032	0.004	4	0.112 +/- 0.069	0.019	3	0.467 +/- 0.067	0.013
309	4	0.051 +/- 0.02	0.002	4	0 +/- 0	0	4	0.812 +/- 0.043	0.007
310	4	0.072 +/- 0.025	0.003	4	0.018 +/- 0.018	0.001	3	0.883 +/- 0.117	0.041
313	4	0.195 +/- 0.075	0.023	4	0.051 +/- 0.02	0.002	4	0.762 +/- 0.055	0.012
315	4	0.067 +/- 0.054	0.012	4	0.05 +/- 0.038	0.006	4	0.75 +/- 0.065	0.017
317	4	0.044 +/- 0.03	0.003	4	0 +/- 0	0	3	0.75 +/- 0.029	0.002
318	4	0.382 +/- 0.161	0.104	4	0.031 +/- 0.031	0.004	3	0.6 +/- 0.076	0.018
319	4	0.062 +/- 0.062	0.016	4	0.019 +/- 0.019	0.001	3	0.633 +/- 0.109	0.036
320	4	0.065 +/- 0.016	0.001	4	0.012 +/- 0.012	0.001	3	0.567 +/- 0.093	0.026
321	3	0.893 +/- 0.054	0.009	4	0.528 +/- 0.137	0.075	2	0.782 +/- 0.018	0.001
324	3	0.837 +/- 0.089	0.024	3	0.802 +/- 0.125	0.047	4	0.767 +/- 0.039	0.006
332	3	0.413 +/- 0.125	0.047	3	0.186 +/- 0.116	0.041	-	-	-
335	-	-	-	-	-	-	3	0.739 +/- 0.039	0.005
336	-	-	-	-	-	-	2	0.65 +/- 0.05	0.005
338	4	0.312 +/- 0.105	0.044	3	0.409 +/- 0.228	0.156	4	0.671 +/- 0.089	0.032
340	4	0.082 +/- 0.037	0.006	4	0 +/- 0	0	3	0.883 +/- 0.044	0.006
348	4	0.04 +/- 0.024	0.002	4	0.05 +/- 0.032	0.004	4	0.812 +/- 0.085	0.029
350	4	0.055 +/- 0.036	0.005	4	0 +/- 0	0	4	0.66 +/- 0.138	0.076
352	4	0.712 +/- 0.195	0.152	4	0.638 +/- 0.212	0.181	4	0.774 +/- 0.076	0.023
354	4	0.188 +/- 0.012	0.001	4	0.036 +/- 0.012	0.001	-	-	-
356	-	-	-	-	-	-	3	0.75 +/- 0.076	0.018
357	4	0.182 +/- 0.068	0.019	4	0.129 +/- 0.059	0.014	4	0.762 +/- 0.08	0.026
358	4	0.094 +/- 0.094	0.035	3	0 +/- 0	0	3	0.4 +/- 0.1	0.03
359	4	0.399 +/- 0.236	0.223	3	0.317 +/- 0.317	0.301	4	0.686 +/- 0.112	0.05
360	4	0.096 +/- 0.041	0.007	3	0.044 +/- 0.003	0	3	0.867 +/- 0.038	0.004
361	4	0.062 +/- 0.04	0.006	4	0 +/- 0	0	3	0.833 +/- 0.017	0.001
362	4	0.08 +/- 0.08	0.026	4	0.071 +/- 0.025	0.003	2	0.758 +/- 0.158	0.05
365	4	0.139 +/- 0.034	0.005	4	0.148 +/- 0.017	0.001	3	0.836 +/- 0.102	0.031
367	4	0.1 +/- 0.079	0.025	4	0 +/- 0	0	3	0.676 +/- 0.091	0.025
370	4	0.888 +/- 0.112	0.051	3	0.667 +/- 0.333	0.333	3	0.889 +/- 0.059	0.01
371	4	0.214 +/- 0.214	0.184	4	0.143 +/- 0.143	0.082	3	0.883 +/- 0.033	0.003
373	3	0.983 +/- 0.017	0.001	4	0.326 +/- 0.115	0.053	3	0.722 +/- 0.04	0.005
375	3	0.264 +/- 0.132	0.052	-	-	-	3	0.8 +/- 0.153	0.07
377	4	1.00 +/- 0	0	4	0.981 +/- 0.019	0.001	3	0.65 +/- 0.076	0.018
379	4	0.343 +/- 0.221	0.196	4	0.279 +/- 0.242	0.234	3	0.7 +/- 0.029	0.002
380	4	0.488 +/- 0.255	0.261	3	0.55 +/- 0.275	0.228	4	0.478 +/- 0.097	0.038
381	4	0.359 +/- 0.22	0.193	4	0.076 +/- 0.035	0.005	-	-	-
382	3	0.171 +/- 0.097	0.028	4	0.084 +/- 0.059	0.014	-	-	-
383	3	0.333 +/- 0.067	0.013	4	0.144 +/- 0.059	0.014	3	0.806 +/- 0.071	0.015
385	4	0.172 +/- 0.094	0.035	3	0.069 +/- 0.069	0.014	3	0.833 +/- 0.033	0.003
386	3	0.048 +/- 0.048	0.007	3	0.151 +/- 0.125	0.047	3	0.85 +/- 0.029	0.002
387	4	0.288 +/- 0.133	0.071	3	0.037 +/- 0.037	0.004	-	-	-
390	-	-	-	-	-	-	3	0.733 +/- 0.169	0.086
391	4	0.015 +/- 0.015	0.001	4	0.05 +/- 0.035	0.005	3	0.733 +/- 0.073	0.016
392	4	0.964 +/- 0.036	0.005	4	0.849 +/- 0.092	0.034	3	0.896 +/- 0.023	0.002

