GENETIC AND ENVIRONMENTAL FACTORS THAT MEDIATE SURVIVAL OF PROLONGED OXYGEN DEPRIVATION IN THE NEMATODE *Caenorhabditis elegans*

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Ischemic events of even a very short duration are not tolerated Ill in humans. The human cost of ischemia, when looked at as combined cardiovascular disease, dwarfs all other causes of death in the United States. Annually, CVD kills as many people in the US as does cancer, chronic lower respiratory disease, accidents, and diabetes mellitus combined. In 2005 (the latest year for which final statistics are available), CVD was responsible for 864,480 deaths or 35.3 percent of total deaths for the year. In my study, I have used the nematode *Caenorhabditis elegans* to determine genetic and environmental modulators of oxygen deprivation a key component of ischemia. I have found that animals with mutations in insulin like signaling pathways, neuronal function, electron transport chain components, germline function, and animals that are preconditioned by being raised on a diet of *E. coli HT115* bacteria at 25°C have an enhanced ability to survive long-term (>72 hours) anoxia (<.005 kPa O_2) at 20°C. The enhanced anoxia survival phenotype partially correlates with increased levels of carbohydrate stores in the nematodes. Suppression of this enhanced anoxia survival phenotype is possible by altering expression of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, the FOXO transcription factor DAF-16, and 5'-AMP kinase.

ACKNOWLEDGEMENTS

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CHAPTER 1

INTRODUCTION

Human and Economic Costs of Ischemia

Ischemic events of even a very short duration are not tolerated well in humans. The human cost of ischemia, when looked at as combined cardiovascular disease (CVD), dwarfs all other causes of death in the United States. Annually, CVD kills as many people in the US as does cancer, chronic lower respiratory disease, accidents, and diabetes mellitus (DM) combined. In 2005 (the latest year for which final statistics are available), CVD was responsible for 864,480 deaths or 35.3 % of total deaths for the year compared to 559,000 for all types of cancer. Currently the approximate number of Americans with one or more types of CVD is roughly 80 million, or one third of all people living in the United States. The estimated total of direct and indirect monetary cost for CVD in the United States is $475.3 billion for 2009, which is approximately 3.3 % of the entire annual gross domestic product of the US for the same period. 1 By any standard, ischemia related health problems are a major problem in the United States.

Ischemia, Oxygen, and Metabolic Demand

Ischemic tissues face many problems. Tissues with ablated blood flow as

in the case of cerebrovascular incident, hypovolemic blood loss, and myocardial infarction have a reduced ability to clear metabolic waste, a reduction in the delivery of metabolites, and a reduced oxygen tension. 2-3

Oxygen deprivation is a key component of ischemia. Studies with anesthetized dog hearts have demonstrated that high flow anoxia is only slightly advantageous to ischemia resulting in a delay of infarct, but not an amelioration of overall damage. 3 This improvement is primarily a consequence of a renewed supply of glycolytic precursors, and a clearance of metabolic end products. The real issue, the decline of high energy phosphate molecules, is a direct result of oxygen deprivation. To better understand the problem of oxygen deprivation, it is helpful to understand the role that oxygen plays in the electron transport system of the mitochondria.

In the electron transport system (ETS or ETC), the final enzyme (ATP synthase), utilizes a proton gradient to produce ATP from ADP and phosphate. The first four enzymes in the system (NADH dehydrogenase, succinate dehydrogenase, UQ-cytochrome c reductase, and cytochrome c oxidase) ultimately establish a proton gradient based on the oxidation of NADH and FADH2. 4 Cytochrome c oxidase plays an additional role of catalyzing the transfer of the electrons that work their way through the chain, via various acceptor molecules, to the terminal receptor, molecular oxygen, which is reduced to water. In this role, oxygen allows the various intermediate carrier molecules of

the electron transport system to become “recycled” by returning them to their oxidized state. In effect, this transfer of electrons allows the chain to return to its initial state, and repeat the process in a continuous cycle. [2,4-5] In the end, the theoretical yield of glucose metabolism utilizing the electron transport system is around 17 times more efficient than substrate-level phosphorylation alone[5]. In vivo (estimated to have a ~54% efficiency in some tissues) this approaches a 9.2 fold increase over glycolysis, or almost an order of magnitude difference. [2,4-5]

The electron transport system recycles NAD^+ and $FADH^+$ pools required for glycolysis and the citric-acid cycle. The recycling of these pools of electron acceptors is another crucial function of oxygen, vis a vis the process of recycling the ETS intermediates, in energy metabolism. Without this oxygen driven process, the alternate pathway for regenerating NAD^+ pools involves either ethanol production; the generation of octopine, alanopine, and stombine from arginine, alanine, and glycine; or the generation of lactate from pyruvate (the case in mammals). In all cases, there is a penalty in the form of a drop in pH, coupled with the aforementioned almost ten-fold decrease in energy production. Metabolic strategy in anaerobic conditions then shifts to maximum ATP production per mole of H^+ generated, and energy must be expended to buffer the increase in H^+ concentration to maintain intracellular homeostasis. [2,4-5]

Another complication that arises in anaerobic conditions is the restrictions placed on substrates available for metabolism. Lipids, in the form of triacylglycerols, are a major energy storage molecule, and can constitute over

80% of metabolic energy stores in an adult human. [4] In oxygen poor environments, lipids, which enter the energy metabolism cycle via β-oxidation are too reduced to be available for catabolism. [5] So in addition to a reduction in metabolic efficiency, and an increase in metabolic demand to maintain homeostasis, there is also the added strain of being limited to relatively inefficient metabolic fuel stores (proteins and carbohydrates) to meet these additional challenges to maintaining cellular ATP pools.

Due to feedback inhibition, the products generated by anaerobic metabolism can inhibit crucial enzymes in glycolysis, which can also add to the problems faced in anoxia. For example, glyceraldehyde-3-P-dehydrogenase is inhibited by a decrease in pH, and NADH. Phosphofructokinase is also inhibited by a lack of ATP. These inhibitions diminish an already compromised energy production system. [2,4-5]

Anaerobic conditions continue to place stress on the cell even after the anoxic conditions pass. The electron transport chain converts approximately 1-4% of all oxygen consumed to reactive oxygen species (ROS) via electron leak. This leak increases as the reduced state of the system increases due to the accumulation of electrons, as is the case when there is no oxygen present to be reduced to water via cytochrome c oxidase. Upon reoxygenation, the accumulated electrons cause a burst of superoxide radicals when in contact with the flood of molecular oxygen, as is the case in reperfusion. These free radicals cause chain reactions with biologically important lipids, proteins, and nucleic

acids, which in turn propagate the reaction until quenched by antioxidant mechanisms in the cell. These reactions can alter or ablate the function of proteins, disrupt membrane integrity, and cause mutations in important genes which can result in necrosis or apoptosis. [2-3,5]

As mentioned earlier, humans are particularly ill-adapted to handle the stress of oxygen deprivation. Even brief periods of ischemia/hypoxia/anoxia are debilitating or lethal, and the tissues that are the most susceptible (cardiac and central nervous system), are also some of the most important for survival, and cannot be regenerated. However, some organisms have mechanisms that allow them to endure longer periods of hypoxia or anoxia quite well.

