ADAPTATION AND SURVIVAL OF THE NEMATODE *Caenorhabditis elegans* DURING OSMOTIC-ANOXIC STRESS

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A dissertation

submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

University of Washington

2014

Reading Committee:

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Program Authorized to Offer Degree:

Molecular and Cellular Biology

University of Washington

Abstract

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Oxygen is an absolute requirement for multicellular life. Animals that are deprived of oxygen for sufficient periods of time eventually become injured and die. This is largely due to the fact that, without oxygen, animals are unable to generate sufficient quantities of energy. In human diseases triggered by oxygen deprivation, such as heart attack and stroke, osmotic stress and cell swelling (edema) arise in affected tissues as a direct result of energetic failure. Edema independently enhances tissue injury in these diseases by incompletely understood mechanisms, resulting in poor clinical outcomes. This dissertation presents investigations into the effects of osmotic stress during complete oxygen

deprivation (anoxia) in the genetically tractable nematode *Caenorhabditis elegans.* In these investigations, it is shown that osmotic stress promotes tissue swelling and reduces animal survival during anoxia. In addition, several genetic pathways, including insulin-like signaling and aquaporin-mediated water transport, are identified that affect survival in this environment. These pathways are shown to impact the ability of the nematode to store and utilize glycogen, providing evidence that energetic supply and demand are factors for nematode survival of osmotic-anoxic stress.

Acknowledgements

I would like to express my deepest gratitude to my committee chair and primary mentor, Dr. Mark Roth. This dissertation would not have been possible without his unwavering support over the last four years. Additionally, I would like to thank members of my thesis advisory committee, Dr. Marshall Horwitz, Dr. Charles Murry, Dr. David Hockenbery and Dr. Nancy Maizels, for positively influencing my development as a scientist. Dr. Harold Frazier provided a conceptual and experimental foundation for much of this dissertation research. Dr. Jason Pitt, Dr. Mark Budde and Dr. Mike Morrison also each played significant roles in my scientific training.

Dedication

To Andrea, *por todo*

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Part 1: Introduction

1.1 The necessity of oxygen to metazoan life

Oxygen is an essential and defining element of multicellular (metazoan) life. Nearly all known metazoan species[1] require oxygen to complete their life cycles, a fact that is easily understood through oxygen's fundamental role in aerobic metabolism (respiration). The biological mechanisms required to maintain proper control of oxygen metabolism have evolved in such a way that animals can survive in many different environments of varying oxygen availability. These same biological mechanisms may also be useful in improving outcomes following injuries that deprive tissues of oxygen. What follows is an introduction to oxygen metabolism and a survey of model animal systems that have been used to identify important mechanisms for maintaining proper and sustainable oxygen metabolism. By reviewing these subjects, the stage will be set to introduce the central goal of this thesis, which is to use animal models to better understand mechanisms of survival in extremely low-oxygen environments.

During respiration, mitochondria within the cell convert raw energy from nutrients into adenosine triphosphate (ATP), a high-energy molecule that is required to power most cellular processes (ERNSTER and SCHATZ 1981; LANGEN and HUCHO 2008; LEHNINGER *et al.* 2008). Mitochondrial ATP production (oxidative phosphorylation) is driven by the transfer of electrons from nutrients through a series of carrier molecules on the inner mitochondrial membrane that

[1] Species of *Loricifera* finely illustrate how the only rule in biology is that there is an exception to every rule. These small (<0.1 mm in size) metazoans have recently been discovered to inhabit deep, oxygen-free basins in the Mediterranean Sea, making them the only known example of anaerobic multicellular life to date (DANOVARO *et al.* 2010).

are collectively referred to as the electron transport chain (ETC) (SARASTE 1999). The energy released in each of these electron transfers is utilized to create an electrochemical proton gradient across the inner membrane (MITCHELL 1961). This gradient is then used to power ATP synthesis in a process conceptually similar to the generation of electricity by hydroelectric dams. At the terminus of the ETC, oxygen serves as a final electron acceptor and is reduced to water in the process. The large amount of free energy that is released upon oxygen's reduction results in oxidative phosphorylation being fifteen times more efficient at generating ATP than by anaerobic fermentation (LEHNINGER *et al.* 2008). This added efficiency results in a theoretical five-fold increase in the amount of energy that can be devoted to cellular growth, an increase that likely fueled the rise of multicellurity and increases in maximum animal size, anatomical specialization and higher-order behaviors such as movement and philosophical dissertations (CATLING *et al.* 2005).

There is extensive evidence in the geological and phylogenetic records that oxygenation of the earth's atmosphere was a primary driver of metazoan evolution (SESSIONS *et al.* 2009; LYONS *et al.* 2014). Geochemical proxies for oxygen levels suggest that initial oxygenation occurred about two billion years ago, following the advent of O_2-producing photosynthesis. During this period, which has been termed the Great Oxidation Event, atmospheric oxygen levels increased from <0.1% of present atmospheric levels (PAL) to approximately 5-10% PAL (SESSIONS *et al.* 2009). This coincided with the appearance of

eukaryotic life and was quickly followed by the emergence of mitochondria. After a 1.5 billion year period of relative stability, atmospheric oxygen increased once more to levels closer to that of present day. This increase was accompanied by the appearance of metazoan life in a period referred to as the Cambrian Explosion. More recent rises in atmospheric oxygen within the last 500 million years have overlapped in time with large increases in animal body size, as well as the emergence of terrestrial metazoans, large predatory fish and placental mammals (CATLING *et al.* 2005; DAHL *et al.* 2010; FALKOWSKI *et al.* 2005). These rises have also coincided with the diversification of biochemical reactions and expansion of possible protein structures (JIANG *et al.* 2012; WANG *et al.* 2011). In sum, the coincidence of oxygenation events with periods of rapid animal evolution strongly supports the idea that oxygen was a fundamental requirement for the development of metazoan life on earth.

Throughout evolutionary history, oxygen also likely provided a large negative selective pressure to those organisms that could not effectively manage the toxicity that O_2 presents to cells (RAYMOND and SEGRÈ 2006). Oxygen's high level of reactivity with other compounds leads to formation of reactive oxygen species (ROS), such as peroxide and superoxide, which further react with and damage biomolecules such as DNA, proteins and membrane lipids (VALKO *et al.* 2007). Cells must possess mechanisms for both the direct detoxification of ROS as well as for the repair of ROS-induced damage. In contemporary metazoans, the former is accomplished by both small molecule antioxidant systems like

glutathione or vitamin E, or by enzyme classes such as the superoxide dismutases and peroxidases. The latter is accomplished at the molecular level by DNA repair mechanisms and protein/lipid degradation pathways, at the cellular level by mechanisms of programmed cell death and at the tissue level by innate immune responses (SIMON *et al.* 2000).

Unlike other nutrients, such as carbohydrates, fats and proteins, oxygen cannot be stored in cells for long periods of time. Therefore, it must be continuously acquired from the surrounding environment. While the simplest of animals are small enough to rely on passive diffusion for the acquisition of oxygen, larger animals contain tissues that lack direct access to the atmosphere and therefore possess specialized anatomic structures that enhance O_2 extraction from the respiratory medium (water or air) and facilitate O_2 delivery throughout the body (MAINA 2002). Gas exchangers (lung or tracheal systems for air-breathers, gills for water-breathers) provide direct interfaces with the surrounding respiratory medium that are thin, large in surface area and heavily vascularized to allow for maximal gas exchange and efficient O_2 intake. Circulatory systems facilitate the oxygen's transport to the tissues via heavily branched networks of blood or haemolymph-containing vessels. The capacity of this transport is greatly increased at a molecular level by the cooperative binding of oxygen to specialized hemoglobin carrier proteins.

Fluctuations in oxygen levels are common in animal habitats. Accordingly, mechanisms for sensing O_2 changes are found throughout metazoa. Molecular

mechanisms of oxygen sensing are found in both vertebrates as well as lower animals (KAELIN and RATCLIFFE 2008; SEMENZA 2001), suggesting that cellular adaptation to changes in environmental oxygen predated the evolution of gas exchangers and circulatory systems. Among these are the prolyl-4-hydroxylase (PHD) enzymes, which function as direct oxygen sensors due to the fact that the protein hydroxylation reactions they catalyze are modulated by O_2 concentration. Decreases in O_2 levels lead to reduced PHD activity; this, in turn, leads to the activation of regulatory proteins such as hypoxia inducible factor (HIF) that effect gene expression changes which lead to changes in cellular processes such as glucose metabolism (WANG *et al.* 1995; SEMENZA 2000). In animals with developed circulatory systems, oxygen sensing also takes place at the tissue level through the carotid body organs, which detect decreases in arterial oxygenation through specialized chemoreceptors and relay this information to the central nervous system (GONZALEZ *et al.* 1994).

1.2 Oxygen deprivation in metazoans

Highlighting oxygen's essentiality, animals that are oxygen deprived for long enough periods of time eventually experience irreversible injury that can lead to death. Given that different animal species inhabit a diverse array of environments containing oxygen levels that vary by more than an order of magnitude (VAN VOORHIES and WARD 2000), however, it is critical to emphasize that "oxygen deprivation" does not imply an absolute oxygen concentration, but

rather implies a significant decrease in oxygen relative to that at which a particular species is adapted (normoxia) (BICKLER and BUCK 2007). Severity of oxygen deprivation, then, is defined as the magnitude of decrease in oxygen level. At one extreme, “anoxia” refers to environments in which oxygen levels are undetectable (here, defined as <0.001% by volume). Intermediate to anoxia, “hypoxia” refers to environments containing detectable levels of oxygen that are still lower than normoxia. Interestingly, although degree of injury is often correlated with the severity of oxygen deprivation, this is not always the case. The naturally soil-dwelling nematode *Caenorhabditis elegans,* for example, can survive periods longer than 24 h in environments containing either >5% or <0.01% O_2 with no apparent injury, but survival rates of *C. elegans* after 24 h in 1% O_2 are less than 5% (NYSTUL and ROTH 2004). For animals with developed oxygen extraction and delivery systems, degree of injury is also influenced by whether the oxygen deprivation arises from inadequate oxygenation of the blood (hypoxemia) or from lack of blood flow (ischemia). In the latter case, injury is often more severe due to a lack of washout of catabolites produced during ischemia, such as lactate and protons, leading to increased intracellular osmolarity (JENNINGS *et al.* 1986; KUMAR *et al.* 2005). A final determining factor for severity of injury, which is related to the cause, is the specific tissue that is affected. In general, tissues that are more metabolically active, such as heart and brain, are significantly more susceptible to the energetic consequences of oxygen deprivation and therefore exhibit greater injury (ELTZSCHIG and ECKLE 2011).

In addition to the large range of oxygen levels present in animal habitats, there is also wide variation among animal species in tolerance for oxygen deprivation (hypoxia/anoxia). Specific reasons for this variation are not completely understood, but in some cases may reflect the degree of fluctuation in oxygen levels present in the species' natural habitat. For instance, coral reef habitats are frequently cut off from the surrounding ocean at low tide, resulting in the enclosed water becoming hypoxic due to respiration of the coral and associated organisms. While many fish species are sensitive to hypoxia, those species that inhabit coral reefs display widespread hypoxia tolerance (NILSSON and OSTLUND-NILSSON 2004). Hypoxia/anoxia tolerance is common in terrestrial species inhabiting soil environments, where large fluctuations in oxygen are common (HOBACK and STANLEY 2001; RAMIREZ *et al.* 2007).

Specific oxygen habitats aside, however, mammals and birds are with few exceptions the most hypoxia/anoxia-intolerant of animals compared to lower vertebrates and invertebrates (HOCHACHKA 1986; BICKLER and BUCK 2007). While the African naked mole rat *(Heterocephalus glaber)* is considered to be one of the most hypoxia/anoxia-tolerant mammals and is able to withstand up to 30 minutes of anoxia before apparent brain injury (LARSON and PARK 2009), most turtles, as well as many fish, amphibian and reptile species, are capable of withstanding anoxia for multiple days and even months without injury (BICKLER and BUCK 2007). This large difference in hypoxia/anoxia tolerance is incompletely understood, but may reflect a fundamental difference in the physiology of warm-

blooded (endothermal) versus cold-blooded (ectothermal) animals, as metabolic rates of endotherms are an order of magnitude greater than those of ectotherms of equal body size and temperature (RAMIREZ *et al.* 2007). In humans, the burden of injury due to oxygen deprivation is especially heavy, as diseases triggered by hypoxia/anoxia are responsible for over 60% of all deaths (SEMENZA 2011). While a significant number of these cases arise from long-term (chronic) and sudden (acute) hypoxemia, the overwhelming majority are due to sudden (acute) ischemia in the heart and brain (HOYERT *et al.* 2012). Acute myocardial infarction (heart attack), for instance, occurs when major blood vessels supplying the heart (usually one or more of the coronary arteries) are blocked following *in situ* thrombosis of an atherosclerotic plaque (KUMAR *et al*. 2005). The resulting ischemia and reperfusion injury leads to massive cell death, inflammation and ultimately compromised heart function. In the brain, stroke is triggered by sudden ischemia resulting from an embolic event. While rapid reperfusion of affected tissues in these diseases does lead to improved clinical outcomes, therapies designed to protect tissues from oxygen-deprivation during ischemia do not currently exist, in spite of hundreds of completed clinical trials (GINSBERG 2009; KLONER 2013). This strongly suggests that our current understanding of the basic mechanisms by which oxygen deprivation leads to cell and tissue death is incomplete.

In clinical ischemia, injury is often enhanced and even directly triggered by increases in intracellular water volume (edema) and cell swelling. This edema is

a consequence of reduced ATP supply and a direct result of failure of ATP-dependent (active) mechanisms of ion transport such as the Na/K ATPase (KHANNA *et al.* 2014; SIMARD *et al.* 2007; GARCIA-DORADO *et al.* 2012). Because the cytoplasm contains relatively high concentrations of biomolecules that are negatively charged, there is an electrochemical gradient favoring the influx of positively charged ions from the ECF. This influx increases total intracellular osmolarity, resulting in the formation of an osmotic gradient that favors the inward movement of water (HOFFMANN *et al.* 2009). Under steady-state, normoxic conditions, mechanisms of active transport move Na^+, K^+ and other positively charged ions across their electrochemical gradients to prevent water influx and maintain cell volume. In ischemic diseases like stroke, however, failure of these mechanisms results in rapid loss of ion homeostasis and subsequent swelling of brain cells, a process that is referred to as cytotoxic cerebral edema (CCE). Because CCE is the result of water moving from extracellular to intracellular compartments within the brain, there is no net increase in total brain volume or intracranial pressure. If reperfusion is not initiated and CCE is allowed to progress, however, the depletion of water volume from the ECF in the brain creates a tissue-level osmotic gradient favoring fluid movement across the blood brain barrier (BBB) and into the brain, a process referred to as vasogenic cerebral edema (VCE). If allowed to progress, VCE leads to increases in intracranial pressure that further impede brain perfusion and perpetuate a downward cycle of ischemic-edema (KAHLE *et al.* 2009). Ischemia-triggered

edema is associated with poor clinical outcomes in acute myocardial infarction as well (GARCIA-DORADO *et al.* 2012), suggesting that therapies targeting ion and cell volume homeostasis during ischemia would be of enormous clinical benefit.