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395	4	0.271 +/- 0.107	0.046	3	0.033 +/- 0.033	0.003	3	0.917 +/- 0.043	0.005
397	3	0.133 +/- 0.133	0.053	3	0.037 +/- 0.037	0.004	3	0.65 +/- 0.104	0.032
399	3	0.056 +/- 0.056	0.009	3	0 +/- 0	0	3	0.833 +/- 0.033	0.003
405	4	0.278 +/- 0.208	0.173	4	0.282 +/- 0.077	0.024	3	0.817 +/- 0.044	0.006
409	4	0.224 +/- 0.211	0.178	4	0.01 +/- 0.01	0	3	0.685 +/- 0.01	0
426	4	0.095 +/- 0.017	0.001	4	0.068 +/- 0.024	0.002	-	-	-
427	4	0.116 +/- 0.068	0.018	4	0.013 +/- 0.013	0.001	3	0.914 +/- 0.048	0.007
437	4	0.176 +/- 0.154	0.095	4	0.295 +/- 0.172	0.118	3	0.667 +/- 0.133	0.053
439	4	0.196 +/- 0.053	0.011	4	0.061 +/- 0.035	0.005	4	0.672 +/- 0.094	0.035
440	3	0.875 +/- 0.067	0.014	3	0.65 +/- 0.223	0.149	3	0.883 +/- 0.073	0.016
441	3	0.306 +/- 0.106	0.034	4	0.109 +/- 0.069	0.019	3	0.776 +/- 0.174	0.091
443	4	0.862 +/- 0.055	0.012	4	0.904 +/- 0.063	0.016	3	0.7 +/- 0.076	0.018
461	-	-	-	-	-	-	3	0.772 +/- 0.122	0.045
486	4	0.054 +/- 0.054	0.011	4	0.026 +/- 0.026	0.003	3	0.622 +/- 0.08	0.019
491	4	0.329 +/- 0.125	0.062	4	0.083 +/- 0.028	0.003	3	0.65 +/- 0.058	0.01
492	4	0.181 +/- 0.082	0.027	4	0.136 +/- 0.085	0.029	-	-	-
502	4	0.095 +/- 0.036	0.005	4	0.111 +/- 0.028	0.003	3	0.867 +/- 0.038	0.004
505	-	-	-	-	-	-	3	0.933 +/- 0.033	0.003
508	4	0.116 +/- 0.096	0.037	4	0.121 +/- 0.094	0.035	3	0.867 +/- 0.033	0.003
509	4	0.083 +/- 0.037	0.006	4	0.121 +/- 0.043	0.007	-	-	-
513	4	0.035 +/- 0.02	0.002	4	0 +/- 0	0	3	0.767 +/- 0.088	0.023
517	4	0.181 +/- 0.108	0.046	4	0.155 +/- 0.116	0.054	2	0.7 +/- 0.05	0.005
528	4	0.016 +/- 0.016	0.001	4	0 +/- 0	0	-	-	-
530	4	0.038 +/- 0.038	0.006	4	0.027 +/- 0.016	0.001	3	0.983 +/- 0.017	0.001
531	4	0 +/- 0	0	4	0 +/- 0	0	2	0.45 +/- 0.05	0.005