Strategies for Surviving Oxygen Deprivation

Several organisms have adapted mechanisms that allow them to mitigate the stress associated with oxygen deprivation. For example, pinnipeds, which spend extended periods underwater on dives for prey, turtles, which overwinter in ponds that are frozen at the surface, soil nematodes, who encounter low oxygen tensions caused by bacterial blooms, and other organisms are able to survive very low oxygen tensions much better than humans. To cope with reduced oxygen in their respective environments, they use different mechanisms that are constrained by similar biochemical pathways. In all of these cases, however, a few basic strategies arise to compensate for the stress of oxygen deprivation.

First the organism could somehow store oxygen or metabolites for use in

low oxygen conditions. Pinnipeds have an abnormally large blood volume that serves as an oxygen store during extended dives. [5] Myoglobins and neuroglobins are capable of doing this at the level of the individual cells, and have been shown to be neuroprotective in vertebrates. [6] Also, it has been shown that pinnipeds and turtles have a much greater concentration of glycogen than other similarly sized organisms of their respective class. [7-8]

As previously mentioned, the metabolic byproducts of anaerobic metabolism can be problematic, so another adaptation is to mitigate the effects of these end products. One example of this is the decrease in pH associated with anaerobic metabolism. The turtle *Chrysemys pictabelli* is able to utilize the calcium carbonate in its shell to buffer the H+ ions generated by fermentation. [9] Another example is in carp and goldfish, which are able to extract one additional mole of ATP per mole of glucose by converting pyruvate to ethanol rather than lactate. The toxic ethanol is then easily passed off of the gills which doesn't allow an accumulation of lactate (decrease in pH) to occur in the tissues. [2,5]

Another strategy is to downregulate unnecessary processes or activity. Organisms are able to reduce bloodflow to non-essential areas, and reduce their heart rate to slow down the use of oxygen. [5] Evidence also suggests that a downregulation of genes that are not involved directly with maintaining the physical integrity of the cell (protein synthesis, protein turnover, glycogen storage, proliferation, etc.) are downregulated to conserve high energy phosphate pools. Additionally, ion channels and other sources of ion leak are,

are inhibited competitively, non-competitively, or allosterically. [2,5,10-11]

Conversely, just as downregulating genes can be helpful, upregulating genes that are particularly beneficial in low oxygen conditions is also a strategy for adapting to these conditions. The hypoxia inducible factor, or HIF, has been shown to upregulate a number of genes to aid in glucose transfer, glycolysis, increased blood volume, and an increase in vasculature across a wide variety of taxa. [12-14] Some signaling associated with hypoxia and glycolytic enzymes have been shown to have a HIF-independent component, and in *Caenorhabditis elegans* it has been demonstrated that other pathways exist that are required for survival at very low oxygen tensions (< .005 kPa O_2). [15-16]

Caenorhabditis elegans: A Model System for Studying Oxygen Deprivation

Model systems are used to answer basic questions in biology that may have applications to human health. Model systems can answer these types of questions in a less-expensive, faster, and more ethical manner. In the area of model systems, *Caenorhabditis elegans* has been used to further our understanding of processes such as apoptosis, [17] synaptic function, [18] and gene silencing/RNA processing. [19] As a model system, *C. elegans* has a wealth of available research literature and established tools that make it ideal for research.

The use of *C. elegans* as a model system was pioneered by Sydney Brenner in the early 1970s. He was looking for a more appropriate model system for genetic interactions, developmental biology, and neuroscience. [20] From his

original publication, the field of *C. elegans* biology has exploded, with over 15,000 publications listed for "*Caenorhabditis elegans* " on Pubmed.gov currently, and 229 individual research labs listed according to the *Caenorhabditis elegans* WWW Server. [21] It was also the first metazoan to be fully sequenced, [22] and has had its entire invariant somatic cell lineage traced. [23]

Brenner's original genetic screen isolated approximately 300 mutants, and the number of known mutants has since grown. There are now over 3000 mutants available for a nominal fee from the publicly funded *C. elegans* Genetics Consortium, or CGC at The University of Minnesota at St. Paul. This provides a repository and distribution system for mutants that are maintained as freezer stocks for the community as a whole. [24]

In addition to a multitude of traditional mutants, tools for reverse genetics are quite convenient in *C. elegans* . Several RNA interference (RNAi) screens, which can be accomplished by feeding the worms commercially available bacterial strains expressing the dsRNA of interest, have been used to look for phenotypes for greater than 80% of the 19,000 predicted genes in the genome. [25-29] These libraries were created using a commercially available molecular vector kit from the Fire lab which also simplifies the introduction of reporter genes (which are easily visible through the transparent nematode). [30]

C. elegans has many characteristics that make it ideal as a model for studying oxygen deprivation. First of all, it has a life cycle of three days at 20^{o}C. It is transparent, so morphological abnormalities can be observed with a

relatively inexpensive low-powered stereomicroscope. Also it has a relatively high fecundity rate, with an average hermaphrodite producing approximately 300 self-fertilized offspring in the first 3 days of adulthood. Furthermore, its somatic cells are post-mitotic, and, similarly to the CNS and myocardium of mammals, are not replaced following death. With only 959 somatic nuclei, if cells die, it is possible to observe the cell corpse in the transparent worm via Nomarski imaging.

More specific for oxygen deprivation however, *Caenorhabditis elegans* is a psuedocoelomate. It uses simple diffusion to supply its cells with oxygen. [31] In such an organism, the flow of nutrients, the delivery of oxygen, and the clearance of metabolic waste are somewhat decoupled. This is not possible in systems that are sufficiently more advanced, because in organisms that are much larger, simple diffusion would not meet oxygen delivery demands.

Furthermore, *C. elegans* is a unique organism for studying oxygen deprivation due to its natural history as a soil nematode. It is particularly resistant to oxygen deprivation, and it has been previously demonstrated that wild type animals are capable of surviving 24 hours of anoxia (defined here as <.001kPa of O_2) at 20^{o}C at all stages of life. The animals are able to survive, by entering into a reversible state of suspended animation where all observable movement, development, and reproductive processes arrest. Following 24 hours of exposure, wild type adult hermaphrodite survival decreases to the point that they are non-viable beyond 72 hours of anoxia. [32-33]

Potential Targets for Altering Anoxia Phenotypes

As mentioned earlier, hypoxia response via canonical hypoxia inducible factor (HIF) signaling has been demonstrated in *C. elegans,* [13] although some responses to low oxygen have been shown to be HIF independent. [33] It has also been shown that *hif-1*, the *C. elegans* homologue of HIF- 1α is involved in heat stress resistance, [34] and is involved in lifespan determination. [35] If altering HIF signaling alters lifespan and stress resistance, then possibly stress resistant and long-lived mutants might have an enhanced ability to survive low oxygen tensions.