1.3 Research in Hypoxia/Anoxia-Tolerant Animals

Animal species that are tolerant of oxygen deprivation (hypoxia/anoxia) compared with mammals can be used to identify basic adaptations required for survival of this stress (BUCK and HOCHACHKA 1993; HOCHACHKA *et al.* 1996). Physiologic and metabolic changes in these animals during hypoxia/anoxia that differ substantially from those in hypoxia/anoxia-sensitive species may be indicative of specific mechanisms for survival. Once characterized, these mechanisms may then be employed as the basis for the development of therapies for human hypoxia/anoxia-triggered diseases (BICKLER 2004). Among vertebrates, some species of freshwater turtles are exceptionally tolerant of anoxia, in spite of the fact that metabolic rates in these species are comparable to those of mammals at similar temperatures. The Western Painted turtle *(Chrysemys picta belli),* for example, is capable of surviving in a dormant state for up to four months in complete anoxia while submerged under ice during winter (ULTSCH 1985). Some fish species are also quite tolerant of anoxia and are, impressively, even capable of remaining active during such periods. The crucian carp *Carassius carassius,* for example, sustain cardiac pumping and autonomic cardiovascular regulation in anoxia for several days (STECYK *et al.* 2004).

Studies in anoxia-tolerant species have revealed that animal survival during oxygen deprivation is highly correlated with the ability to avoid energy failure due to depletion of cellular ATP (HOCHACHKA 1986). This can be accomplished in two distinct ways: (1) by increasing the supply of nutrients used to generate ATP and (2) by suppressing the demand of ATP-consuming processes (HOCHACHKA *et al.* 1996). Regarding (1), fermentation of glucose is the principle means of ATP production in the absence of oxygen. Glycogen, the storage form of glucose, is depleted in a time-dependent manner from animal tissues during anoxia (ROSE *et al.* 1965; DAW *et al.* 1967; HEMS and BROSNAN 1970). In the tissues of many anoxia-tolerant animals, quantities of glycogen are far greater than those found in most mammals, suggesting that increases in glycogen supply fuel ATP production in anoxia for longer periods of time, thereby extending survival limits (BICKLER and BUCK 2007). Regarding (2), it is clear that many anoxia-tolerant species suppress ATP demand through coordinated, reversible suppression of physiologic processes. This process, generally referred to as "hypometabolism," is associated with states of dormancy in which movement and other energetically expensive processes are significantly reduced (BICKLER and BUCK 2007). During hypometabolism, preferential suppression of cellular processes that consume large quantities of ATP likely lead to large reductions in total ATP demand (HOCHACHKA *et al.* 1996).

Suppression of ATP demand has been observed in vertebrate anoxia-tolerant species during oxygen deprivation. Liver cells of the anoxia-tolerant

turtle, for example, exhibit a greater than 90% reduction in the rate of ATP consumption by active ion transport, a process that is responsible for nearly half of cellular ATP consumption in normoxia (BUCK and HOCHACHKA 1993). In contrast to anoxia-sensitive mammalian cells, however, turtle cells do not exhibit signs of pump failure (ion gradient disturbances, cell swelling, etc.) during anoxia, suggesting that other mechanisms exist to prevent loss of ion homeostasis. According to the channel hypothesis, decreases in membrane ion permeability could compensate for reduced active ion transport to maintain cell volume in anoxia. However, these decreases have not been directly linked to animal survival in anoxia owing to the fact that they have not been demonstrated in an intact animal.

1.4 The nematode Caenorhabditis elegans as a model system for hypoxia/anoxia research

C. elegans is an attractive model for hypoxia/anoxia research at a molecular level for a variety of reasons (BRENNER 1974). First, in contrast to vertebrate species, nematodes are relatively small (adults ~1 mm in length) and have a rapid generation time (~4 days), making it possible to perform experiments with large populations and with fast turnaround time. Second, *C. elegans* possess bodies that are optically clear, making it easy to visualize tissues and internal body structures with a microscope. Third, in spite of *C. elegans* only possessing ~1000 somatic cells, these cells are organized in an

invariant fashion into discrete nervous, digestive, excretory, muscular, epithelial and reproductive systems, allowing the investigator to study tissue-specific physiology. Fourth, and perhaps most importantly for the study of molecular mechanisms, *C. elegans* is genetically tractable. Genome-wide screens can be conducted in which unknown mutations are mapped to defined phenotypes (forward genetics) or in which the activity of defined genes are altered and resulting phenotypes subsequently analyzed (reverse genetics). Additionally, the *C. elegans* genome was the first metazoan genome to be sequenced and is exceptionally well-annotated (C. ELEGANS SEQUENCING CONSORTIUM 1998). A high percentage of *C. elegans* genes have human homologs, allowing the disease-oriented researcher to focus on genetic pathways that are known to be present in humans.

Prior work has demonstrated that *C. elegans* is highly tolerant of oxygen deprivation. Early studies showed that *C. elegans* survives and continues to develop in atmospheres containing as little as 5% oxygen with no measurable changes in metabolic rate. In atmospheres containing less than 5% O_2, nematode developmental and metabolic rates decline as a function of oxygen concentration. These declines are reversed, however, upon the animals' return to normoxia (NYSTUL and ROTH 2004). *C. elegans* also survives for more than 2 days in anoxia (VAN VOORHIES and WARD 2000). If adjusted for average nematode lifespan (~3 weeks), this is equivalent to a human surviving for 6-8 years without taking a single breath. During anoxia, *C. elegans* enters an altered

physiologic state referred to as “suspended animation,” in which observable processes such as locomotion, feeding and cell division cease (PADILLA *et al.* 2002). Entry into suspended animation is required for nematode anoxia survival (NYSTUL *et al.* 2003), yet the specific changes to nematode physiology and metabolism that occur during suspended animation remain poorly understood. In contrast to anoxia-tolerant vertebrates, which exhibit a stabilization of cellular ATP levels during oxygen deprivation, nematodes in suspended animation display a ten-fold reductions in cellular ATP during suspended animation (PADILLA *et al.* 2002). Nematode glycogen stores are also depleted in a time-dependent manner by roughly three-fold after 24 h in anoxia (FÖLL *et al.* 1999; FRAZIER and ROTH 2009).

In spite of the impressive ability of *C. elegans* to survive oxygen deprivation, nematodes do eventually become injured and die if oxygen is not restored. Similar to other ectothermic animals, nematode survival during oxygen deprivation is correlated with environmental temperature. In anoxia, increasing the temperature from 20 to 28°C for the duration of the exposure results in an 80% decrease in survival after 24 h (MENDENHALL *et al.* 2006). Similar effects are also seen between these temperatures in severe hypoxia (SCOTT *et al.* 2002; ANDERSON *et al.* 2009). A major focus of *C. elegans* hypoxia research has therefore been to identify genes whose activity regulates nematode survival in high temperature (28°C) hypoxia/anoxia, as well as in extended (>72 h) hypoxia/anoxia. Investigators have used these conditions in genome-wide

screens as well as directed experiments to identify genetic pathways affecting nematode survival during oxygen deprivation. Insulin/IGF signaling and protein synthesis, two cellular processes that have been found to greatly affect nematode survival of oxygen deprivation, are discussed in greater depth below.

Insulin-like signaling regulates survival of oxygen deprivation in *C. elegans*

Nematode insulin/IGF signaling occurs through a conserved PI-3 kinase pathway that is known to regulate several key aspects of animal physiology, including longevity, development, learning and metabolism (KENYON *et al.* 1993; VAN VOORHIES and WARD 1999; TOMIOKA *et al.* 2006; DEPUYDT *et al.* 2014). In this pathway, signals from the insulin-like receptor homolog *daf-2* lead to an activation of the PI-3 kinase homolog *age-1* and subsequent activation of the downstream protein kinases homologs *akt-1* and *akt-2*. As a result, the FOXO transcription factor homolog *daf-16* is phosphorylated, preventing its entry into the nucleus and blocking *daf-16*-dependent changes in gene expression. *Daf-2* signaling is negatively regulated at the level of *age-1* by the PTEN homolog *daf-18* (OGG and RUVKUN 1998).

Reduction of function mutations in the *daf-2* gene have been found to confer large increases in survival of both high-temperature hypoxia as well as extended (>72 h) anoxia, suggesting that *daf-2* signaling represses nematode survival during oxygen deprivation (SCOTT *et al.* 2002; MENDENHALL *et al.* 2006). Consistent with this, mutations in *daf-18* that increase *daf-2* signaling result in significant decreases in survival of high-temperature hypoxia. In *daf-2* mutants,

loss of functional *daf-16* completely suppresses the enhanced survival phenotypes of these animals, suggesting that *daf-16* activity is necessary for survival in oxygen deprivation in these mutants (SCOTT *et al.* 2002). *Daf-16* is responsible for the regulation of nearly 200 genes, most of which are upregulated in hypoxia/anoxia-resistant *daf-2* mutants, making them candidates for antagonizing death in anoxia/hypoxia (MABON *et al.* 2009). Considerable focus has been given to identifying which of these genes are responsible for mediating *daf-2/daf-16*-dependent survival of oxygen deprivation, with the hope that this may illuminate the specific physiologic processes necessary for nematode survival in oxygen deprivation. Surprisingly, however, the results of these studies have been inconclusive, due mainly to the fact that (1) only a small number (<10) produce hypoxia/anoxia sensitivity when mutated or knocked down and (2) of these, all are either unrelated in function or have no clear human orthologs (MABON *et al.* 2009). In contrast, mechanisms of *daf-2*-mediated hypoxia/anoxia survival that are independent of *daf-16* activity have not been thoroughly investigated.

Suppression of protein synthesis during oxygen deprivation in *C. elegans*

Decreases in protein synthesis rates have long been associated with survival increases during oxygen deprivation in a variety of experimental models (FÄHLING 2009). Although this was presumed to be due to the large ATP cost that protein synthesis incurs upon the cell, this hypothesis has not been adequately

tested. In *C. elegans*, multiple studies have found that functional loss of genes involved in protein synthesis, such as those coding for aminoacyl-transfer RNA (tRNA) synthetase enzymes and translation factors, significantly increases nematode survival during oxygen deprivation (ANDERSON *et al.* 2009; SCOTT *et al.* 2013). Global protein synthesis rates were reduced by more than 50% in the most anoxia/hypoxia-resistant of these mutants. Several of these mutations are also strongly correlated with resistance to toxicity due to misfolded proteins.

1.5 Central Hypothesis

To summarize the major themes presented in this dissertation so far:

1. Oxygen is essential for animal life; oxygen deprivation leads to animal injury and death.
2. During clinical forms of oxygen deprivation such as heart attack and stroke, injury is often enhanced by osmotic stress (edema) by poorly understood mechanisms.
3. Mechanisms promoting survival during oxygen deprivation, while incompletely understood, may be further elucidated through the comparative study of anoxia-tolerant species.

4. Anoxia-tolerant animals avoid energetic failure during periods of oxygen deprivation by increasing energetic supply (glycogen storage) and decreasing energetic demand (hypometabolism).

The central hypothesis of this dissertation is: **Combining osmotic stress with anoxia in a *C. elegans* survival model will result in decreased animal survival time (sensitization) and will allow for the identification of novel survival mechanisms that may be relevant to the treatment of human ischemic diseases such as heart attack and stroke.**

Part 2: Results and Discussion

2.1 Hyposmotic-Anoxia: A C. elegans model of ischemic injury

Loss of membrane ion homeostasis during periods of clinical ischemia leads to osmotic imbalances and swelling (edema) in affected tissues as a result of water influx (TRANUM-JENSEN *et al.* 1981; SIMARD *et al.* 2007; GARCIA-DORADO *et al.* 2012; WEERASINGHE and BUJA 2012). Edema contributes significantly to the degree of injury and death in ischemic diseases such as heart attack and stroke, yet specific cytoprotective therapies targeting osmotic-ischemic stress have not been developed (MORETTI *et al.* 2014). In contrast, tissues of anoxia-tolerant turtles exhibit no signs of edema formation or changes in membrane ion gradients during oxygen deprivation (DOLL *et al.* 1991; PAMENTER *et al.* 2012). Instead, these tissues display significant reductions in membrane ion permeability by unknown mechanisms (BUCK and HOCHACHKA 1993; BUCK and BICKLER 1998). We sought to model osmotic-ischemic stress in the nematode *Caenorhabditis elegans* for the purpose of identifying genetic mechanisms that regulate animal survival in this environment. During periods of anoxia, *C. elegans* enters a state of suspended animation in which animal movement, including feeding, ceases (PADILLA *et al.* 2002). Therefore, although nematodes do not possess developed circulatory systems, the nematode in anoxia recapitulates two defining elements of ischemia: oxygen deprivation and nutrient starvation.