535	3	0.945 +/- 0.032	0.003	-	-	-	4	0.753 +/- 0.055	0.012
551	4	0.03 +/- 0.03	0.004	4	0 +/- 0	0	2	0.825 +/- 0.075	0.011
555	4	0.029 +/- 0.029	0.003	4	0.017 +/- 0.017	0.001	3	0.867 +/- 0.033	0.003
559	4	0.392 +/- 0.209	0.174	4	0.213 +/- 0.154	0.094	4	0.781 +/- 0.062	0.016
563	4	0.95 +/- 0.05	0.01	4	0.84 +/- 0.095	0.036	3	0.7 +/- 0.115	0.04
566	4	0.037 +/- 0.022	0.002	4	0.079 +/- 0.036	0.005	4	0.723 +/- 0.016	0.001
584	4	0.176 +/- 0.086	0.03	4	0.238 +/- 0.09	0.033	4	0.911 +/- 0.018	0.001
589	4	0.874 +/- 0.109	0.047	4	0.539 +/- 0.128	0.066	2	0.859 +/- 0.041	0.003
595	4	0.095 +/- 0.047	0.009	4	0.112 +/- 0.048	0.009	4	0.862 +/- 0.043	0.007
596	4	0.06 +/- 0.023	0.002	4	0.015 +/- 0.015	0.001	-	-	-
627	4	0.016 +/- 0.016	0.001	4	0 +/- 0	0	3	0.889 +/- 0.022	0.001
630	4	0.04 +/- 0.026	0.003	4	0.114 +/- 0.035	0.005	4	0.635 +/- 0.088	0.031
634	4	0.352 +/- 0.13	0.067	4	0.263 +/- 0.13	0.068	4	0.45 +/- 0.092	0.034
639	6	0.538 +/- 0.083	0.042	6	0.118 +/- 0.044	0.012	4	0.8 +/- 0.05	0.01
646	4	0.126 +/- 0.062	0.015	4	0.024 +/- 0.024	0.002	2	0.2 +/- 0	0
703	4	0.065 +/- 0.036	0.005	4	0.049 +/- 0.028	0.003	4	0.538 +/- 0.134	0.072
705	3	0.584 +/- 0.244	0.178	3	0.597 +/- 0.202	0.122	3	0.833 +/- 0.06	0.011
707	3	0.759 +/- 0.021	0.001	4	0.571 +/- 0.182	0.133	3	0.967 +/- 0.017	0.001
712	4	0.01 +/- 0.01	0	4	0 +/- 0	0	3	0.667 +/- 0.067	0.013
714	3	0.169 +/- 0.101	-	4	0.068 +/- 0.025	-	-	-	-
716	3	0.452 +/- 0.293	0.257	4	0.315 +/- 0.156	0.097	3	0.8 +/- 0.115	0.04
721	3	0.171 +/- 0.079	0.019	3	0.013 +/- 0.013	0.001	3	0.756 +/- 0.022	0.001
727	4	0.098 +/- 0.064	0.016	4	0.029 +/- 0.017	0.001	-	-	-
730	3	1.00 +/- 0	0	3	0.801 +/- 0.1	0.03	3	0.933 +/- 0.044	0.006
732	4	0.218 +/- 0.143	0.081	4	0.45 +/- 0.263	0.277	3	0.9 +/- 0.076	0.018