A number of existing *C. elegans* mutants have an improved resistance to a variety of other stresses. These include exposure to reactive oxygen species generating compounds such as paraquat, [36] heat stress, [37] heavy metals, [38] ultraviolet radiation, [39] and hyperoxia. [40] In addition to these stresses, aging itself can be thought of as a series of stresses that accumulate in the cells and tissues of the organism. There are a number of mutants and conditions that through one mechanism or another are capable of a prolonged lifespan when compared to the wild type animal under non-stressed conditions. [41-45]

Dauer Signaling Pathways: Longevity, Stress Resistance, and Insulin-Signaling

C. elegans has an entire alternate developmental stage specific for stressful conditions. During larval development, if the larvae encounter crowding,

starvation, high concentrations of a secreted pheromone (an ascaroside, called daumone), [46-48] or other stressful conditions they enter an alternate larval stage called the dauer (German for enduring) stage. Dauer larvae are characterized by a thickened cuticle, sealed pharyngeal orifices, and stress resistance. [49]

Dauer larvae enter an ageless state that can last for several times the length of an ordinary *C. elegans* lifespan, and then exit the dauer stage when food is present and daumone is low. Once the animals exit dauer, they continue their developmental lifespan as if they had progressed through development normally, and are morphologically indistinguishable from non-dauer adults. [49-51]

A number of known signaling pathways have been shown to influence dauer formation. Among the known involved pathways are the Insulin/Insulin-like growth factor pathway, the TGF-β pathway, and a retinoic acid-like nuclear hormone receptor, along with a neuronal based system involving sensory amphids for daumone. Modulation of these pathways is known to occur via the TOR pathway, c-jun kinase pathway and AMP associated kinase. Mutant animals in these pathways can have phenotypes as adults if the animals are able to progress to adulthood without arrest. Mutant animals in the I/IGF-like pathway, *daf-2(e1370)*, were shown to be very long-lived as adults. [43] Subsequent studies discovered that it was an insulin-like cell surface receptor. [52] Adults animals carrying the mutation were also shown to be resistant to heat shock, paraquat, and have a reduced fecundity and pharyngeal pumping rate. [37]

Other mutants in the classic IGF-like signaling pathway also display

increased stress resistance and longevity. [53-55] At least some of the longevity and stress resistance effects of this pathway are attributable to the FOXO (Forkhead box O) transcription factor DAF-16. [56-58] However, as illustrated in Figure 1, there is some cross-talk of the I/IGF-like pathway with other pathways

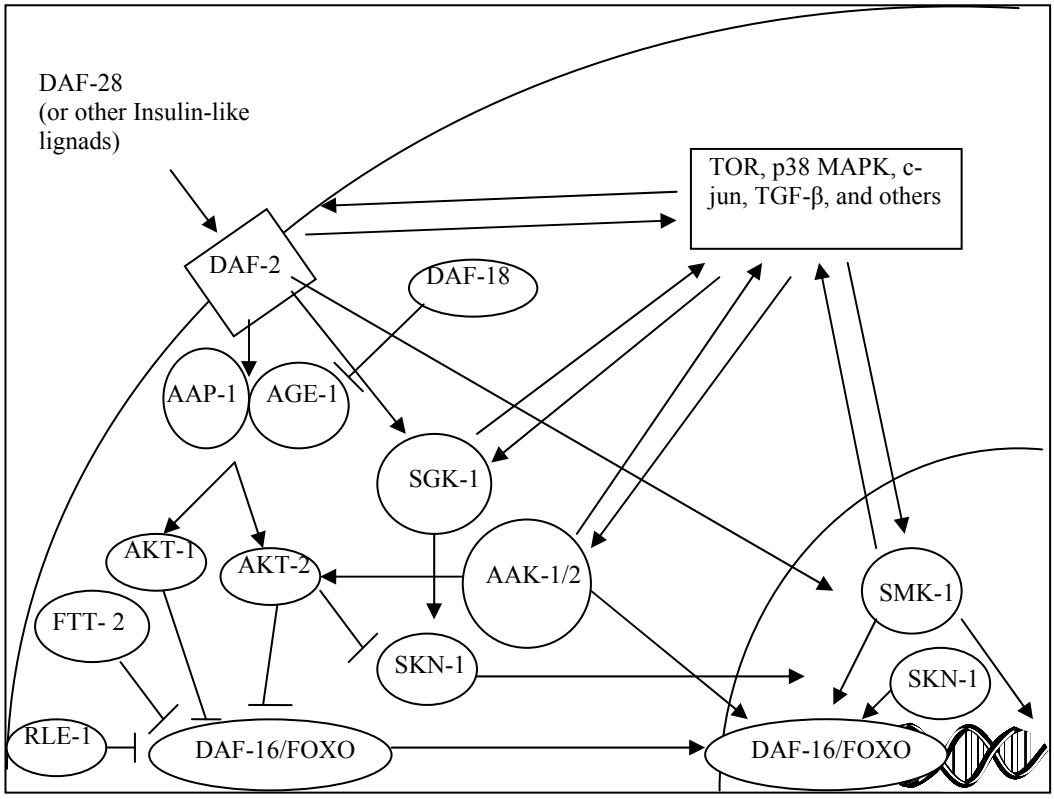


Figure 1. An illustration of some of the known effectors of the Insulin/IGF-like signaling pathway involved in dauer formation in *C. elegans*.

that has been evidenced by the genetic interaction of AMPK, [59] a lipase gene, [60] steroid hormone pathways, [61] the TOR pathway, [62] *ras* signaling, [63] and TGF-β signaling,[64]

Microaaray data suggests that DAF-16 upregulates the expression of 40 genes that are involved with longevity. Among them are known and putative enzymes involved in glycolysis, the TCA cycle, fatty acid metabolism, steroid synthesis, detoxification, and heat shock proteins among others. The same study demonstrated that 15 genes were downregulated which conferred longevity. Among these were genes involved with peptide transport, vitellogenin, an insulin homolog, and nucleases among others. [65]

Germ-Line Signaling and Lifespan Extension

Some longevity mutants were determined to have an altered fecundity phenotype. [37,44] Using the germ-line mutant *glp-1(e2141)*, it was also determined that signals from the germ-line could affect longevity and stress resistance as well. [66-69] These phenotypes appears to function through DAF-16 , a predicted lipase, KRI-1, require the somatic germ-line , and to be independent of the AMPK. [59,68,70-72] However, the role of AMPK is controversed, and a separate study demonstrated that germ-line signaling was dependent on AMPK. [73]

The Role of AMPK in Intracellular Energy Signaling

AMPK, or AMP-activated protein kinase, plays an intracellular role that is homologous to the extracellular role played by classic insulin signaling. AMPK serves as a metabolic checkpoint that is activated when AMP levels are high in the cell, a condition indicative of low nutrient levels in the cell. The signal

modulates many non-proliferative signals in yeast, mice, flies and *C. elegans* , such as I/IGF like signaling, the TOR pathway, and TGF-β signaling. [74-75]

AMPK exists as a heterotrimer consisting of the α catalytic subunit, a polysaccharide binding β subunit, and the AMP-binding γ subunit which is a dimer in the holoenzyme structure. While phosphorylation of the α subunit by the tumor-suppressor gene LKB1is required for function, binding of AMP is thought to facilitate the accessibility of this phosphorylation site, and also to allosterically inhibit the dephosphorylation of the site of LKB1 activation (a threonine at position 172 in the α subunit). The regulatory role of the β subunit is thought to be a bimodal sensor for carbohydrate stores as well as the nucleotide sensing capabilities of the γ subunits. [75]