We found that exposing *C. elegans* to combined osmotic-anoxic stress results in significantly decreased animal survival compared to osmotic stress or anoxia alone. While wild type (N2) animals on isosmotic media are capable of

>95% survival after 24 h in anoxia, survival decreases by ten- to twenty-fold if animals are placed on hyposmotic or hyperosmotic media for the duration of anoxia **(Figure 1A, 1B).** To determine whether adaptation to altered osmotic environments can precondition animals to survive oxygen deprivation in that environment, we cultured wild type animals on hyposmotic, isosmotic or hyperosmotic media for 24 h prior to anoxia exposure. We found that preconditioning animals in hyposmotic or hyperosmotic media increased their survival of the same osmotic condition while in anoxia by nearly 10-fold **(Figure 2).** The same type of osmotic stress (hyposmotic or hyperosmotic) was required for effective preconditioning, suggesting that animals were adapting to the direction of the osmotic gradient. Interestingly, the vast majority of animals (>90%) that die in hyposmotic-anoxia display visible herniation of intestinal and gonadal tissue through the vulva or outer cuticle **(Figure 1C)**. This “popping” phenotype, which can be used as a convenient visual marker to accurately determine survival rates *in situ* during an oxygen deprivation experiment, is highly indicative of tissue swelling due to water influx. Consistent with this, time-lapse photography of animals in hyposmotic-anoxia revealed gradual increases in animal body volume prior to death **(Figure 3).**

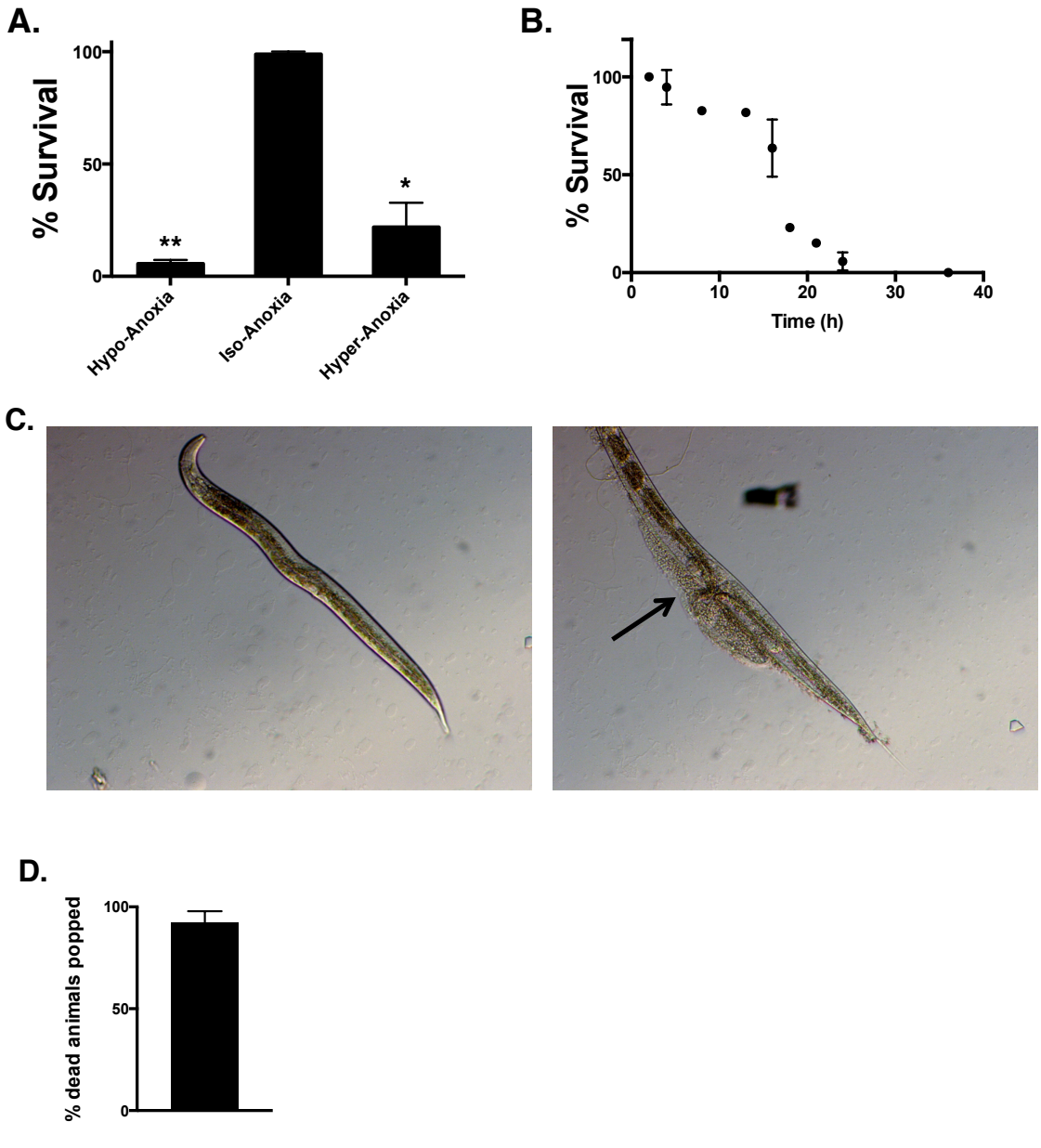

Figure 1: Osmotic stress sensitizes wild type *(N2)* animals to anoxia. **(A)** Percent survival of *N2* animals after exposure to 24 h anoxia while on media that was hyposmotic (hypo; 10 mOsm), isosmotic (iso; 200 mOsm), or hyperosmotic (hyper; 600 mOsm) relative to conventional Nematode Growth Medium (~200 mOsm) on which animals were raised. **(B)** Percent survival of *N2* animals in hyposmotic-anoxia at indicated time points. **(C)** Representative micrographs of *N2* L4 animals on hyposmotic media before (left) and after (right) lethal anoxic exposure. Arrow points to herniation of intestinal and gonadal tissue through vulva ("popping" phenotype). **(D)** Percentage of dead *N2* L4 animals that exhibit popped phenotype following a 24 h exposure of hyposmotic-anoxia. All experimental data represent a minimum of three independent trials with at least 20 animals. Errors bars represent +/- standard error of the mean. **P* <0.05, ***P* <0.01 (two-tailed *t* test).

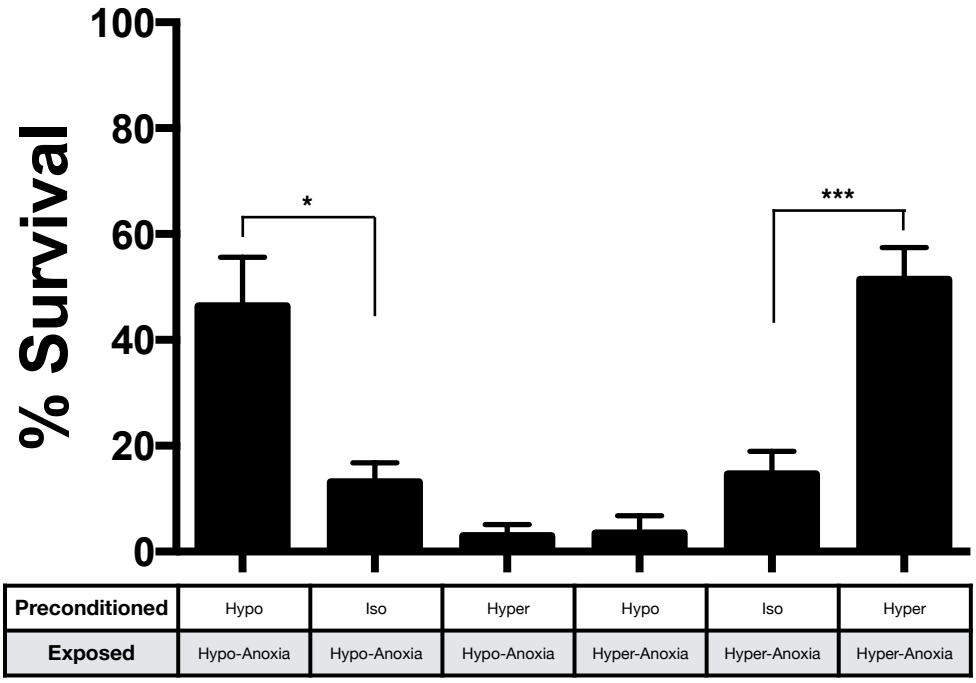

Figure 2: Osmotic preconditioning in normoxia significantly alters survival rates in anoxia. Wild type (N2) L4 animals were preconditioned for 24 h in normoxia on hyposmotic (hypo), isosmotic (iso), or hyperosmotic (hyper) media. Shown are percent survival rates for these animals after 24 h in anoxia while on hyposmotic (hypo), isosmotic (iso), or hyperosmotic (hyper) media. Experimental data represent a minimum of three independent trials, each with at least 10 animals. Errors bars denote +/- standard error of the mean. $^{*}P < 0.05$, $^{***}P < 0.001$ (two-tailed *t* test).

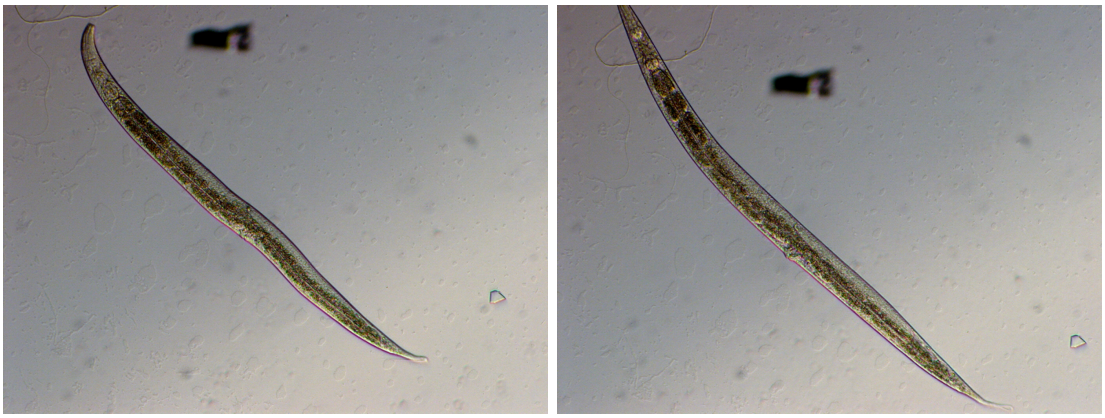

Figure 3: Nematode body increases in size during hyposmotic-anoxia. Representative micrographs (taken at same magnification) of a wild type (N2) L4 animal taken at 2 h (left) and 8 h (right) during hyposmotic-anoxia exposure. Images were taken at the same magnification. Note that at 8 h, substantial increase in animal length is apparent, such that a portion of the animal's head has shifted out of the field of view.

We find that osmotic (hyposmotic or hyperosmotic) stress significantly sensitizes nematodes to periods of anoxia, such that 24 h animal survival rates are reduced up to twenty-fold compared to anoxia without osmotic stress. In hyposmotic-anoxia, animals exhibit time-dependent increases in body volume and eventual herniation of internal tissues, suggesting that failure to maintain osmotic homeostasis contributes to animal lethality in this environment.

To our knowledge, *C. elegans* hyposmotic-anoxia represents the first animal survival model in which osmotic stress is combined with oxygen deprivation. Because injuries that arise during ischemic diseases such as stroke and heart attack are often the combined result of oxygen deprivation and edema,

mechanisms of survival during hyposmotic-anoxia identified in *C. elegans* may contribute to our understanding of the pathogenesis of these diseases and may offer insight into novel therapeutic strategies.

2.2 C. elegans aquaporin water channel homologs regulate animal survival of hyposmotic-anoxia

Reductions in membrane permeability have been hypothesized to facilitate the entry into reversible hypometabolic states in anoxia-tolerant animals, likely because such reductions allow for decreased rates of ATP-dependent ion transport in a process referred to as "channel arrest" (BUCK and HOCHACHKA 1993; HOCHACHKA *et al.* 1996; BUCK and BICKLER 1998). Previous studies of channel arrest have focused primarily on the membrane permeabilities of ions such as Na^+, Ca^{2+} and K^+ and have not considered whether changes in the permeabilities of other biologically relevant molecules contribute to hypometabolism during oxygen deprivation. While cell swelling can be triggered by changes in intracellular and/or extracellular osmolarities, it is always a direct result of water influx (MCMANUS *et al.* 1995; HOFFMANN *et al.* 2009). We therefore hypothesized that reductions in membrane water permeability would blunt cell swelling and increase survival in the nematode during hyposmotic-anoxia. To test this, we obtained *C. elegans* genetic loss-of-function mutants for four nematode aquaporin water channel homologs: *aqp-2, aqp-4, aqp-8* and *aqp-11.* Aquaporins represent a functionally conserved class of membrane-bound proteins that

facilitate rapid, bidirectional water flux driven by the osmotic gradient (AGRE *et al.* 1993). Experiments carried out in the *Xenopus* oocyte model have demonstrated that genetic removal of aquaporin channels decreases membrane water permeability by as much as ten-fold (PRESTON *et al.* 1992; HUANG *et al.* 2007). We found that the *aqp-2(ok2159)* and *aqp-4 (ok2587)* mutants exhibited significant increases in survival rates relative to wild type following 24 h hyposmotic-anoxia exposure, although the *aqp-8(ok2800)* and *aqp-11(ok3578)* mutants did not **(Figure 4A).** To rule out the possibility that the enhanced survival phenotypes of the *ok2159* and *RB1967* mutants were due to linked, epistatic mutations elsewhere in the genome, we treated wild type (N2) animals with RNAi targeting *aqp-2* or *aqp-4*. We found that both of these knockdowns resulted in significantly increased survival rates following hyposmotic-anoxia, confirming that expression of *aqp-2* and *aqp-4* impede survival in this environment **(Figure 4B).**

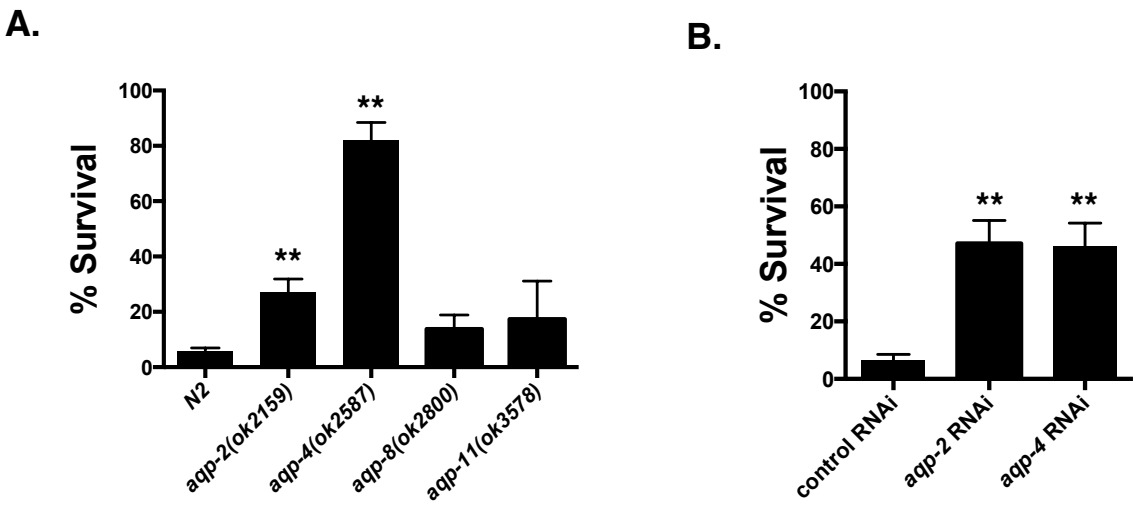

Figure 4: Removal of aquaporin water channels from major nematode-environment interfaces promotes survival in hyposmotic-anoxia. **(A)** Percent survival of several aquaporin *(aqp)* loss-of-function mutant L4 animals following 24 h exposure to hyposmotic-anoxia. **(B)** Percent survival of *control(RNAi)*, *aqp-2(RNAi)* and *aqp-4(RNAi)* L4 animals following 24 h exposure to hyposmotic-anoxia. All experimental data represent a minimum of four independent trials with at least 20 animals. Errors bars represent +/- standard error of the mean. $^{**}P < 0.01$ (two-tailed *t* test).