Continued on next page

737	4	0.42 +/- 0.116	0.053	4	0.256 +/- 0.202	0.164	3	0.683 +/- 0.136	0.056
738	5	0.32 +/- 0.173	0.149	6	0.252 +/- 0.138	0.114	3	0.75 +/- 0.132	0.052
748	4	0.372 +/- 0.218	0.19	4	0.683 +/- 0.23	0.212	3	0.733 +/- 0.073	0.016
757	3	0.026 +/- 0.026	0.002	4	0.041 +/- 0.026	0.003	-	-	-
761	4	0.033 +/- 0.033	0.004	4	0 +/- 0	0	3	0.567 +/- 0.06	0.011
763	3	0.112 +/- 0.068	0.014	3	0.094 +/- 0.025	0.002	-	-	-
765	4	0.152 +/- 0.09	0.033	4	0.062 +/- 0.024	0.002	2	0.859 +/- 0.041	0.003
774	4	0.293 +/- 0.237	0.225	4	0.249 +/- 0.223	0.199	3	0.667 +/- 0.038	0.004
776	3	0.95 +/- 0.029	0.002	4	0.575 +/- 0.217	0.189	3	0.922 +/- 0.04	0.005
783	4	0.4 +/- 0.232	0.215	4	0.578 +/- 0.196	0.154	2	0.855 +/- 0.055	0.006
786	4	0.739 +/- 0.218	0.191	4	0.481 +/- 0.205	0.168	4	0.763 +/- 0.048	0.009
787	4	0.312 +/- 0.164	0.107	4	0.241 +/- 0.159	0.101	3	0.617 +/- 0.044	0.006
790	3	0.893 +/- 0.034	0.003	3	0.571 +/- 0.297	0.265	2	0.889 +/- 0.111	0.025
796	4	0.321 +/- 0.112	0.05	4	0.189 +/- 0.109	0.047	3	0.272 +/- 0.106	0.034
799	-	-	-	-	-	-	3	0.436 +/- 0.108	0.035
801	-	-	-	-	-	-	4	0.515 +/- 0.062	0.015
802	4	0.129 +/- 0.105	0.044	4	0.267 +/- 0.096	0.037	3	0.644 +/- 0.044	0.006
804	4	0.015 +/- 0.015	0.001	4	0 +/- 0	0	3	0.567 +/- 0.088	0.023
805	4	0.84 +/- 0.068	0.019	4	0.294 +/- 0.031	0.004	3	0.467 +/- 0.102	0.031
808	4	0.083 +/- 0.032	0.004	4	0.191 +/- 0.052	0.011	3	0.633 +/- 0.088	0.023
810	4	0.57 +/- 0.209	0.175	3	0.053 +/- 0.053	0.009	3	0.772 +/- 0.086	0.022
812	4	0.147 +/- 0.034	0.005	4	0.025 +/- 0.025	0.002	-	-	-
818	4	0.061 +/- 0.047	0.009	4	0.039 +/- 0.026	0.003	3	0.289 +/- 0.111	0.037
819	4	0.197 +/- 0.074	0.022	4	0 +/- 0	0	-	-	-
820	4	0.381 +/- 0.11	0.048	4	0.011 +/- 0.011	0.001	3	0.917 +/- 0.044	0.006
821	4	0.468 +/- 0.196	0.153	3	0.345 +/- 0.106	0.034	3	0.533 +/- 0.067	0.013
822	4	0.381 +/- 0.231	0.214	4	0.406 +/- 0.239	0.228	4	0.575 +/- 0.014	0.001
832	3	0.671 +/- 0.137	0.056	-	-	-	3	0.833 +/- 0.033	0.003
843	4	0.201 +/- 0.172	0.119	4	0.25 +/- 0.25	0.25	3	0.822 +/- 0.022	0.001
849	4	0.096 +/- 0.034	0.005	4	0.106 +/- 0.054	0.012	-	-	-
850	4	0.255 +/- 0.131	0.069	4	0 +/- 0	0	3	0.617 +/- 0.109	0.036
852	5	0.308 +/- 0.173	0.15	4	0.253 +/- 0.217	0.189	3	0.924 +/- 0.014	0.001
853	4	0.167 +/- 0.06	0.014	4	0 +/- 0	0	3	0.617 +/- 0.142	0.061
855	3	0.605 +/- 0.055	0.009	3	0.142 +/- 0.118	0.041	3	0.933 +/- 0.044	0.006
857	3	0.979 +/- 0.021	0.001	3	0.93 +/- 0.035	0.004	3	0.744 +/- 0.082	0.02
859	6	0.152 +/- 0.127	0.097	3	0 +/- 0	0	3	0.833 +/- 0.093	0.026
861	-	-	-	-	-	-	2	0.8 +/- 0.15	0.045
879	4	0.25 +/- 0.25	0.25	4	0.145 +/- 0.095	0.036	-	-	-
882	3	0.941 +/- 0.03	0.003	4	0.804 +/- 0.152	0.093	2	0.892 +/- 0.058	0.007
884	4	0.214 +/- 0.038	0.006	4	0.167 +/- 0.047	0.009	3	0.967 +/- 0.017	0.001
890	4	0.133 +/- 0.047	0.009	4	0.014 +/- 0.014	0.001	3	0.75 +/- 0.05	0.008
892	4	0.245 +/- 0.102	0.041	4	0.114 +/- 0.049	0.01	-	-	-
894	4	0.019 +/- 0.019	0.001	4	0.025 +/- 0.025	0.002	-	-	-
897	4	0.267 +/- 0.141	0.079	4	0.071 +/- 0.057	0.013	3	0.95 +/- 0	0
900	4	0.057 +/- 0.043	0.007	4	0.029 +/- 0.029	0.003	3	0.711 +/- 0.156	0.073
907	4	0.361 +/- 0.142	0.081	4	0.105 +/- 0.06	0.015	3	0.8 +/- 0.029	0.002
908	4	0.734 +/- 0.218	0.19	4	0.672 +/- 0.16	0.103	3	0.752 +/- 0.148	0.066
911	3	0.767 +/- 0.12	0.043	-	-	-	3	0.667 +/- 0.044	0.006
913	3	0.152 +/- 0.152	0.069	4	0.015 +/- 0.015	0.001	3	0.883 +/- 0.044	0.006