In *C. elegans,* there are 5 putative genes for the γ regulatory subunits and two isoforms each of the catalytic α and regulatory β subunits. One of the catalytic subunit isoforms, AAK-2, has been shown to be involved with regulation of the TOR pathway, regulation of the SKN-1 co-transcription factor, lipid metabolism, and the NAD+ dependent deacetylase SIRT1, in addition to the aforementioned role in I/IGF signaling. [76-79] That particular subunit has also been shown to be required for resistance to paraquat, a superoxide generating compound used as a pesticide. [80]

Dietary Restriction, Mitochondrial Mutants, and Longevity

Caloric restriction, or dietary restriction as it is referred to in *C. elegans* ,

has been known to increase longevity in animals for decades starting with Osborne et al's work with rats early in the 20th century. [81] Caloric restriction is sufficient to extend longevity and increase stress resistance in *C. elegans* as evidenced by studies on nutrient poor media, using heat killed bacteria as a food source, and the pharyngeal pumping mutant *eat-2(ad1116).* [45,82]

One possible consequence of caloric restriction which doesn't directly involve the dauer signaling pathways is that of reduced overall energy metabolism. The mutant *clk-1(e2519)* is deficient in an enzyme needed to synthesize coenzyme Q. It displays a slowed developmental time frame, and an extended lifespan. [39,42,83-85] Lifespan extension in *eat-2* and *clk-1* animals has been shown to function in the same pathway and there is evidence to support that it functions through DAF-16. [42]

Other players may have a role in caloric restriction mediated longevity. There is evidence that another forkhead-type transcription factor, *pha-4*, plays a role in this lifespan extension via caloric restriction. [86] Also Greer and Brunet demonstrated that differing methods of caloric restriction function through different pathways. [87]

Other Effectors of Llifespan

Animals with the *daf-2* mutation are known to be resistant to bacterial pathogens. [88] The *E. coli* strain OP50, the standard laboratory food source for *C. elegans* , is usually not considered pathogenic. Yet, *C. elegans* lived longer

when grown on a common soil organism, *Bacillus subtilis*, than on the *E. coli* strain OP50. [88] Evidence has also been given that intestinal packing of OP50 is a major contributor of death in aged animals. [89]

Additionally heat stress has been shown to effect lifespan. By cycling through 10 minute periods of lower temperatures and higher temperatures, it was possible to increase the lifespan of wild type animals. Lifespan extension was also possible in *clk-1*, *daf-2*, and *eat-2* mutant animals, but not in *daf-16* null mutant animals. Expression studies showed that under these conditions heat shock proteins are upregulated that are also upregulated in conditions of reduced insulin-like signaling. This evidence suggests that the process occurs through DAF-16 in a manner consistent with other known mediators of stress and lifespan in the I/IGF-like pathway. [41]

CHAPTER 2

GENETIC ENHANCERS OF LONG-TERM ANOXIA SURVIVAL IN *Caenorhabditis elegans* [1]

As discussed earlier it is already known that adult hermaphrodite *C. elegans* wild type N2 animals are able to survive 24 hours of 20^{o}C anoxia. It is also known that this ability to survive 20^{o}C anoxia lasted out to about 72 hours. We hypothesized that some of the known longevity and stress resistant mutant alleles would possibly be able to survive beyond this threshold. In a collaborative effort with Dr. Pamela Padilla and Alex Mendenhall, we set out to find mutant strains that would be able to survive long-term anoxia defined here as greater than three days of anoxia.

Insulin-Like Signaling and Anoxia Survival

daf-2(e1370) mutants are temperature sensitive dauer formation constitutive mutants that are 100% dauer at 25^{o}C, yet can be raised to adulthood when grown at 20^{o}C . Loss of function alleles cause mutant animals to form dauer larvae at the permissive temperature. *daf-2(e1370)* is also what is known as a class 2 allele, because it exhibits other phenotypes in addition to being

[1] Portions of this chapter have been published with permission of the Genetics Society of America. 90. Mendenhall AR, Larue B, Padilla PA. Glyceraldehyde-3-phosphate dehydrogenase mediates anoxia response and survival in Caenorhabditis elegans. Genetics 2006(Journal Article).

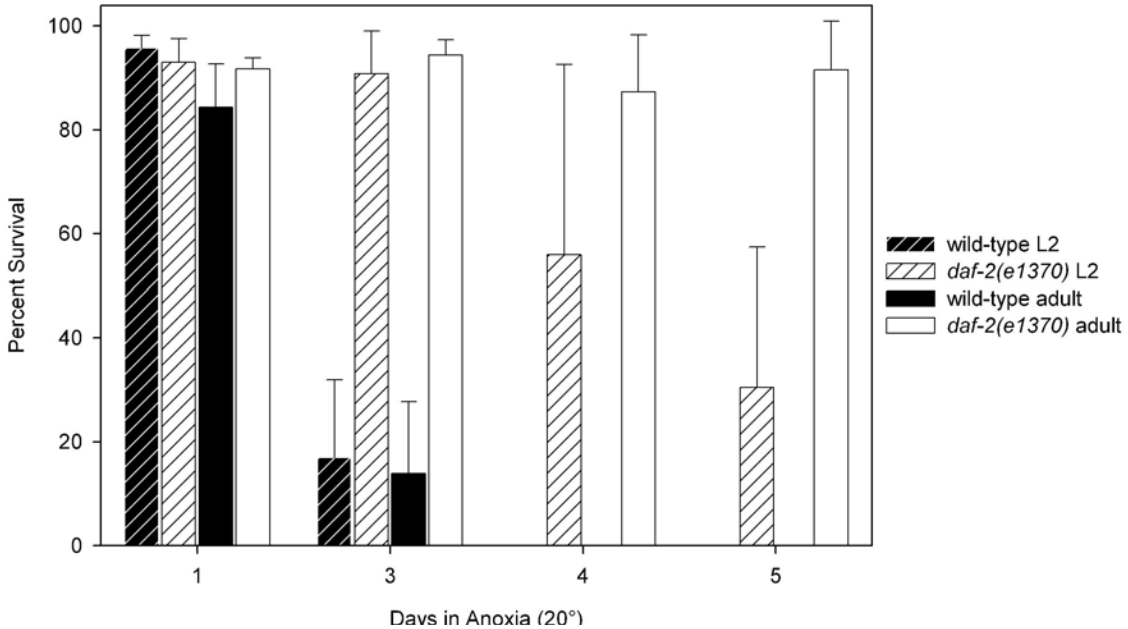
dauer constitutive. These phenotypes include an increased lifespan, slowed developmental timescale, a reduced pharyngeal pumping rate, a resistance to a myriad of stresses (heat shock, paraquat, hypoxia, hyperoxia, etc…), and a lengthened reproductive period.