We chose to further investigate the mechanisms responsible for the high level of resistance to hyposmotic-anoxia observed in the *RB1967* mutant. To determine if *RB1967* animals are generally anoxia resistant, we exposed them to long-term (48 h) anoxia while on isosmotic media, thereby uncoupling anoxic stress from osmotic stress. We found that *RB1967* animals possess levels of resistance in this assay that are similar to that seen in hyposmotic-anoxia **(Figure 5)**, suggesting that loss of functional *aqp-4* confers resistance to anoxia that is not solely dependent on the external osmotic milieu.

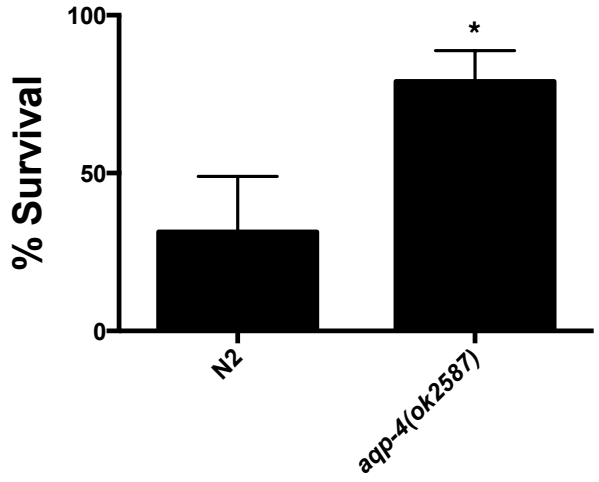

Figure 5: The *aqp-4(ok2587)* mutant is resistant to long-term anoxia. Percent survival of N2 or *aqp-4(ok2587)* L4 animals following 48 h in anoxia on conventional Nematode Growth Medium (NGM). All experimental data represent a minimum of five independent trials, each with at least 20 animals. Errors bars denote +/- standard error of the mean. $*P < 0.05$ (two-tailed t test).

To determine if nematode *aqp-4* expression is regulated by hyposmotic environments, we raised wild type animals from embryo to adulthood on hyposmotic media, then measured *aqp-4* mRNA levels by quantitative PCR (qPCR). We found that *aqp-4* expression is reduced in this environment by nearly three-fold **(Figure 6)**, suggesting that a component of the nematode adaptive response to cell swelling during hyposmotic stress involves reducing membrane water permeability. Consistent with this, we found that *aqp-4(RB1967)* animals are deficient in their ability to undergo hyposmotic preconditioning prior to hyposmotic-anoxia exposure **(Figure 7).** Previously, it has been has demonstrated that nematode *aqp-4* is expressed at the apical membrane of the intestine, a major interface between the nematode and its external environment (HUANG *et al.* 2007). Changes in *aqp-4* expression could, therefore, significantly alter the rate at which water moves into or out of the animal. To determine the

effect of *aqp-4* loss on cell/tissue swelling, we measured body volumes of *RB1967* animals during hyposmotic-anoxia and found that, compared to wild type, *RB1967* animals exhibit significantly smaller increases in body volume during hyposmotic-anoxia exposure **(Figure 8).**

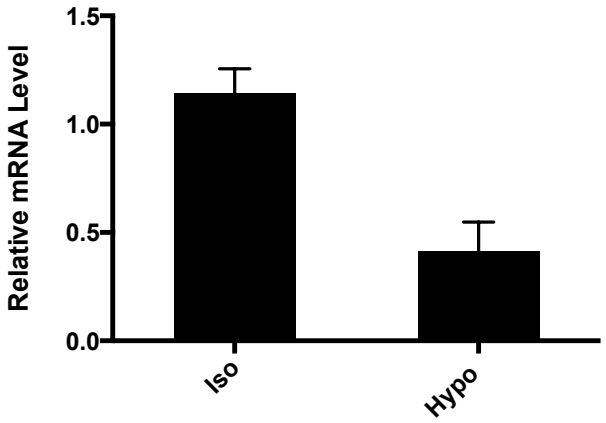

Figure 6: Expression of *aqp-4* is downregulated in hyposmotic environments. Levels of *aqp-4* mRNA were quantitated by real-time reverse transcription-PCR in wild type *(N2)* L4 animals following 24 h on either isosmotic (iso; 200 mOsm) or hyposmotic media (hypo; 10 mOsm) in normoxia. The cDNA of *aqp-4* is shown as the average increase relative to the geometric mean of the cDNA levels of the housekeeping genes *act-1* and *gpd-2.* Errors bars represent +/- standard error of the mean.

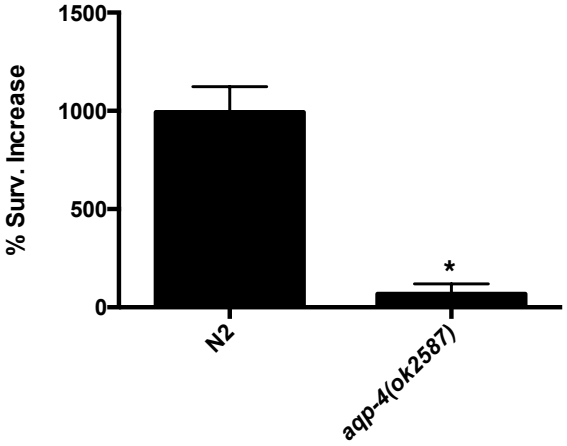

Figure 7: Hyposmotic preconditioning to hyposmotic-anoxia requires functional *aqp-4. N2* and *aqp-4(ok2587)* L4 animals were placed on either isosmotic or hyposmotic media for 24 h, then exposed to hyposmotic-anoxia for 24 h. Percent survival increase was calculated as the percent difference in survival following hyposmotic-anoxia of animals preconditioned on hyposmotic media relative to those preconditioned on isosmotic media. Experimental data represent a minimum of four independent trials, each with at least 20 animals. Errors bars denote +/- standard error of the mean. *$P < 0.05$ (two-tailed *t* test).

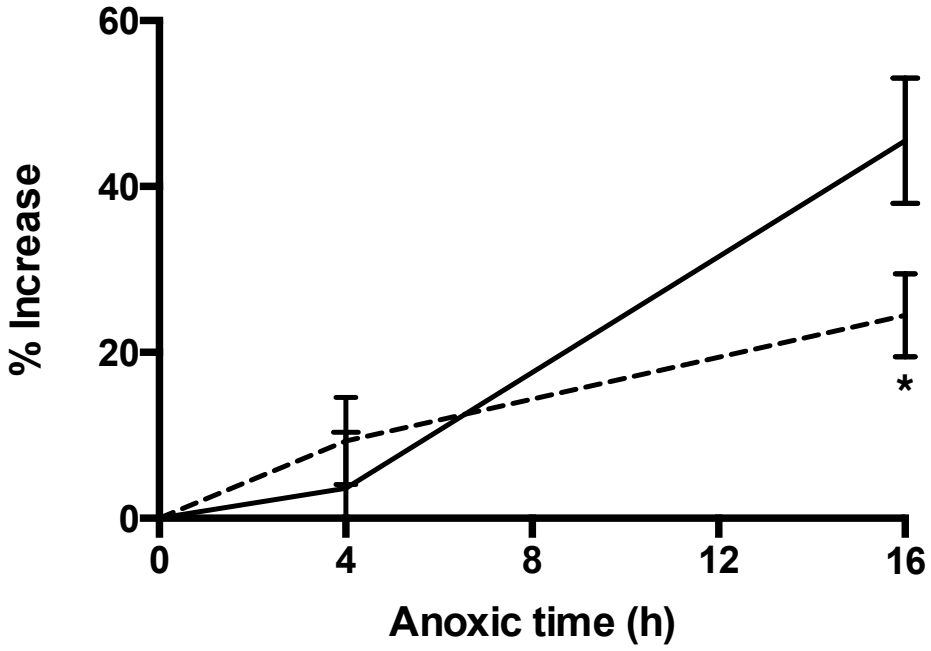

Figure 8: Body swelling during hyposmotic-anoxia is blunted in the aqp-4(ok2587) mutant. Quantification of whole-animal body volume for N2 L4 animals (solid line) and aqp-4(ok2587) L4 animals (dashed line). Each data point represents measurements of at least 5 animals normalized to volume at t = 0. Error bars denote +/- standard error of the mean. *$P < 0.05$ (two-tailed *t* test).

During hyposmotic stress, cells rely on ATP-consuming (active) mechanisms of ion transport to limit swelling. We considered a model for *aqp-4*-mediated anoxia survival in which loss of functional *aqp-4* results in decreased rates of active transport due to reduced membrane water permeability. While previous studies have associated suppression of active transport with reduced energetic demand (hypometabolism) during anoxia (BUCK and HOCHACHKA 1993; EBENSPERGER *et al.* 2005), it remains unclear if this suppression is sufficient for the hypometabolic changes observed. We therefore investigated the effects of *aqp-4* loss on nematode energy consumption during hyposmotic-anoxia. To accomplish this, we quantified animal glycogen content at different time points during hyposmotic-anoxia exposure. Glycogen is the primary energy source utilized during anoxia and is not regenerated in *C. elegans* until normoxia is restored (FRAZIER and ROTH 2009). Therefore, the rate of decrease in glycogen content during anoxia is proportional to the rate of energy consumption. We found that this rate in *RB1967* animals during hyposmotic-anoxia exposure was less than half that of wild type *(N2)* **(Figure 9)**. This indicates that the presence of a functional AQP-4 channel in wild type animals increases energetic demand during hyposmotic-anoxia. Interestingly, decreases in glycogen content during hyposmotic-anoxia coincided temporally with decreases in survival for both *N2* and *RB1967* animals, suggesting that the probability of surviving anoxia is related to body glycogen content at that point in time.

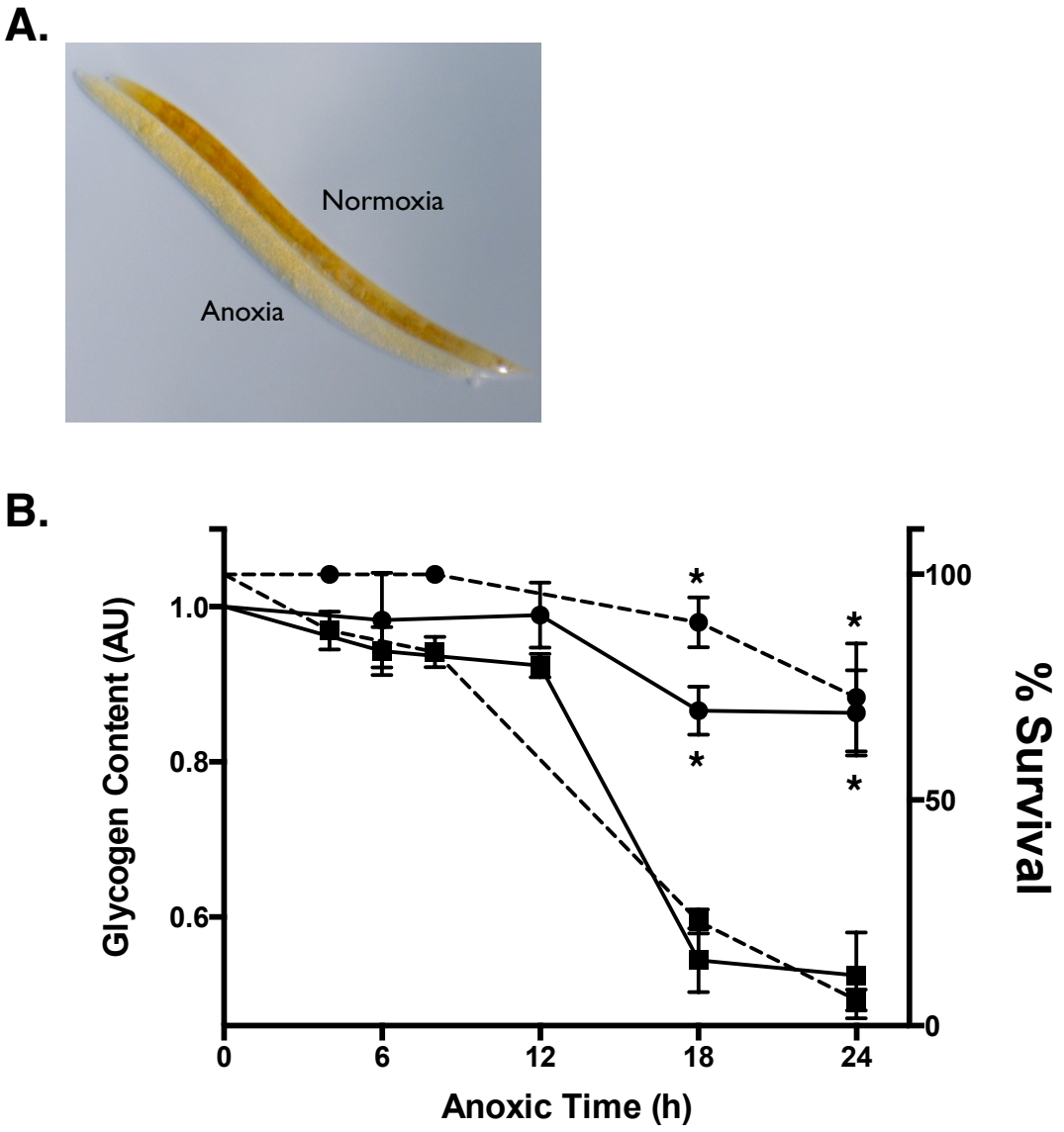

Figure 9: Rate of glycogen consumption in hyposmotic-anoxia is reduced in the *aqp-4(ok2587)* mutant and is correlated temporally with survival. **(A)** Representative micrographs of N2 L4 animals stained for glycogen following either normoxia or anoxia. **(B)** Quantification of glycogen stain (solid lines) in N2 (squares) and *aqp-4(ok2587)* (circles) animals at indicated time points of hyposmotic-anoxia exposure. Dashed lines represent percent survival for respective strains at indicated time points. Each data point represents measurements of at least five H-A-exposed animals normalized to normoxic controls. Error bars denote +/- standard error of the mean. *$P <$ 0.05 *aqp-4(ok2587)* vs. N2 (two-tailed *t* test).

Over two decades ago, the channel arrest hypothesis was proposed to explain how anoxia-tolerant animals are able to maintain ion homeostasis and cell volume while simultaneously suppressing mechanisms of active ion transport during oxygen deprivation. Most studies of channel arrest have intuitively focused on mechanisms of ion transport as effectors of this process and have not investigated possible roles for water transport. Here we present evidence that reduction of aquaporin-mediated water transport leads to decreased tissue swelling and increased survival in anoxia. Removal of aquaporin channels from the membrane also leads to reduced rates of glycogen utilization in anoxia, suggesting that suppression of aquaporin function may be a mean of inducing nematode hypometabolism in this environment.