APPENDIX B

CORRELATIONS BETWEEN ANOXIA TOLERANCE AND OTHER PHENOTYPES MEASURED IN THE DGRP

Trait	Male			Female			Pooled Sex		Larvae			Citation
	cor	*p*		*cor*	*p*		*cor*	*p*	*cor*	*p*		
sensitivity to oxidative stress (negative geotaxis)	0.057	0.443		0.126	0.051				0.317	0.000	***	Jordan et al. (2012)
sensitivity to oxidative stress (startle response)	-0.157	0.035	*	-0.135	0.037	*			0.020	0.799		
mito function (P:O ratio)	-0.131	0.426		0.099	0.510				-0.031	0.856		Jumbo-Lucioni et al. (2012)
mito function (State 3)	0.022	0.893		-0.047	0.753				0.415	0.011	*	
mito function (State 4)	0.190	0.246		-0.198	0.182				-0.141	0.406		
chill coma recovery	0.207	0.006	**	0.160	0.016	*			0.047	0.561		Mackay et al (2012)
startle response	-0.016	0.827		0.020	0.754				-0.006	0.941		
starvation resistance	0.156	0.029	*	0.143	0.022	*			-0.057	0.453		
alcohol tolerance	0.186	0.009	**	0.159	0.011	*			-0.002	0.978		Morozova et al (2015)
resistance to fungal infection (MA549)	0.058	0.431		0.061	0.348				0.087	0.266		Wang et al (2017)
resistance to fungal infection (PA14)	0.184	0.089		0.424	0.001	***			0.201	0.080		
oxidative stress paraquat	0.125	0.110		0.021	0.761				0.012	0.884		Weber et al (2012)
oxidative stress msb	0.109	0.166		0.281	0.001	***			0.184	0.026	*	
food intake	-0.083	0.273		-0.194	0.003	**			0.124	0.119		Garlapow et al 2015
boric acid toxicity				0.098	0.167							Najarro et al (2017)
mated lifespan				-0.051	0.439							Durham et al (2014)
fecundity				0.037	0.573							
traumatic brain injury				0.052	0.446							Katzenberger et al (2015)
susceptibility to enteric infection				-0.291	0.001	***						Bou Sleiman et al. (2015)
tolerance to bacterial infection				0.117	0.093							Howick and Lazzaro (2017)
bacterial load				-0.089	0.201							
genotypic deviation of tolerance from bacterial infection				-0.023	0.742							
virgin female lifespan				0.284	0.001	***						Ivanov et al. (2015)
endoplasmic ret stress (hazard ratio)	-0.177	0.060										Chow et al (2013)
endoplasmic ret stress (LT50)	-0.317	0.001	***									
male aggression	0.027	0.707										Shorter et al (2015)
radiation resistance	-0.076	0.345										Vaisnav et al. (2014)
glucose pooled	-0.119	0.162										Unckless et al (2015)
glucose high glucose	-0.078	0.358										
glucose low glucose	-0.088	0.294										
glycerol pooled	-0.194	0.022	*									
glycerol high glucose	-0.224	0.007	**									
glycerol low glucose	-0.104	0.216										
glycogen pooled	-0.040	0.640										
glycogen high glucose	-0.084	0.320										
glycogen low glucose	0.017	0.838										
triglyceride pooled	-0.163	0.055										
triglyceride high glucose	-0.128	0.129										
triglyceride low glucose	-0.128	0.128										
protein pooled	0.009	0.919										
protein high glucose	-0.023	0.791										
protein low glucose	0.020	0.811										
meanweight (ug) pooled	-0.026	0.758										
meanweight (ug) high glucose	-0.052	0.540										
meanweight (ug) low glucose	-0.004	0.966										
low glucose PC1	0.046	0.583										
low glucose PC2	-0.039	0.645										
low glucose PC3	0.063	0.455										
low glucose PC4	-0.204	0.014	*									
low glucose PC5	-0.017	0.840										
high glucose PC1	-0.163	0.053										
high glucose PC2	-0.139	0.101										
high glucose PC3	0.046	0.588										