Our initial interest, was to determine if *daf-2(e1370)* would also be able to survive 72 hours (or more) of 20^{o}C anoxia better than wild type. We refer to this as a long-term anoxia phenotype, and unless otherwise noted, it will mean 72 hours at 20^{o}C anoxia (defined as <.001kPa of O_2). We were also interested if only class 2 alleles were able to show an enhanced long-term anoxia phenotype, or if all *daf-2* mutants displayed this phenotype.

The results of this collaborative work were the subject of our 2006 publication in the journal *Genetics.* [90] We found that not only could *daf-2(e1370)* survive anoxia better than wild type, but that it could survive well beyond 72 hours in anoxia at 20^{o}C. *daf-2(e1370)* animals are able to survive 110 hours in anoxia (Figure 2 A) and maintain reproductive viability. They are able to survive out to 9 days at a low level, but are severely injured by the prolonged anoxia exposure (data not shown).

daf-2(e1370) animals are also able to survive 24 hours of anoxia at 28^{o}C (Figure 2B). Wild type worms are unable to survive this dual (cont. on pg. 22)

A



B

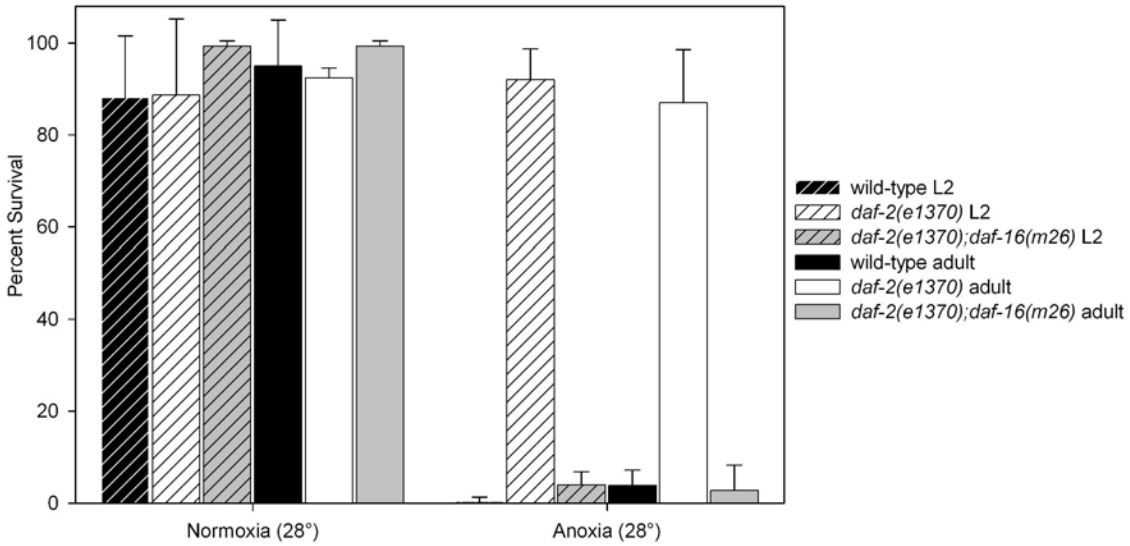


Figure 2. *daf-2(e1370)* animals have an enhanced ability to survive anoxic stress. A) The survival rate of *daf-2(e1370)* and wild type animals exposed to long-term anoxia at 20°C. Wild type L2 larvae, wild type adults, *daf-2(e1370*) L2 larvae, and *daf-2(e1370)* adults were exposed to 1, 3, 4 and 5 days of anoxia at 20°C. The data shown are representative of at least three independent experiments with a total of at least 150 animals. Error bars represent standard deviation. B) The survival rate of *daf-2(e1370)*, wild type and *daf-16(m26); daf-2(e1370)*

animals exposed to high-temperature (28°C) anoxia. Wild type L2 larvae, *daf-2(e1370)* L2 larvae, *daf-16(m26); daf-2(e1370)* L2 larvae, wild type adults, *daf-2(e1370)* adults and *daf-16(m26); daf-2(e1370)* adults were exposed to 1 day of anoxia at 28°C. The data shown are representative of at least three independent experiments with a total of more than 150 nematodes. Error bars represent standard deviation. [90]

stress on the organism. These data support the idea that 28°C is indeed a stressful temperature for the organism, and agrees with a common husbandry technique for cultivating males for breedings from stock populations which involves shifting the parental generation of hermaphrodites to an elevated temperature for short periods of time to generate male offspring [91]. Interestingly enough, male *C. elegans* are XO offspring generated from a non-disjunction event, and usually occur at an incidence of < 1% [31] which indicates that raising the organism at 28°C is stressful enough to affect meiosis. [90]

We also were able to characterize, in wild type animals, abnormal morphologies associated with long-term anoxia. In general, there was a large amount of disorganization in the tissues of wild type animals exposed to long-term anoxia compared to *daf-2(e1370)*. This is very evident in the area of the pharynx. Large “vacuolar-like” cavities appear in the muscles, but do not uniformly coincide with necrotic cells (figure 3). [90]

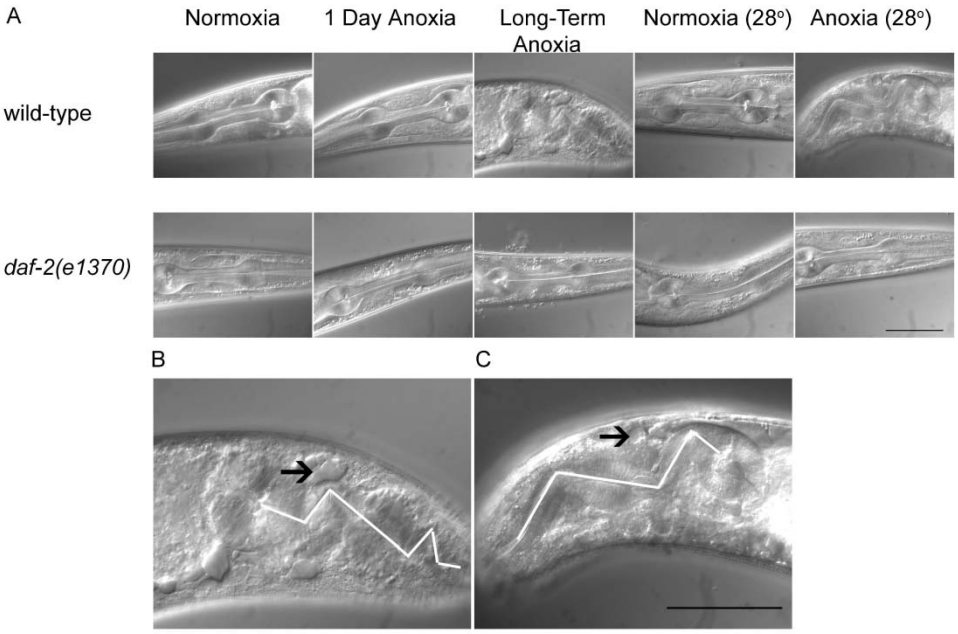


Figure 3. Tissue morphology of wild type and *daf-2(e1370)* animals exposed to long-term anoxia (20°C) and high-temperature anoxia (28°C). The anterior head region of wild type and the *daf-2(e1370)* adult hermaphrodites were examined using DIC microscopy. (A) The animals were exposed to normoxia, 1 day, 3 days (wild type) or 5 days (*daf-2(e1370))* of anoxia at 20°C or 1 day of normoxia or anoxia at 28°C. Animals exposed to anoxia were allowed to recover in air for 1-4 hrs before images were obtained. Representative animals for each genotype and condition are shown. Scale bar equals 50 µm. (B) Enlarged image of a wild type adult hermaphrodite exposed to 3 days of anoxia. (C) Enlarged image of wild type adult hermaphrodite exposed to 1 day of anoxia at 28°C. For B and C the arrow points to a cavity and the white line is drawn along the lumen. Scale bar equals 50µm. [90]