2.3 Glycogen promotes survival of hyposmotic-anoxia

Previous studies in anoxia-tolerant species have demonstrated that tissue glycogen levels decrease in a time-dependent manner during anoxia exposure (ROSE *et al.* 1965; BUCK *et al.* 1993; BICKLER and BUCK 2007; FRAZIER and ROTH 2009). We therefore hypothesized that *C. elegans* glycogen content would be directly correlated with survival time in hyposmotic-anoxia. To test this, we treated wild type *(N2) C. elegans* with RNAi targeting the nematode homolog of glycogen synthase (*gsy-1*). Previous work in our lab has demonstrated that knockdown of *gsy-1* in *C. elegans* significantly reduces animal glycogen content (FRAZIER and ROTH 2009); confirming this, *gsy-1(RNAi)* L4 animals exhibit

dramatically reduced glycogen staining by iodine compared to animals that had been treated with empty RNAi vector *(control(RNAi))* **(Figure 10A)**. We also found that, after a 16 h exposure to hyposmotic-anoxia, the survival rate of *gsy-1(RNAi)* animals was reduced by five-fold compared to controls **(Figure 10B)**. This suggests that glycogen synthesis is necessary for nematode survival in hyposmotic-anoxia.

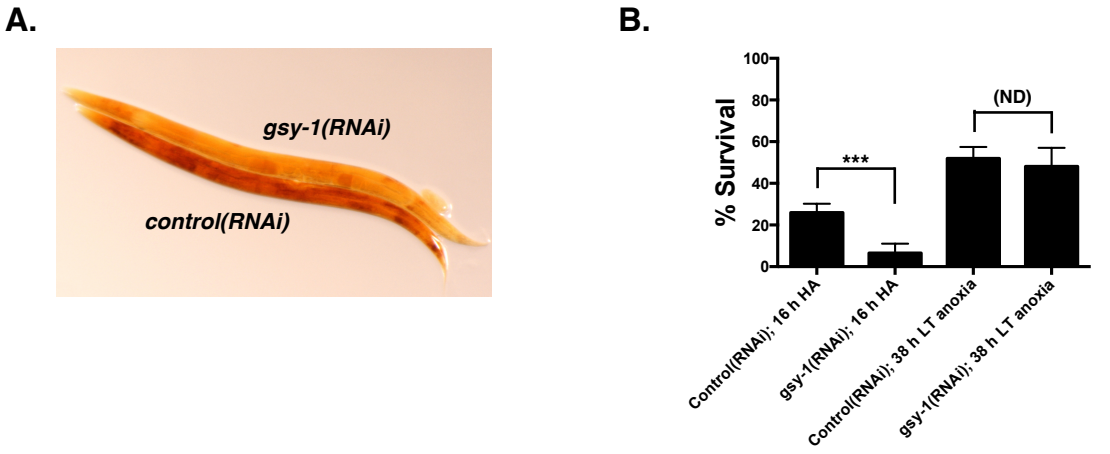

Figure 10: Glycogen synthesis is required for survival of anoxia in hyposmotic, but not isosmotic environments. **(A)** Representative micrograph of *control(RNAi)* and *gsy-1(RNAi)* L4 animals stained for glycogen (glycogen visualized as dark rust color). **(B)** Survival of control(RNAi) and *gsy-1(RNAi)* L4 animals following 16 h exposure to hyposmotic-anoxia or to 38 h isosmotic-anoxia. Data represent means of at least three independent experiments. Error bars denote +/- standard error of the mean. ***P <0.001 (two-tailed t-test).

Long-term (>3 d) survival rates of *gsy-1(RNAi)* animals on hyposmotic media while in normoxia are greater than 95% and not significantly different than control(RNAi) animals. Additionally, *gsy-1(RNAi)* appear phenotypically normal in this environment, with no observable defects in locomotion, feeding, or reproduction (data not shown). Together, these observations strongly suggest

that *gsy-1(RNAi)* sensitivity in hyposmotic-anoxia is not exclusively due to these animals being vulnerable to hyposmotic stress. Surprisingly, however, we observed that the survival rate of *gsy-1(RNAi)* animals in 38 h anoxia while on isosmotic media is indistinguishable from that of *control(RNAi)* animals after 38 h **(Figure 10B)**. This suggests that, in animals lacking an ability to perform glycogen synthesis, hyposmotic stress is more effective at sensitizing to anoxia than in wild type animals. Interestingly, previous reports in several mammalian experimental systems indicate that hyposmotic environments promote post-translational activation of glycogen synthase by cell-swelling-dependent mechanisms (BAQUET *et al.* 1990; AL-HABORI *et al.* 1992; LOW *et al.* 1996). Activation of glycogen synthesis is thought to reduce further swelling by removing free glucose from intracellular pools, thereby reducing intracellular osmolarity. We reasoned that if the *C. elegans* GSY-1 enzyme were activated by similar mechanisms, a measurable increase in glycogen content would be observed in animals raised on hyposmotic media. Interestingly, however, we observed no significant increase in glycogen staining of wild type *(N2)* animals raised from embryo to adulthood on hyposmotic media relative to those raised on isosmotic media **(Figure 11)**. Animals raised on hyposmotic media are, however, resistant to subsequent exposure to 24 h hyposmotic-anoxia **(Figure 2),** suggesting that hyposmotic preconditioning to this environment may occur by mechanisms that do not involve glycogen synthesis/accumulation.

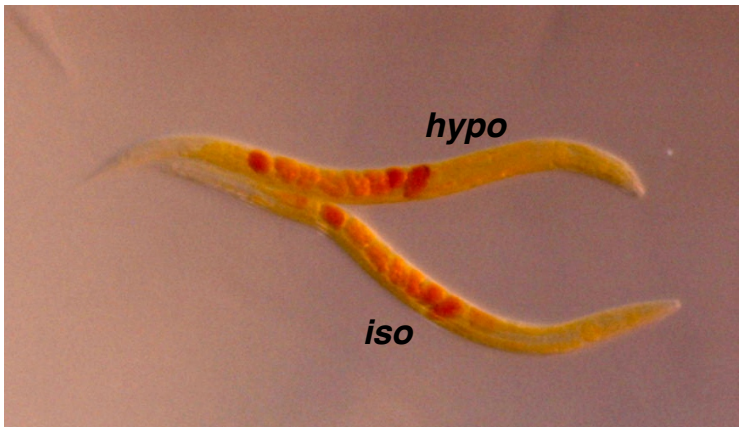

Figure 11: Representative micrograph of wild type (N2) first day adult animals stained for glycogen (glycogen visualized as dark rust color) after being raised on either hyposmotic *(hypo)* or isosmotic *(iso)* media.

In *C. elegans*, *daf-2* codes for an insulin/IGF receptor homolog that signals through a conserved PI-3 kinase pathway to regulate gene expression (KENYON *et al.* 1993; MORRIS *et al.* 1996; OGG *et al.* 1997; LIN *et al.* 2001). The PTEN homolog *daf-18* negatively regulates *daf-2* activity (OGG and RUVKUN 1998; GIL *et al.* 1999). Reductions in *daf-2* signaling have been associated with resistance to long-term anoxia and high temperature hypoxia but the mechanisms underlying this resistance are unclear (MABON *et al.* 2009). We found that the hypomorphic *daf-2(e1370)* mutant exhibits an enhanced survival rate in hyposmotic-anoxia, such that the survival of *e1370* animals is more than 15 times greater than that of N2 animals after 24 h **(Figure 12A).** We also found that the loss-of-function *daf-18(e1375)* mutant displays significantly reduced survival relative to wild type following 16 h exposure to hyposmotic-anoxia **(Figure 12B).** These data strongly suggest that reductions in *daf-2* signaling promote survival in hyposmotic-anoxia.

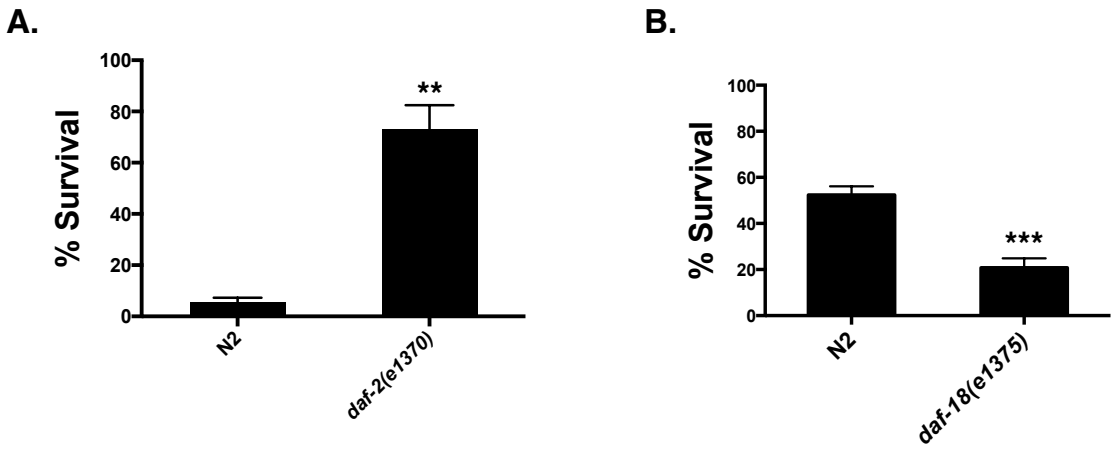

Figure 12: Nematode insulin-IGF signaling suppresses survival in hyposmotic-anoxia. **(A)** Percent survival of *daf-2(e1370)* mutant L4 animals following 24 h exposure to hyposmotic-anoxia. **(B)** Percent survival of *daf-18(e1375)* mutant animals following 16 h exposure to hyposmotic-anoxia. All experimental data represent a minimum of four independent trials with at least 20 animals. Errors bars represent +/- standard error of the mean. ** P <0.01, ***P <0.001 (two-tailed t test).

Previous studies have shown that *daf-2(e1370)* animals exhibit a decreased metabolic rate (VAN VOORHIES and WARD 1999), increased body glycogen content (FRAZIER and ROTH 2009) and altered expression of genes involved in metabolism (LEE *et al.* 2003; MURPHY *et al.* 2003). To test the hypothesis that the *daf-2(e1370)* enhanced survival phenotype depends on glycogen synthesis, we treated *daf-2(e1370)* animals with RNAi targeting *gsy-1* and measured the survival rates of these animals in hyposmotic-anoxia. We found that *gsy-1* knockdown reduced anoxia survival in *daf-2(e1370)* animals to that of wild-type **(Figure 13),** suggesting that glycogen synthesis is required for *daf-2(e1370)* enhanced survival in anoxia. Interestingly, we also found that rates of glycogen utilization during hyposmotic-anoxia were correlated directly with insulin/IGF signaling, such that the rate of glycogen consumption relative to wild

type was significantly decreased in *daf-2(e1370)* animals while significantly increased in *daf-18(e1375)* animals **(Figure 14)**. This suggests that insulin-like signaling in the nematode regulates glycogen demand during anoxia and that this demand is a major determinant of nematode survival limits in anoxia.

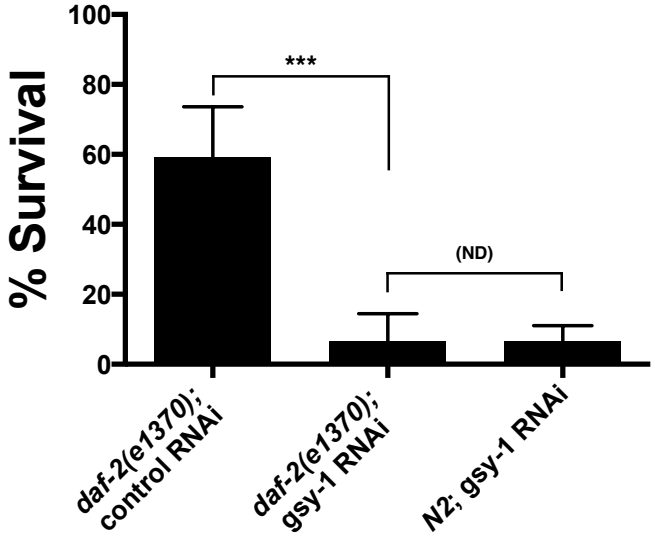

Figure 13: The *daf-2(e1370)* enhanced survival phenotype in hyposmotic-anoxia depends on glycogen synthesis. Survival of *daf-2(e1370)*;*control(RNAi)* and *daf-2(e1370)*;*gsy-1(RNAi)* L4 animals following 24 h exposure to hyposmotic-anoxia. Data represent means of at least five independent experiments. Error bars denote +/- standard error of the mean. ***P <0.001 (two-tailed t-test).

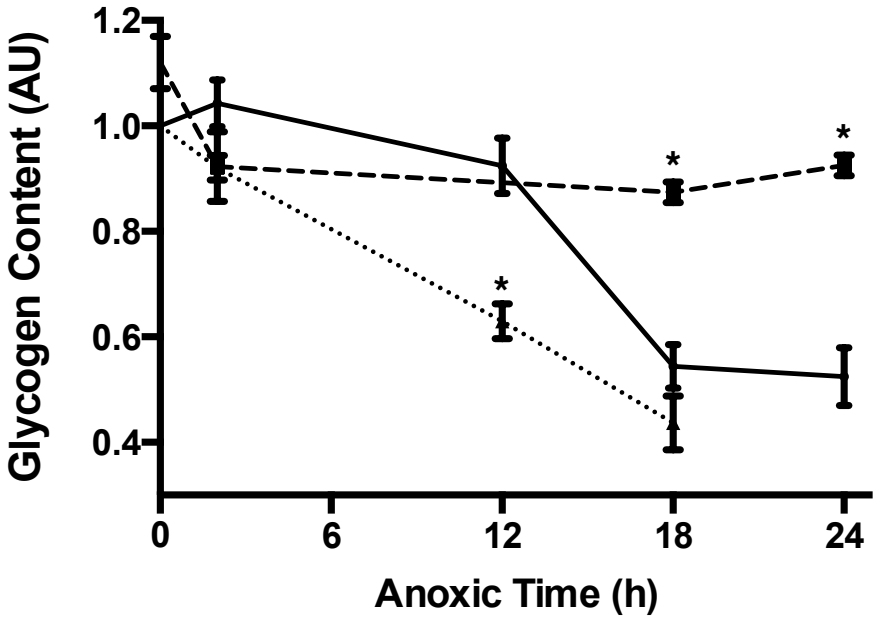

Figure 14: Rate of glycogen consumption in hyposmotic-anoxia (H-A) correlates directly with *daf-2*-mediated insulin/IGF signaling. Quantification of glycogen staining in N2 (solid line), *daf-2(e1370)* (dashed line) *and daf-18(e1375)* (dotted line) animals at indicated time points during hyposmotic-anoxia (H-A) exposure. Each data point represents measurements of at least five H-A-exposed animals normalized to normoxic controls. Error bars denote +/- standard error of the mean. **P* < 0.05 *daf-2(e1370)* or *daf-18(e1375)* vs. N2 (two-tailed *t* test).