Significance at p<0.001***, p<0.01**, p<0.05*

Trait	Male		Female			Pooled Sex			Larvae			Citation
	cor	*p*	*cor*	*p*		*cor*	*p*		*cor*	*p*		
high glucose PC4	-0.151	0.075										Unckless et al (2015)
high glucose PC5	-0.004	0.961										
starvation resistance mean (nutrient restrictive)			0.010	0.885								Nelson et al (2016)
starvation resistance mean (nutrient rich)			0.065	0.324								
starvation resistance median (nutrient restrictive)			0.037	0.575								
starvation resistance median (nutrient rich)			0.045	0.492								
starvation resistance max (nutrient restrictive)			0.033	0.616								
starvation resistance max (nutrient rich)			0.028	0.668								
body mass (nutrient restrictive)			0.073	0.266								
body mass (nutrient rich)			-0.012	0.855								
lipid (nutrient restrictive)			-0.343	0.001	***							
lipid (nutrient rich)			-0.234	0.001	***							
protein (nutrient restrictive)			-0.057	0.388								
protein (nutrient rich)			-0.011	0.872								
glucose (nutrient restrictive)			-0.106	0.105								
glucose (nutrient rich)			-0.121	0.065								
lead tolerance	0.081	0.264	0.120	0.058								Zhou et al (2016)
lead development time	-0.018	0.811	-0.076	0.244					0.020	0.793		
DDT mortality (LD50)			0.036	0.582								Schmidt et al (2017)
embryo development time						0.027	0.867		0.139	0.393		Horvath et al (2016)
egg viability						-0.025	0.875		-0.118	0.467		
Larval amanitin resistance LC50						-0.086	0.619		-0.345	0.046	*	Mitchell et al (2017)
Larval amanitin resistance (0.2 ppm)						-0.004	0.965		0.117	0.194		
Larval amanitin resistance (2 ppm)						-0.006	0.947		0.207	0.020	*	
larval insecticide resistance (0.25 ppm)						0.175	0.022	*	0.003	0.973		Battlay et al (2016)
larval insecticide resistance (0.5 ppm)						0.064	0.402		-0.011	0.889		
larval insecticide resistance (1 ppm)						-0.047	0.541		-0.073	0.364		
larval insecticide resistance (2 ppm)						-0.041	0.595		-0.059	0.459		
larval insecticide resistance (LD50)						-0.028	0.712		0.050	0.535		
larval methylmercury survival (0 M)						-0.074	0.344		0.028	0.731		Montegomery et al (2014)
larval methylmercury survival (5 M)						-0.049	0.529		0.029	0.719		
larval methylmercury survival (10 M)						-0.068	0.381		0.006	0.946		
larval methylmercury survival (15 M)						0.044	0.575		0.117	0.148		
larval methylmercury + caffiene survival (0 M + 2 mM)						0.143	0.064		0.160	0.049	*	
larval methylmercury + caffiene survival (10 M + 2 mM)						-0.014	0.854		0.027	0.742		