The alimentary canal in the area of the pharynx also develops characteristic bends in both long-term anoxia and 24 hours of 28°C anoxia (Figure 3 A, B, and C). Wild type organisms develop these bends in 24 hours on anoxia, but are able to recover upon reoxygenation. In long-term anoxia, or 28°C anoxia, the pharynx becomes convoluted, and upon reoxygenation, does not recover its original morphology even in organisms that survive the anoxia exposure. [90]

Conversely, *daf-2(e1370)* animals however do not develop tissue malformations such as “vacuolar-like” cavities (Figure 3 A) in either 72 hours of 20^{o}C anoxia, or 24 hours of 28^{o}C anoxia. They also do not develop permanent bends in their pharynx. Upon reoxygenation and recovery, the mutant animals appear to have tissue morphology similar to normoxia controls. [90]

Additionally, wild type animals exposed to long-term anoxia or 28^{o}C anoxia develop an abnormal motility phenotype. *C. elegans* usually moves back in forth in a sinusoidal motion. When exposed to long-term anoxia or 28^{o}C anoxia, wild type animals display a “tail dragging” phenotype. The tail of the organism seems to follow behind the anterior listlessly, and the organism is ineffectual at traveling in reverse. Once again *daf-2(e1370)* animals do not display this motility defect. [90]

Our next question, was if the enhanced anoxia survival phenotypes were specific for *daf-2(e1370)* animals, or if other *daf-2* alleles were also able to survive as well as the *daf-2(e1370)* mutants. We looked at the survival rates of three other daf-2 alleles, *daf-2(e1371)* a class 1 mutant, and two additional class 2 mutants one that is considered a weaker allele, *daf-2(m596)*, and one that is considered similar to *daf-2(e1370)*, *daf-2(m579)*. [90]

While the other robust class 2 allele, *daf-2(m579),* was able to survive both 28^{o}C anoxia and long-term anoxia (albeit at a more modest level), the other two alleles, *daf-2(e1371)* and *daf-2(m596)* did not display an enhanced anoxia survival phenotype (Table 1). [90]

Table 1. Survival of various *daf-2* alleles in high-temperature anoxia and long-term anoxia. (n=total number of animals for 3 independent trials.) [90]

Strain	Allele Class	28°C 24 hour anoxia survival	20°C 72 hour anoxia survival
daf-2(e1370)	2	95.2 ± 3.3 (n = 150)	91.6 ± 9.4 (n = 150)
daf-2(e1371)	1	4.5 ± 5.3 (n = 200)	0.0 ± 0.0 (n = 200)
daf-2(m596)	2	0.0 ± 0.0 (n = 200)	0.0 ± 0.0 (n = 200)
daf-2(m579)	2	77.6 ± 17.7 (n = 300)	75.3 ± 32.1 (n = 300)

We next turned to downstream messengers of the DAF-2 receptor, and with known modulators of I/IGF-like signaling in *C. elegans* . The first of such messengers is the PI3K homolog *age-1*. *age-1* loss of function mutants mimic the ageing and slowed developmental phenotypes associated with *daf-2* mutants. It has also been shown to be epistatic of *daf-2* mutants, and the longevity phenotypes associated with the caloric restriction (CR), such as the *eat-2* and mitochondrial function mutants. All of these have been shown to be at least partially dependent upon DAF-16 the FOXO transcription factor homolog in *C. elegans* . We utilized the robust loss of function *age-1(hx456)* mutant allele to determine if PI3K and canonical I/IGF signaling is solely responsible for the increase in anoxia survival associated with *daf-2(e1370)*. No *age-1(hx456)* animals were observed to survive long-term anoxia (n= 200 in 4 independent trials). It is possible that the allele is only epistatic to DAF-2 in a way that does not promote an enhanced long-term anoxia phenotype, but it is also likely that *daf-2(e1370)* alters anoxia survival through a bifurcation in the pathway. For

example the *C. elegans* homolog of AMPK, *aak-2* has been shown to modulate DAF-16 independent of AKT/PKB [92] (see Figure 1).

Animals with Some Neuronal Defects Have an Enhanced Long-Term Anoxia Survival Phenotype

Insulin-like signaling and other pathways involved with energy signaling, are important in many cell types, but some cell types are particularly vulnerable to anoxia. The cells that are particularly susceptible in mammals to ischemia, cardiomyocytes and neurons have unique problems due to the energy demands associated with their function, and their method of action (a constant depolarization/repolarization cycle that is energy expensive).

C. elegans has a well characterized nervous system with a host of mutants that are known to possess defined deficiencies in synaptic function. Answering questions in neuroscience was one of Brenner's original goals when he established the worm as a model system, and in his original screen he isolated several mutants (particularly in the "*unc*" class) that were later used to help identify components of synaptic function. Yet excitotoxicity, a phenomenon known to effect neurons in other systems has not been fully described in the worm.

Briefly, excitotoxicity occurs during the low energy conditions encountered in anoxia/hypoxia/ischemia. These low energy conditions don't allow for the clearance of excitatory neurotransmitters from the synaptic cleft via glial or

neuronal re-uptake mechanisms. One consequence of this abundance of excitatory neurotransmitter in the synaptic cleft is the continuous binding of neurotransmitters to ligand-gated ion channels. The resulting flood of calcium and other ions from these ligand-gated channels can start signaling cascades that result in the death of the cell due to apoptotic mechanisms. [93]

Glutamate has been shown to be an especially potent mediator of excitotoxicity, via the NMDA-type ionotropic calcium channels, and to a lesser extent the metabotropic quisqualalte-type recptors which can trigger release of internal calcium stores. *In vivo* and *in vitro* studies have demonstrated that both competitive and non-competitive antagonists of the NMDA receptor are capable of short-term neuroprotection to hypoxia and ischemia in vertebrates. The effects of glutamate mediated neurotoxicity are particularly pronounced in non-myelinated portions of the cell, and less damaging in axonal regions. [93]

C. elegans, as an invertebrate, does not have myelinated neurons. It also has a unique locomotory circuit where opposite sides of the worm are out of phase with regards to waves of excitatory and inhibitory neuronal signals that propagate the length of the worm. The motor neurons utilize GABA as an inhibitory signal, and acetylcholine as an excitatory signal to propagate a wave of contraction along the length of the worm to create a sinusoidal fluid movement across the medium. These “out-of-phase” relaxation/contraction signals are coordinated by glutamatergic interneurons. [94]