A genome-wide RNAi-based screen previously performed in our lab identified over 200 genes whose activity affects glycogen content and localization in *C. elegans* (Harold Frazier, unpublished data). These glycogen storage genes (GSGs) were subdivided into four major groups based on glycogen staining patterns: increased glycogen with normal localization (+), decreased glycogen with normal localization (-), more glycogen, including prominent glycogen in hypodermal or gut cells (G/H), and abnormal localization of glycogen (Ab). Based on our data linking glycogen synthesis by *gsy-1* to survival in hyposmotic-anoxia, we hypothesized that GSG knockdowns would also exhibit survivorship differences in hyposmotic-anoxia relative to controls. To test this, we performed

RNAi in wild type *(N2) C. elegans,* targeting each of the 33 (+) GSGs as well as a randomly selected subset of the (-) GSGs, and then assayed these knockdowns for survival in hyposmotic-anoxia. We found a strong correlation between survival and gene activity that alters glycogen content (*$P<0.001$ *versus* control RNAi, one-way ANOVA with Dunnett's multiple comparison correction), such that 18 out of the 33 (+) GSG knockdowns displayed significant increases in survival relative to controls **(Figure 15A)** and all seven of the (-) GSG knockdowns that were tested displayed significant survival decreases **(Figure 15B)**. Because the GSGs represent a functionally diverse set of genes **(Table 1)**, it is unlikely that they converge on another unknown process in parallel to glycogen storage. Therefore, these survival data strongly suggest that nematode glycogen content is a major determinant of survival in hyposmotic-anoxia.

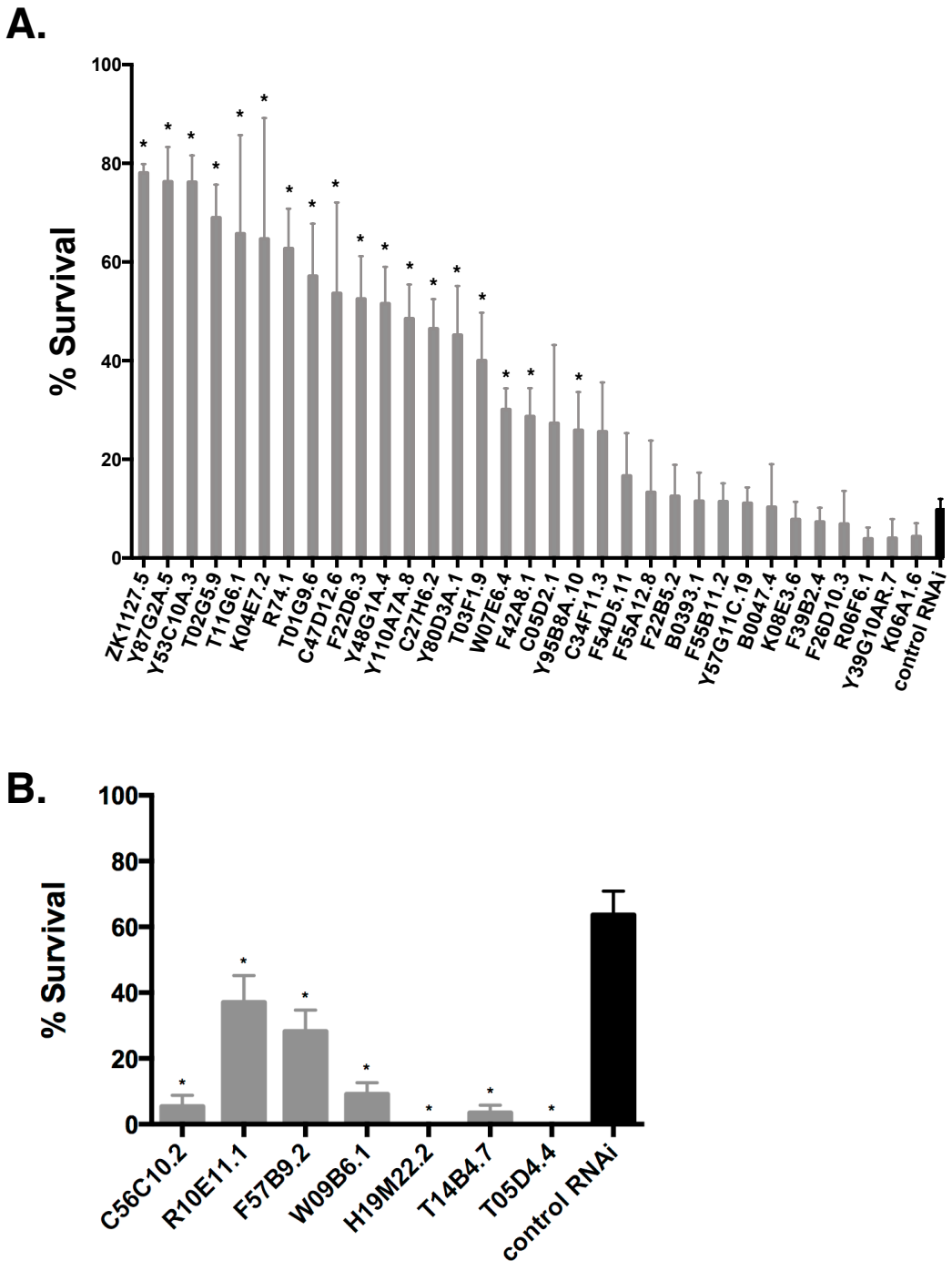

Figure 15: Animal glycogen content is directly correlated with survival of hyposmotic-anoxia. **(A)** Survival after 24 h hyposmotic-anoxia of wild type *(N2)* L4 animals that had been treated with RNAi targeting (+) glycogen storage genes. **(B)** Survival after 16 h hyposmotic-anoxia of wild type *(N2)* L4 animals that had been treated with RNAi targeting a randomly selected subset of (-) glycogen storage genes. Each bar represents at least three independent experiments, each with >10 animals. Error bars denote +/- SEM. **P* <0.05 vs. vector control (two-tailed t-test).

We also found that when the seven (-) GSG knockdowns mentioned above were performed in *daf-2(1370)* animals, five of them significantly decreased 24 h hyposmotic-anoxia survival relative to a *daf-2(e1370)* control RNAi knockdown **(Figure 16)**. In all cases, however, survival rates of (-) GSG knockdowns were higher in *daf-2(e1370)* animals than in wild type, suggesting that while glycogen content is an important aspect of the *daf-2(e1370)* mutant's resistance in hyposmotic-anoxia, there likely exist other mechanisms by which reduced insulin/IGF signaling leads to increased survival in this environment.

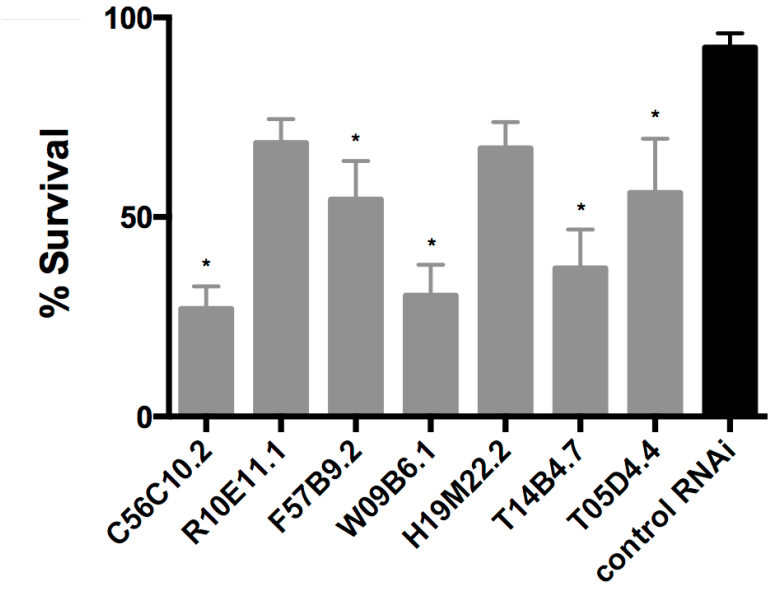

Figure 16: Survival after 24 h hyposmotic-anoxia of *daf-2(e1370)* animals that had been treated with RNAi targeting several (-) glycogen storage genes. For each RNAi target, data represent at least five independent experiments, each with >10 animals. Error bars denote +/- standard error of the mean. **P* <0.05 vs. RNAi control (two-tailed t-test).

Previous studies in other model systems have shown that decreases in tissue glycogen stores are correlated with the length of oxygen deprivation (ROSE *et al.* 1965; UNIACKE and HILL 1972; HEMS and WHITTON 1980). Apart from canonical pathways directly involved in glycogen synthesis and destruction, however, the genetic regulation of glycogen metabolism in metazoans during oxygen deprivation has remained relatively unexplored. Here, we present evidence in *C. elegans* that glycogen is both necessary and sufficient for promoting animal survival during oxygen deprivation. We also show that RNAi targeting of several genes whose activity increases tissue glycogen content, including the glycogen synthase homolog *gsy-1,* results in a suppression of the *daf-2(e1370)* mutant's anoxia resistance phenotype. Prior studies have suggested that *daf-2(e1370)* mutants possess a metabolic profile that is different from that of wild-type (MURPHY *et al.* 2003) (HOUTHOOFD *et al.* 2005); (MENDENHALL *et al.* 2006). However, specific *daf-2*-regulated processes most relevant to nematode survival of oxygen deprivation, metabolic or otherwise, have remained unclear. For instance, while knockdown of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase *(gpd-2/3)* was shown to partially suppress *daf-2*-dependent anoxia survival, knockdown of other glycolytic enzymes did not produce altered survival phenotypes (MENDENHALL *et al.* 2006). By showing that the increased anoxia survival observed in *daf-2(e1370)* animals depends on functional glycogen synthase *(gsy-1)*, we suggest a potential mechanism for *daf-2*-mediated anoxia survival that involves altered glycogen

metabolism. Future studies will attempt to clarify whether *daf-2(e1370)* animals simply possess increased glycogen content, as some have suggested previously (FRAZIER and ROTH 2009), or whether they also possess a decreased rate of glycogen consumption.

Several knockdowns from our screen, all of which possess increased glycogen content, have previously been reported to increase nematode survival in hypoxia (MABON *et al.* 2009) (ANDERSON *et al.* 2009). We show that, in addition to these, the majority of knockdowns identified as having increased glycogen also displayed increased survival in hyposmotic-anoxia. Consistently, all knockdowns with decreased glycogen that were assayed displayed reduced survival in this environment. Together, these strongly suggest that the presence of glycogen is a major determinant of animal survival. A variety of cellular processes are represented amongst the gene knockdowns that produce increases in glycogen content and hypoxia survival, including protein translation, transcription and cell division. While knockdown of tRNA synthetases in *C. elegans* was previously shown to blunt the unfolded protein response and increase animal survival in hypoxia (ANDERSON *et al.* 2009), this survival was not significantly associated with decreased animal oxygen consumption (SCOTT *et al.* 2013). Our data suggest that alterations in anaerobic metabolism, as opposed to respiration, may serve as the basis for increased hypoxia survival.

It is interesting to speculate on potential reasons why a significant number of increased glycogen knockdowns did not lead to increases in survival. Given

the diversity in known function of these genes **(Table 1)**, there is no obvious evidence for a particular cellular process underlying this apparent "exceptions to the glycogen rule." However, it is possible that the glycogen content increases produced by these knockdowns are the result of an inability to access glycogen, either through constitutive inhibition of enzymes required for glycogen breakdown (such as glycogen phosphorylase and debranching enzyme) or through other means like aberrant subcellular glycogen localization. Previous studies have indicated that hyposmotic environments can lead to an inhibition of glycogen breakdown in order to maintain low intracellular osmolarity (BAQUET *et al.* 1990; AL-HABORI *et al.* 1992; LOW *et al.* 1996). Future experiments should therefore investigate whether these (+) knockdowns lead to an inability of the nematode to utilize glycogen as a energetic source during hyposmotic anoxia.

2.4 Summary of Investigations

Experimental work presented in this dissertation focuses on how osmotic stress impacts survival of the nematode *Caenorhabditis elegans* during periods of oxygen deprivation (anoxia). Although a considerable amount of effort has been made in the past to identify mechanisms for *C. elegans* adaptation and survival during oxygen deprivation, prior to this work the extent to which changes in environmental osmolarity affected nematode survival in anoxia were completely unknown. Here we show that osmotic (hyposmotic or hyperosmotic) stress lowers nematode survival time in anoxia, an effect that can be partially rescued with osmotic preconditioning in normoxia. In particular, hyposmotic

stress in anoxia (hyposmotic-anoxia) is interesting because this condition leads to tissue swelling in the nematode, a phenotype that is similar in certain aspects to edema formation during clinical ischemia.

We identify two general processes, aquaporin-mediated water transport and glycogen storage, which play roles in extending nematode survival limits in hyposmotic-anoxia. Although seemingly unrelated in function, both of these processes appear to help the nematode balance the energetic supply and demand equation during oxygen deprivation. On one side of the equation, suppression of aquaporin function results in decreased glycogen consumption during hyposmotic-anoxia. This is ostensibly a reflection of decreases in ATP demand, owing to the fact that glycogen is the primary fuel source for ATP production in anoxia. On the other side, enhanced glycogen storage results in increased survival rates during hyposmotic-anoxia. Because depletion of nematode glycogen stores correlates temporally with animal death, increases in glycogen stores likely function to support ATP production in anoxia for longer periods of time, thereby increasing ATP supply.

Our work has also shown that functional removal of genes regulating protein synthesis and insulin/IGF signaling leads to increased nematode survival in hyposmotic-anoxia. While this is consistent with prior reports of these pathways being important for nematode survival of oxygen deprivation, we go further to show that glycogen metabolism is altered upon genetic mutation or RNAi knockdown of genes in these pathways. Knockdown of tRNA synthetase

homologs leads to increased nematode glycogen storage, suggesting that the ATP demand of protein synthesis limits the storage glycogen. Insulin/IGF pathway mutants (*daf-2* and *daf-18)* possess altered rates of glycogen consumption in hyposmotic-anoxia that correlate with the survival rates of these animals. Removal of glycogen content from *daf-2* mutants reduces survival rates in these animals, suggesting that nematode insulin/IGF signaling increases energetic demand in anoxia. Together, these data suggest a model in the nematode that places glycogen in the center of an energetic (ATP) supply and demand network during anoxia. Pathways in this network that consume ATP would ultimately lead to depletion of glycogen stores. Likewise, pathways that increased the efficiency of ATP generation during anoxia would lead to stabilization of glycogen stores. Because of the relative ease with which quantitative glycogen measurements can be made in the nematode, future studies should investigate the feasibility of its use as a reporter for nematode survival in anoxia and other forms of oxygen deprivation.