Significance at p<0.001***, p<0.01**, p<0.05*

APPENDIX C

ANOVAS FOR THE EFFECTS OF WOLBACHIA INFECTION AND INVERSIONS ON ANOXIA TOLERANCE

Analysis	Covariates	Df	SS	RSS	AIC	F	P	
Female	none			12.7010	-420.6000			
	factor(wolbachia)	1	0.0327	12.7340	-422.1600	0.4094	0.5232	
	factor(In_2L_t)	2	0.1788	12.8800	-422.2100	1.1193	0.3291	
	factor(In_2R_NS)	2	0.7344	13.4350	-414.9900	4.5968	0.0115	*
	factor(In_3R_P)	2	0.1523	12.8530	-422.5600	0.9531	0.3877	
	factor(In_3R_K)	2	0.1931	12.8940	-422.0200	1.2088	0.3013	
	factor(In_3R_Mo)	2	0.1431	12.8440	-422.6800	0.8954	0.4105	
Male	none			8.4151	-490.9900			
	factor(wolbachia)	1	0.0346	8.4498	-492.2900	0.6545	0.4197	
	factor(In_2L_t)	2	0.0113	8.4264	-494.7600	0.1064	0.8991	
	factor(In_2R_NS)	2	0.7265	9.1416	-480.8300	6.8631	0.0014	**
	factor(In_3R_P)	2	0.0438	8.4590	-494.1000	0.4140	0.6617	
	factor(In_3R_K)	2	0.1615	8.5766	-491.7400	1.5253	0.2207	
	factor(In_3R_Mo)	2	0.0865	8.5016	-493.2400	0.8169	0.4437	
Average	none			9.5861	-468.7100			
	factor(wolbachia)	1	0.0337	9.6198	-470.1100	0.5583	0.4560	
	factor(In_2L_t)	2	0.0573	9.6434	-471.6900	0.4751	0.6227	
	factor(In_2R_NS)	2	0.7288	10.3149	-460.1800	6.0438	0.0030	**
	factor(In_3R_P)	2	0.0885	9.6747	-471.1400	0.7342	0.4815	
	factor(In_3R_K)	2	0.1755	9.7616	-469.6100	1.4551	0.2365	
	factor(In_3R_Mo)	2	0.1107	9.6968	-470.7500	0.9178	0.4015	
Difference	none			3.8877	-623.0400			
	factor(wolbachia)	1	0.0000	3.8877	-625.0400	0.0011	0.9731	
	factor(In_2L_t)	2	0.1510	4.0387	-620.5200	3.0885	0.0483	*
	factor(In_2R_NS)	2	0.0067	3.8944	-626.7400	0.1365	0.8725	
	factor(In_3R_P)	2	0.0380	3.9257	-625.3700	0.7780	0.4611	
	factor(In_3R_K)	2	0.0073	3.8950	-626.7200	0.1492	0.8615	
	factor(In_3R_Mo)	2	0.0163	3.9040	-626.3200	0.3341	0.7165	
Larvae	none			3.6928	-622.1800			
	factor(wolbachia)	1	0.0124	3.7052	-623.6100	0.5268	0.4690	
	factor(In˙2L˙t)	2	0.0365	3.7293	-624.5100	0.7758	0.4621	
	factor(In˙2R˙NS)	2	0.0742	3.7670	-622.8100	1.5778	0.2097	
	factor(In˙3R˙P)	2	0.0523	3.7450	-623.8000	1.1109	0.3318	
	factor(In˙3R˙K)	2	0.0931	3.7859	-621.9700	1.9792	0.1416	
	factor(In˙3R˙Mo)	2	0.0280	3.7207	-624.9000	0.5943	0.5532	

Significance at p<0.001***, p<0.01**, p<0.05*

APPENDIX D

RESULTS OF THE GWAS FOR ADULTS EXPOSED TO 6 H OF ANOXIA

See Supplementary Excel File

APPENDIX E

RESULTS OF THE GWAS FOR LARVAE EXPOSED TO 1 H OF ANOXIA

See Supplementary Excel File