Fortuitously, we discovered that animals with the mutation *unc-32(f131),* which have altered neuronal function, also have an enhanced ability to survive long-term anoxia. Localization studies have demonstrated that UNC-32 is localized to neurons, and not other tissues in the worm. [95] The mutation is a partial loss of function in a V-H^+ATPase subunit caused by a G to A transition.[95] V-H^+ATPases function by creating a proton gradient via the breakdown of ATP. In the case of UNC-32, the putative function of the V-H^+ATPase is to establish a proton gradient that is used to antiport neurotransmitters into synaptic vesicles. [96]

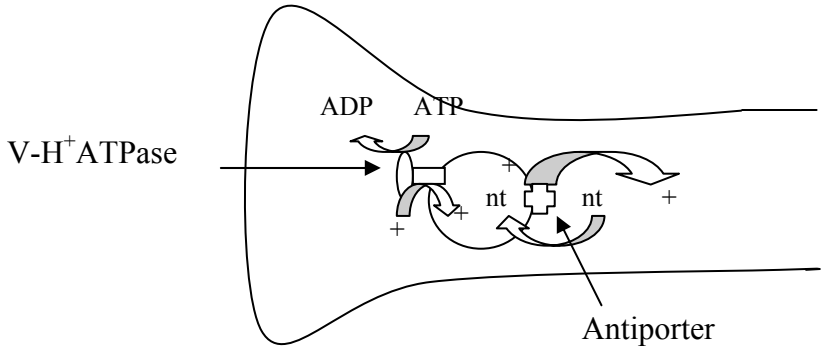


Figure 4. Graphical representation of the role neuronal V-H^+ATPase plays in the neurons of *C.elegans.* A proton gradient is established via the V-H^+ATPase , and an antiporter transports neurotransmitter via the proton motive force*"+"* represents protons and "nt" represents neurotransmitter.

In animals with the *unc-32(f131)* allele, this resultant phenotype is that of a very "strong" coiler (due to low neurotransmitter levels in the pre-synaptic vesicles). Coilers are a type of phenotype, where the usual excitation/relaxation circuit that results in sinusoidal movement is usually frozen into a rigid coil

somewhat similar to a snail's shell. *unc-32(f131)* animals are usually not very motile as adults, and typically remain in one spot unless prodded or starved. They display a varying degree of abnormal morphologies, and could be described as "sickly" compared to wild type. However, animals with this mutation survive 3 day anoxia at greater than 90% on average (Figure 5).

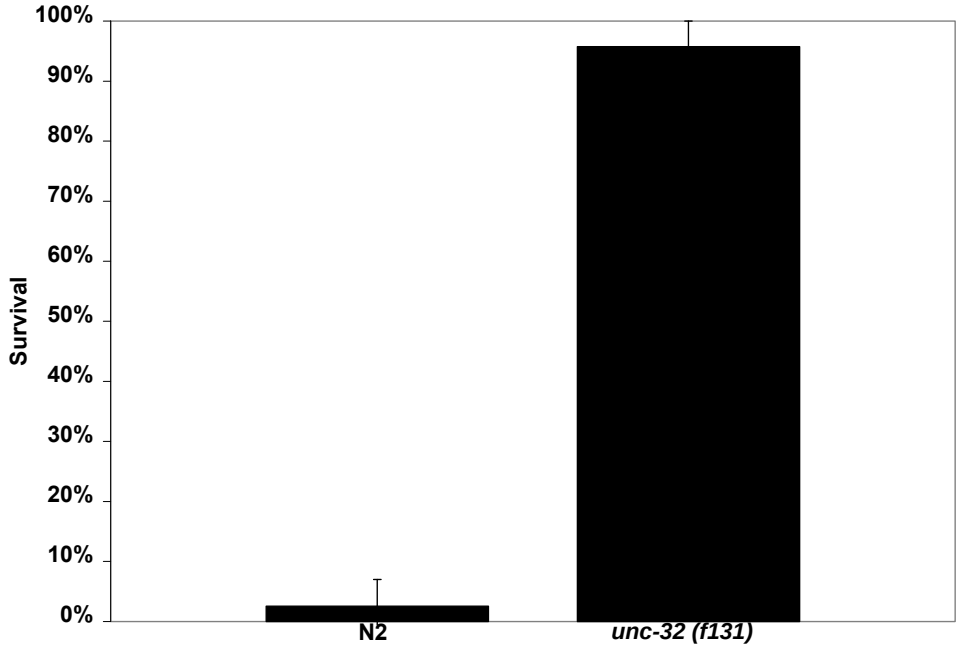
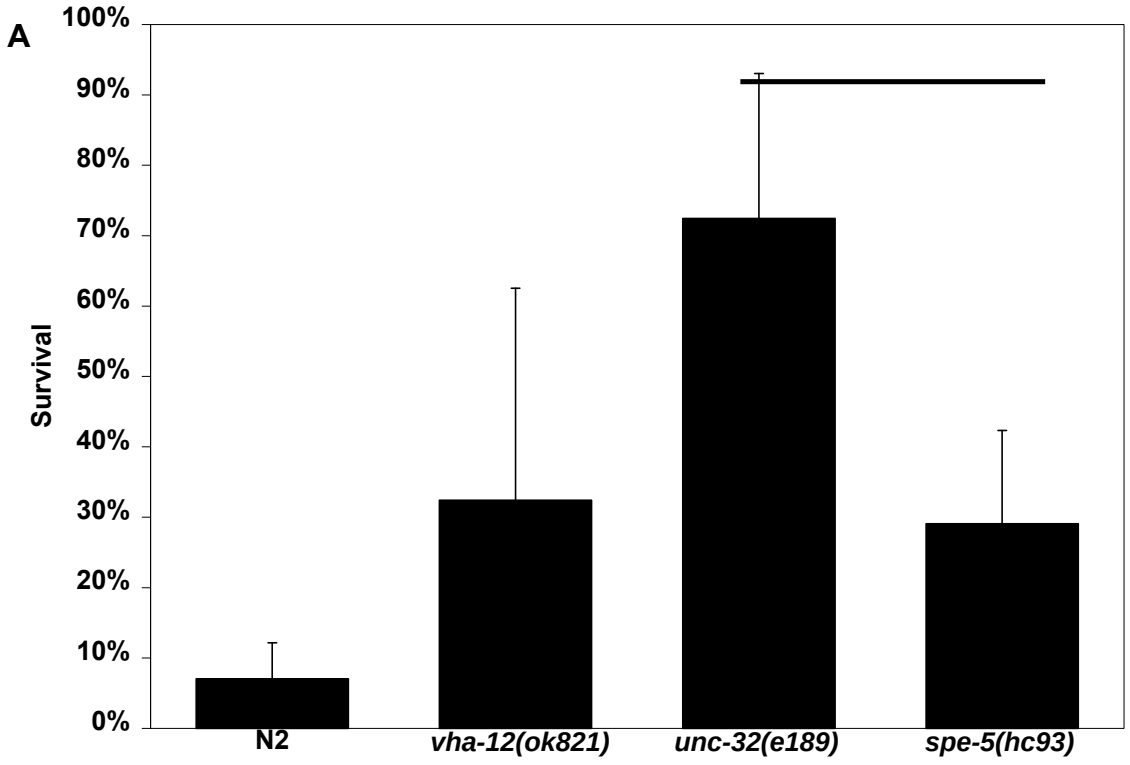


Figure 5. Mean long-term anoxia survival rates of wild type animals versus animals with the *unc-32(f131)* mutation. *unc-32(f131)* animals display an increase in long-term anoxia survival over wild type ($p < .001$, Student's paired one-tailed t-test; n >150 over three independent trials. Error bars represent standard deviation).