Appendix 1: Table 1

Increased Glycogen

Sequence Name	Gene Name	Glycogen Phenotype	Percent survival in Hyposmotic Anoxia (24 h)	Category	Sub-category
R06F6.1	cdl-1	+	3.921568627	Transcription	mRNA processing
Y39G10AR.7		+	4	Other	
K06A1.6	dgk-5	+	4.285714286	Signalling	
F26D10.3	hsp-1	+	6.896551724	Protein Stability	Chaperone
F39B2.4	sur-2	+	7.272727273	Signalling	Mediator
K08E3.6	cyk-4	+	7.843137255	Signalling	G-protein
Control RNAi	**Control RNAi**	**N/A**	**9.76**	N/A	N/A
B0047.4	math-1	+	10.25641026	Other	
F55B11.2		+(slight)	11.42857143	Other	
B0393.1	rps-0	+	11.53846154	Other	
Y57G11C.19		+	11.53846154	Protein synthesis	Ribosome
F22B5.2	eif-3.G	+	12.5	Protein synthesis	Translation
F55A12.8	nath-10	+	13.33333333	Other	
F54D5.11		+	16.66666667	Transcription	Initiation
C34F11.3		+	25.56390977	Metabolism	Nucleotide
Y95B8A.10	pde-6	+(slight)	25.89285714	Signalling	cAMP
C05D2.1	daf-4	+(slight)	27.27272727	Signalling	TGF-b
F42A8.1		+	28.69565	Other	
W07E6.4	prp-21	+	30.12048193	Transcription	mRNA processing
T03F1.9		+	40	Cell cycle	Centromere
Y80D3A.1	wars-1	+	45.20547945	Protein synthesis	Translation
C27H6.2	ruvb-1	+	46.46464646	Transcription	Helicase
Y110A7A.8	prp-31	+	48.51485149	Transcription	mRNA processing
Y48G1A.4		+	51.54639175	Protein synthesis	Nucleolus
F22D6.3	nars-1	+	52.51798561	Protein synthesis	Translation
C47D12.6	tars-1	+	53.65853659	Protein synthesis	Translation
T01G9.6	kin-10	+	57.14285714	Signalling	
R74.1	lars-1	+	62.74509804	Protein synthesis	Translation
K04E7.2	pept-1	++	64.70588235	Trafficking	Peptide
T11G6.1	hars-1	+	65.71428571	Protein synthesis	Translation
T02G5.9	kars-1	+	68.96551724	Protein synthesis	Translation
Y53C10A.3	hsf-2	+	76.19047619	Protein Stability	Chaperone
Y87G2A.5	vars-2	+	76.27118644	Protein synthesis	Translation
ZK1127.5		+	78.04878049	Protein synthesis	Nucleolus

Decreased Glycogen

Gene ID	Gene Name	Glycogen Phenotype	Percent Survival in Hyposmotic Anoxia (16 h)	Category	Sub-category
H19M22.2	let-805	--	0	Body structure	Neuromuscular
T05D4.4	osm-7	-	0	Other	
T14B4.7	dpy-10	-	3.4	Body structure	Cuticle/collagen
C56C10.3	vps-32.1	--	5.357142857	Trafficking	Vesicle
Y46G5A.31	gsy-1	-	6.5	Metabolism	Polysaccharide
W09B6.1	pod-2	--	9.090909091	Metabolism	Lipid
F57B9.2	let-711	--	28.16901408	Transcription	Global regulator
R10E11.1	cbp-1	--	37.03703704	Signalling	TCF
Control RNAi	**Control RNAi**	N/A	63.6	N/A	N/A

Table 1: Glycogen storage gene knockdowns—glycogen phenotypes, survival in hyposmotic-anoxia and functional categorization. For glycogen phenotypes, (+) denotes increased glycogen and (–) decreased glycogen. More severe phenotypes are indicated with (++) and (--), respectively. “Category” and “Subcategory” refer to the respective gene’s predicted biological function (Harold Frazier, unpublished data).

Appendix 2: Materials and Methods

Nematode strains and culture:

Nematodes were grown at 20˚C on NGM agar seeded with OP50 *E. coli* bacteria and manipulated using standard protocols (BRENNER 1974) unless noted otherwise. A combination of visual anatomical markers, such as the gonad and uterine morphology, and time following the L4 molt were used to assess the developmental stage of animals. Synchronous F1 populations were obtained through treating gravid P0 adults with a 20% alkaline hypochlorite solution and recovering their embryos. For all experiments, animals were only used from populations that had not experienced a starvation event in the previous two generations. The following strains were acquired from the *Caenorhabditis* Genetics center: N2 (wild-type var. Bristol); CB1370 *daf-2(e1370);* DR26 *(daf-16(m26);* DR1309 *(daf-16(m26); daf-2(e1370);* RB1169 *daf-18(e1375)*; RB1715 *aqp-2(ok2159) ;* RB1967 *aqp-4(ok2587);* RB 2115 *aqp-8(ok2800);* RB1914 *aqp-9(ok2487);* RB2570 *aqp-11(ok3578);* MT3564 *osm-7(n1515);* MT3643 *osm-11(n1604);* CB128 *(dpy-10(e128)II).* The RB1967 *aqp-4(ok2587)* strain was backcrossed three times into the wild-type N2 strain.

Oxygen deprivation experiments:

All oxygen deprivation experiments were performed at an incubation temperature of 20˚C unless indicated otherwise. Atmospheres were generated using mass flow controllers and airtight chambers as previously described (MILLER and ROTH 2009). Immediately prior to anoxia exposure, L4 *C. elegans* were transferred via wire pick to either Nematode Growth Medium (NGM) with live OP50 bacteria, or to media consisting of 2% agar + 5 mM Potassium Phosphate Buffer (PPB) with the following concentrations of NaCl: for hyposotic media, no NaCl was added; for isosmotic media, 100 mM NaCl; for hyperosotic media, 300 mM NaCl (media was seeded with live OP50 bacteria that had been washed with ddH_2O). Following anoxia exposure, animals were returned to room air, allowed to recover for at least 6 h and scored for survival within 24 h. Animals exhibiting major trauma ("popped" phenotype) were scored as dead. Animals in which visual trauma was not apparent were scored as dead if they were unresponsive to 30 sec. of gentle touch with a platinum wire. For glycogen quantification experiments, animals were returned to room air and immediately stained with iodine vapor.

Glycogen staining of nematodes with iodine:

Whole body glycogen content was visualized and qualitatively assessed using a previously described iodine vapor staining method (FRAZIER and ROTH 2009). Animals were first placed on an agar pad and allowed to roam in order to remove

excess OP50 bacteria associated with the animals. The agar pad was then exposed to iodine vapor for 90 sec by inverting it over an open bottle of 100g bottle of iodine chips (Sigma), after which period the animals were immediately visualized and photographed using a. For quantification of glycogen content, iodine-stained animals were photographed using a Nikon SMZ1500 stereomicroscope. Mean stain intensity and animal area were subsequently calculated from micrographs using the ImageJ program. To account for small variations in stain concentration and animal handling, experimental animals were stained side-by-side with control animals and imaged simultaneously. The linear range of the iodine vapor stain was determined through the generation of a standard curve (Equation 1) using known concentrations of glycogen spotted onto NGM plates. This standard curve was employed in the definition of Glycogen Content (Equation 2).

Equation 1: Glycogen (mg) = ([Area]x[Intensity] +26)/71

Equation 2:

Glycogen Content $(Animal)_{exp} = ([Area]_{exp} \times [Intensity]_{exp} + 26)/(([Area]_{control} \times [Intensity]_{control} + 26)$

RNA interference (RNAi) experiments:

RNAi was achieved as previously described (KAMATH *et al.* 2001) by feeding animals *E. coli* HT115 (DE3) strains expressing double-stranded RNA corresponding in sequence to the gene of interest. These strains were generated by the J. Ahringer laboratory and were obtained as a library from MRC Geneservice. For all experiments, gravid adult animals were placed on agar RNAi feeding plates and allowed to lay embryos for 24 h. Following this period, adults were removed and embryos were allowed to hatch and develop to the L4 stage at 20°C. RNAi was confirmed qualitatively by observing previously described phenotypes of the knockdown or quantitatively by qRT-PCR.

Live animal microscopy:

L4 *C. elegans* were transferred to a thin (<1 mm) agarose pad and placed inverted in glass-bottom atmospheric chamber mounted on a Nikon TE200 inverted microscope. Hypoxic (100% nitrogen) atmospheres were generated using a mass flow controller. Images of whole animals were collected at the designated time points using a Zeiss AxioCam MRc digital camera and analyzed using the ImageJ program. For body volume measurements, animals were treated as cylinders and volume was calculated as $v = \pi r^2 l$ (MCCULLOCH and GEMS 2003).

Quantitative Real-Time PCR (qPCR):

Synchronized L4 *C. elegans* were grown on isosmotic or hyposmotic agar media for 24 h, then washed three times with water and frozen in liquid nitrogen. Trizol reagent (Life Technologies) was added (1 mL/gram worms) and RNA was extracted according to manufacturer's instructions. Total RNA concentration was determined using a Nanodrop spectrophotometer (Thermo Scientific) and cDNA was synthesized using a Superscript III kit (Life Technologies) according to manufacturer's instructions. Quantitative PCR was performed on a ABI 7900HT Real Time PCR System using iTaq SYBR Green (Bio-Rad Laboratories) according to manufacturer's instructions. Standard curves were generated using cDNA and expression levels were determined using the Pfaffl analysis method (PFAFFL 2001). Levels of *aqp-4* cDNA were normalized to the geometric mean of the cDNA levels of the housekeeping genes *act-1* and *gpd-2.* All PCR products were verified to be the correct size by gel electrophoresis.

Adaptation to Altered Culture Medium Osmolarities:

Synchronous populations of nematodes were raised from embryo to the L2/L3 stage on NGM at 20°C and then transferred to hyposmotic (10 mOsm), isosmotic (200 mOsm) or hyperosmotic (600 mOsm) media that had been seeded with live OP50 *E. coli*. After 24 h, animals from these three conditions that had progressed to the L4 stage were transferred by a platinum pick to fresh plates containing

hyposmotic, isosmotic or hyperosmotic media and assayed for 24 h survival in a 100% nitrogen atmosphere as described above.

Statistical Analysis:

All statistical analyses were carried out in either Microsoft Excel or Prism 6 (Graphpad Software). One-way ANOVA analysis was performed (with Dunn's test) comparing experimental conditions in an unpaired fashion with the control condition. All multiple comparisons were reanalyzed individually with the Student's *t* test and confirmed to be statistically significant.

References

AGRE P., PRESTON G. M., SMITH B. L., JUNG J. S., RAINA S., MOON C., GUGGINO W. B., NIELSEN S., 1993 Aquaporin CHIP: the archetypal molecular water channel. Am. J. Physiol. **265**: F463–76.

AL-HABORI M., PEAK M., THOMAS T. H., AGIUS L., 1992 The role of cell swelling in the stimulation of glycogen synthesis by insulin. Biochem J **282 (Pt 3)**: 789–796.

ANDERSON L. L., MAO X., SCOTT B. A., CROWDER C. M., 2009 Survival from hypoxia in C. elegans by inactivation of aminoacyl-tRNA synthetases. Science **323**: 630–633.

BAQUET A., HUE L., MEIJER A. J., VAN WOERKOM G. M., PLOMP P. J., 1990 Swelling of rat hepatocytes stimulates glycogen synthesis. J Biol Chem **265**: 955–959.

BICKLER P. E., 2004 Clinical perspectives: neuroprotection lessons from hypoxia-tolerant organisms. J Exp Biol **207**: 3243–3249.

BICKLER P. E., BUCK L. T., 2007 Hypoxia tolerance in reptiles, amphibians, and fishes: life with variable oxygen availability. Annu. Rev. Physiol. **69**: 145–170.

BRENNER S., 1974 The genetics of Caenorhabditis elegans. Genetics.

BUCK L. T., BICKLER P. E., 1998 Adenosine and anoxia reduce N-methyl-D-aspartate receptor open probability in turtle cerebrocortex. J Exp Biol **201**: 289–297.

BUCK L. T., HOCHACHKA P. W., 1993 Anoxic suppression of Na(+)-K(+)-ATPase and constant membrane potential in hepatocytes: support for channel arrest. Am. J. Physiol. **265**: R1020–5.

BUCK L. T., LAND S. C., HOCHACHKA P. W., 1993 Anoxia-tolerant hepatocytes: model system for study of reversible metabolic suppression. Am. J. Physiol. **265**: R49–56.

C. ELEGANS SEQUENCING CONSORTIUM, 1998 Genome sequence of the nematode C. elegans: a platform for investigating biology. Science **282**: 2012–2018.

CATLING D. C., GLEIN C. R., ZAHNLE K. J., MCKAY C. P., 2005 Why O2 is required by complex life on habitable planets and the concept of planetary

"oxygenation time". Astrobiology **5**: 415–438.

DAHL T. W., HAMMARLUND E. U., ANBAR A. D., BOND D. P. G., GILL B. C., GORDON G. W., KNOLL A. H., NIELSEN A. T., SCHOVSBO N. H., CANFIELD D. E., 2010 Devonian rise in atmospheric oxygen correlated to the radiations of terrestrial plants and large predatory fish. Proceedings of the National Academy of Sciences **107**: 17911–17915.

DANOVARO R., DELL'ANNO A., PUSCEDDU A., GAMBI C., HEINER I., KRISTENSEN R. M., 2010 The first metazoa living in permanently anoxic conditions. BMC Biol **8**: 30.

DAW J. C., WENGER D. P., BERNE R. M., 1967 Relationship between cardiac glycogen and tolerance to anoxia in the western painted turtle, Chrysemys picta bellii. Comp. Biochem. Physiol. **22**: 69–73.

DEPUYDT G., XIE F., PETYUK V. A., SMOLDERS A., BREWER H. M., CAMP D. G. II, SMITH R. D., BRAECKMAN B. P., 2014 LC–MS Proteomics Analysis of the Insulin/IGF-1-Deficient Caenorhabditis elegans daf-2(e1370)Mutant Reveals Extensive Restructuring of Intermediary Metabolism. J. Proteome Res. **13**: 1938–1956.

DOLL C. J., HOCHACHKA P. W., REINER P. B., 1991 Effects of anoxia and metabolic arrest on turtle and rat cortical neurons. Am. J. Physiol. **260**: R747–55.

EBENSPERGER G., EBENSPERGER R., HERRERA E. A., RIQUELME R. A., SANHUEZA E. M., LESAGE F., MARENGO J. J., TEJO R. I., LLANOS A. J., REYES R. V., 2005 Fetal brain hypometabolism during prolonged hypoxaemia in the llama. J. Physiol. (Lond.) **567**: 963–975.

ELTZSCHIG H. K., ECKLE T., 2011 Ischemia and reperfusion--from mechanism to translation. Nat Med **17**: 1391–1401.

ERNSTER L., SCHATZ G., 1981 Mitochondria: a historical review. J Cell Biol **91**: 227s–255s.

FALKOWSKI P. G., KATZ M. E., MILLIGAN A. J., FENNEL K., CRAMER B. S., AUBRY M. P., BERNER R. A., NOVACEK M. J., ZAPOL W. M., 2005 The rise of oxygen over the past 205 million years and the evolution of large placental mammals. Science **309**: 2202–2204.

FÄHLING M., 2009 Surviving hypoxia by modulation of mRNA translation rate. J. Cell. Mol. Med. **13**: 2770–2779.

FÖLL R. L., PLEYERS A., LEWANDOVSKI G. J., WERMTER C., HEGEMANN V., PAUL R.

J., 1999 Anaerobiosis in the nematode Caenorhabditis elegans. Comp. Biochem. Physiol. B, Biochem. Mol. Biol. **124**: 269–280.

FRAZIER H. N., ROTH M. B., 2009 Adaptive sugar provisioning controls survival of C. elegans embryos in adverse environments. Curr Biol **19**: 859–863.

GARCIA-DORADO D., ANDRES-VILLARREAL M., RUIZ-MEANA M., INSERTE J., BARBA I., 2012 Myocardial edema: A translational view. J Mol Cell Cardiol **52**: 931–939.

GIL E. B., MALONE LINK E., LIU L. X., JOHNSON C. D., LEES J. A., 1999 Regulation of the insulin-like developmental pathway of Caenorhabditis elegans by a homolog of the PTEN tumor suppressor gene. Proc Natl Acad Sci USA **96**: 2925–2930.

GINSBERG M. D., 2009 Current Status of Neuroprotection for Cerebral Ischemia: Synoptic Overview. Stroke **40**: S111–S114.

GONZALEZ C., ALMARAZ L., OBESO A., RIGUAL R., 1994 Carotid body chemoreceptors: from natural stimuli to sensory discharges. Physiol Rev **74**: 829–898.

HEMS D. A., BROSNAN J. T., 1970 Effects of ischaemia on content of metabolites in rat liver and kidney in vivo. Biochem J **120**: 105–111.

HEMS D. A., WHITTON P. D., 1980 Control of hepatic glycogenolysis. Physiol Rev **60**: 1–50.

HOBACK W. W., STANLEY D. W., 2001 Insects in hypoxia. J. Insect Physiol. **47**: 533–542.

HOCHACHKA P. W., 1986 Metabolic arrest. Intensive Care Med **12**: 127–133.

HOCHACHKA P. W., BUCK L. T., DOLL C. J., LAND S. C., 1996 Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. Proc Natl Acad Sci USA **93**: 9493–9498.

HOFFMANN E. K., LAMBERT I. H., PEDERSEN S. F., 2009 Physiology of cell volume regulation in vertebrates. Physiol Rev **89**: 193–277.

HOUTHOOFD K., FIDALGO M. A., HOOGEWIJS D., BRAECKMAN B. P., LENAERTS I., BRYS K., MATTHIJSSENS F., DE VREESE A., VAN EYGEN S., MUÑOZ M. J., VANFLETEREN J. R., 2005 Metabolism, physiology and stress defense in three aging Ins/IGF-1 mutants of the nematode Caenorhabditis elegans. Aging Cell **4**: 87–95.

HOYERT D. L., ARIAS E., SMITH B. L., MURPHY S. L., KOCHANEK K. D., 2012

National vital statistics reports. Deaths: Final Data for 1999 **49**.

HUANG C. G., LAMITINA T., AGRE P., STRANGE K., 2007 Functional analysis of the aquaporin gene family in Caenorhabditis elegans. Am J Physiol, Cell Physiol **292**: C1867–73.

JENNINGS R. B., REIMER K. A., STEENBERGEN C., 1986 Myocardial ischemia revisited. The osmolar load, membrane damage, and reperfusion. J Mol Cell Cardiol **18**: 769–780.

JIANG Y.-Y., KONG D.-X., QIN T., LI X., CAETANO-ANOLLÉS G., ZHANG H.-Y., 2012 The impact of oxygen on metabolic evolution: a chemoinformatic investigation. PLoS Computational Biology **8**: e1002426.

KAELIN W. G., RATCLIFFE P. J., 2008 Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. Molecular Cell **30**: 393–402.

KAHLE K. T., SIMARD J. M., STALEY K. J., NAHED B. V., JONES P. S., SUN D., 2009 Molecular Mechanisms of Ischemic Cerebral Edema: Role of Electroneutral Ion Transport. Physiology **24**: 257–265.

KAMATH R. S., MARTINEZ-CAMPOS M., ZIPPERLEN P., FRASER A. G., AHRINGER J., 2001 Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in Caenorhabditis elegans. Genome Biol **2**: RESEARCH0002.

KENYON C., CHANG J., GENSCH E., RUDNER A., TABTIANG R., 1993 A C. elegans mutant that lives twice as long as wild type. Nature **366**: 461–464.

KHANNA A., KAHLE K. T., WALCOTT B. P., GERZANICH V., SIMARD J. M., 2014 Disruption of ion homeostasis in the neurogliovascular unit underlies the pathogenesis of ischemic cerebral edema. Transl Stroke Res **5**: 3–16.

KLONER R. A., 2013 Current state of clinical translation of cardioprotective agents for acute myocardial infarction. Circ Res **113**: 451–463.

LANGEN P., HUCHO F., 2008 Karl Lohmann and the Discovery of ATP. Angewandte Chemie International Edition **47**: 1824–1827.

LARSON J., PARK T. J., 2009 Extreme hypoxia tolerance of naked mole-rat brain. Neuroreport **20**: 1634–1637.

LEE S. S., KENNEDY S., TOLONEN A. C., RUVKUN G., 2003 DAF-16 target genes that control C. elegans life-span and metabolism. Science **300**: 644–647.

LIN K., HSIN H., LIBINA N., KENYON C., 2001 Regulation of the Caenorhabditis elegans longevity protein DAF-16 by insulin/IGF-1 and germline signaling.

Nat Genet **28**: 139–145.

LOW S. Y., RENNIE M. J., TAYLOR P. M., 1996 Modulation of glycogen synthesis in rat skeletal muscle by changes in cell volume. J. Physiol. (Lond.) **495 (Pt 2)**: 299–303.

LYONS T. W., REINHARD C. T., PLANAVSKY N. J., 2014 The rise of oxygen in Earth's early ocean and atmosphere. Nature **506**: 307–315.

MABON M. E., SCOTT B. A., CROWDER C. M., 2009 Divergent mechanisms controlling hypoxic sensitivity and lifespan by the DAF-2/insulin/IGF-receptor pathway. PLoS ONE **4**: e7937.

MAINA J. N., 2002 Structure, function and evolution of the gas exchangers: comparative perspectives. J. Anat. **201**: 281–304.

MCCULLOCH D., GEMS D., 2003 Body size, insulin/IGF signaling and aging in the nematode Caenorhabditis elegans. Exp Gerontol **38**: 129–136.

MCMANUS M. L., CHURCHWELL K. B., STRANGE K., 1995 Regulation of cell volume in health and disease. N Engl J Med **333**: 1260–1266.

MENDENHALL A. R., LARUE B., PADILLA P. A., 2006 Glyceraldehyde-3-phosphate dehydrogenase mediates anoxia response and survival in Caenorhabditis elegans. Genetics **174**: 1173–1187.

MILLER D. L., ROTH M. B., 2009 C. elegans are protected from lethal hypoxia by an embryonic diapause. Curr Biol **19**: 1233–1237.

MITCHELL P., 1961 Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. Nature **191**: 144–148.

MORETTI A., FERRARI F., VILLA R. F., 2014 Pharmacology & Therapeutics. Pharmacol Ther: 1–12.

MORRIS J. Z., TISSENBAUM H. A., RUVKUN G., 1996 A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in Caenorhabditis elegans. Nature **382**: 536–539.

MURPHY C. T., MCCARROLL S. A., BARGMANN C. I., FRASER A., KAMATH R. S., AHRINGER J., LI H., KENYON C., 2003 Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. Nature **424**: 277–283.

NILSSON G. E., OSTLUND-NILSSON S., 2004 Hypoxia in paradise: widespread hypoxia tolerance in coral reef fishes. Proc. Biol. Sci. **271 Suppl 3**: S30–3.

NYSTUL T. G., ROTH M. B., 2004 Carbon monoxide-induced suspended

animation protects against hypoxic damage in Caenorhabditis elegans. Proc Natl Acad Sci USA **101**: 9133–9136.

NYSTUL T. G., GOLDMARK J. P., PADILLA P. A., ROTH M. B., 2003 Suspended animation in C. elegans requires the spindle checkpoint. Science **302**: 1038–1041.

OGG S., RUVKUN G., 1998 The C. elegans PTEN homolog, DAF-18, acts in the insulin receptor-like metabolic signaling pathway. Molecular Cell **2**: 887–893.

OGG S., PARADIS S., GOTTLIEB S., PATTERSON G. I., LEE L., TISSENBAUM H. A., RUVKUN G., 1997 The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. Nature **389**: 994–999.

PADILLA P. A., NYSTUL T. G., ZAGER R. A., JOHNSON A. C. M., ROTH M. B., 2002 Dephosphorylation of cell cycle-regulated proteins correlates with anoxia-induced suspended animation in Caenorhabditis elegans. Mol Biol Cell **13**: 1473–1483.

PAMENTER M. E., HOGG D. W., GU X. Q., BUCK L. T., HADDAD G. G., 2012 Painted turtle cortex is resistant to an in vitro mimic of the ischemic mammalian penumbra. **32**: 2033–2043.

PFAFFL M. W., 2001 A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res **29**: e45.

PRESTON G. M., CARROLL T. P., GUGGINO W. B., AGRE P., 1992 Appearance of water channels in Xenopus oocytes expressing red cell CHIP28 protein. Science **256**: 385–387.

RAMIREZ J.-M., FOLKOW L. P., BLIX A. S., 2007 Hypoxia tolerance in mammals and birds: from the wilderness to the clinic. Annu. Rev. Physiol. **69**: 113–143.

RAYMOND J., SEGRÈ D., 2006 The effect of oxygen on biochemical networks and the evolution of complex life. Science **311**: 1764–1767.

ROSE F. L., ZAMBERNARD J., POGANY G. S., 1965 HEPATIC GLYCOGEN DEPLETION IN AMPHIUMA DURING INDUCED ANOXIA. Science **147**: 1467–1468.

SARASTE M., 1999 Oxidative phosphorylation at the fin de siecle. Science **283**: 1488–1493.

SCOTT B. A., AVIDAN M. S., CROWDER C. M., 2002 Regulation of hypoxic death in C. elegans by the insulin/IGF receptor homolog DAF-2. Science **296**: 2388–

2391.

SCOTT B., SUN C.-L., MAO X., YU C., VOHRA B. P. S., MILBRANDT J., CROWDER C. M., 2013 Role of oxygen consumption in hypoxia protection by translation factor depletion. J Exp Biol **216**: 2283–2292.

SEMENZA G., 2000 HIF-1 and human disease: one highly involved factor. Genes Dev.

SEMENZA G. L., 2001 HIF-1, O(2), and the 3 PHDs: how animal cells signal hypoxia to the nucleus. Cell **107**: 1–3.

SEMENZA G. L., 2011 Oxygen sensing, homeostasis, and disease. N Engl J Med **365**: 537–547.

SESSIONS A. L., DOUGHTY D. M., WELANDER P. V., SUMMONS R. E., NEWMAN D. K., 2009 The continuing puzzle of the great oxidation event. Curr Biol **19**: R567–74.

SIMARD J. M., KENT T. A., CHEN M., TARASOV K. V., GERZANICH V., 2007 Brain oedema in focal ischaemia: molecular pathophysiology and theoretical implications. Lancet Neurol **6**: 258–268.

SIMON H.-U., HAJ-YEHIA A., LEVI-SCHAFFER F., 2000 Role of reactive oxygen species (ROS) in apoptosis induction. Apoptosis **5**: 415–418.

STECYK J. A. W., STENSLØKKEN K.-O., FARRELL A. P., NILSSON G. E., 2004 Maintained cardiac pumping in anoxic crucian carp. Science **306**: 77.

TOMIOKA M., ADACHI T., SUZUKI H., KUNITOMO H., SCHAFER W. R., IINO Y., 2006 The insulin/PI 3-kinase pathway regulates salt chemotaxis learning in Caenorhabditis elegans. Neuron **51**: 613–625.

TRANUM-JENSEN J., JANSE M. J., FIOLET W. T., KRIEGER W. J., D'ALNONCOURT C. N., DURRER D., 1981 Tissue osmolality, cell swelling, and reperfusion in acute regional myocardial ischemia in the isolated porcine heart. Circ Res **49**: 364–381.

ULTSCH G. R., 1985 The viability of nearctic freshwater turtles submerged in anoxia and normoxia at 3 and 10 degrees C. Comp Biochem Physiol A Comp Physiol **81**: 607–611.

UNIACKE C. A., HILL R. M., 1972 The depletion course of epithelial glycogen with corneal anoxia. Arch. Ophthalmol. **87**: 56–59.

VALKO M., LEIBFRITZ D., MONCOL J., CRONIN M. T. D., MAZUR M., TELSER J., 2007 Free radicals and antioxidants in normal physiological functions and human

disease. Int. J. Biochem. Cell Biol. **39**: 44–84.

VAN VOORHIES W. A., WARD S., 1999 Genetic and environmental conditions that increase longevity in Caenorhabditis elegans decrease metabolic rate. Proc Natl Acad Sci USA **96**: 11399–11403.

VAN VOORHIES W. A., WARD S., 2000 Broad oxygen tolerance in the nematode Caenorhabditis elegans. J Exp Biol **203**: 2467–2478.

WANG G. L., JIANG B. H., RUE E. A., SEMENZA G. L., 1995 Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci USA **92**: 5510–5514.

WANG M., JIANG Y.-Y., KIM K. M., QU G., JI H.-F., MITTENTHAL J. E., ZHANG H.-Y., CAETANO-ANOLLÉS G., 2011 A universal molecular clock of protein folds and its power in tracing the early history of aerobic metabolism and planet oxygenation. Molecular Biology and Evolution **28**: 567–582.

WEERASINGHE P., BUJA L. M., 2012 Experimental and Molecular Pathology. Experimental and Molecular Pathology **93**: 302–308.