To determine if the long-term anoxia phenotype of unc-32(f131) animals were allele specific or were a common trait among all V-H^+ATPase mutants we exposed three V-H^+ATPase mutant strains, *unc-32(e189), vha-12(ok821)*, and *spe-5(hc93*) to long-term 20^{o}C anoxia and compared them to wild type controls.

SPE-5 is involved in sperm function, and VHA-12 is shown to be localized to the excretory cell, pharynx, and intestine. I collaborated on this project with Henry D. Greyner.

In 3 days of anoxia *unc-32(e189)* animals survived at a much higher rate than either of the other mutant strain or wild type animals. *vha-12(ok821)* animal's did not survive at a rate that was significantly different than the survival rates for wild type animals (Figure 6 A). In four days of anoxia the *unc-32(e189)* animals did not survive at a significantly different level compared to either *vha-12(ok821)* or wild type animals (Figure 6 B). In contrast *spe-5(hc93)* animals survived at a modest but consistent rate that was significantly different than the rate that wild type animals survived either three or four days of anoxia (Figure 6 A, B).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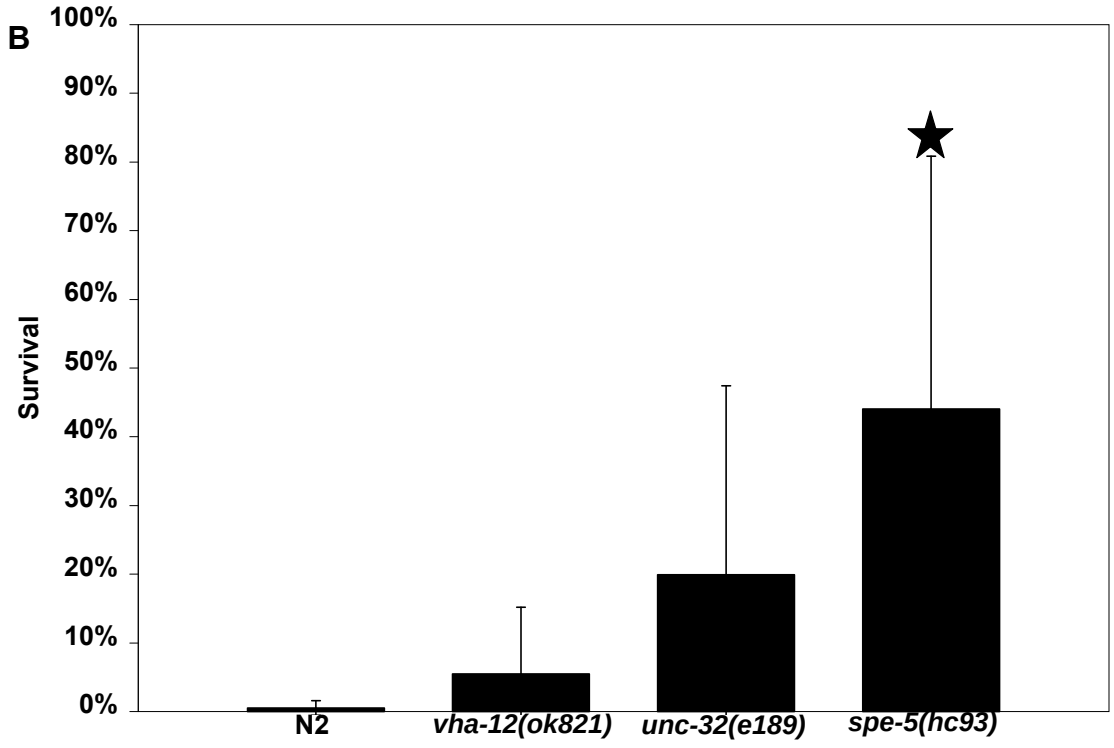
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Survival
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vha-12(ok821)
unc-32(e189)
spe-5(hc93)

Figure 6.
Anoxia survival rates of various V-H^+ATPase mutant animals. A) Long-term anoxia survival rates of V-H^+ATPase mutant animals in 3 days of anoxia at 20^{o}C compared to N2 controls. Stars indicate groups with a statistically significant increase in mean survival rate over N2 (n >180 over 4 independent trials, p< .05 Student's paired one-tailed t-test; error bars indicate standard deviation). B) Long-term anoxia survival rates of V-H^+ATPase mutant animals in 4 days of anoxia at 20^{o}C compared to N2 controls. Star indicates a statistically significant increase in mean survival rate over N2 (n >175 over 4 independent trials, p< .05 Student's paired one-tailed t-test. Error bars indicate standard deviation).

CHAPTER 3

ENVIRONMENTAL ENHANCERS OF LONG-TERM ANOXIA SURVIVAL IN *Caenorhabditis elegans*[2]

Effects of Dietary Restriction on Long-Term Anoxia Survival

As mentioned in the background section, it has been known for decades that caloric restriction (Referrred to as dietary restriction, or DR, in *C. elegans*) can increase lifespan. This has been demonstrated in *C. elegans* , using animals that have reduced pharyngeal pumping rates, are grown on nutrient poor media compared to an OP50 lawn, or have defects in mitochondrial function. Some aspects of this type of lifespan extension are attributed to DAF-16, and mutant animals like *daf-2(e1370)* exhibit a reduced pharyngeal pumping rate (although it is controversed if this phenotype contributes to the longevity and stress resistance phenotypes of *daf-2(e1370)* animals). Evidence does exists that some of the effects are mediated through *pha-4,* the *C. elegans* homolog for the FOXA transcription factor. [86]

To explore the possibility that the factors which extend lifespan associated with DR could also enhance anoxia survival, I analyzed four mutant alleles known to increase lifespan. The first is the classic DR mutant strain *eat-2(ad-1116)*, which has a reduced pharyngeal pump rate. The other three are mutants involved with mitochondrial function. *mev-1(tk22)* is a partial loss of function

[2] Portions of this chapter are in review.

mutant in sub-unit B of succinate dehydrogenase. The remaining two are different alleles of *clk-1*, *e2519* and *qm30*, which is an enzyme involved with Co-enzyme Q synthesis.

eat-2(ad1116) animals have a mean long-term anoxia survival rate of over 70%, while *mev-1(tk22)* animals had a survival rate over 40%. Both strains were significantly different from N2 controls. Both *clk-1(e2519)* and *clk-1(qm30)* mutant animals did not display an enhanced anoxia survival phenotype, and both of their survival rates were not different from wild type N2 control animals (Figure 7).

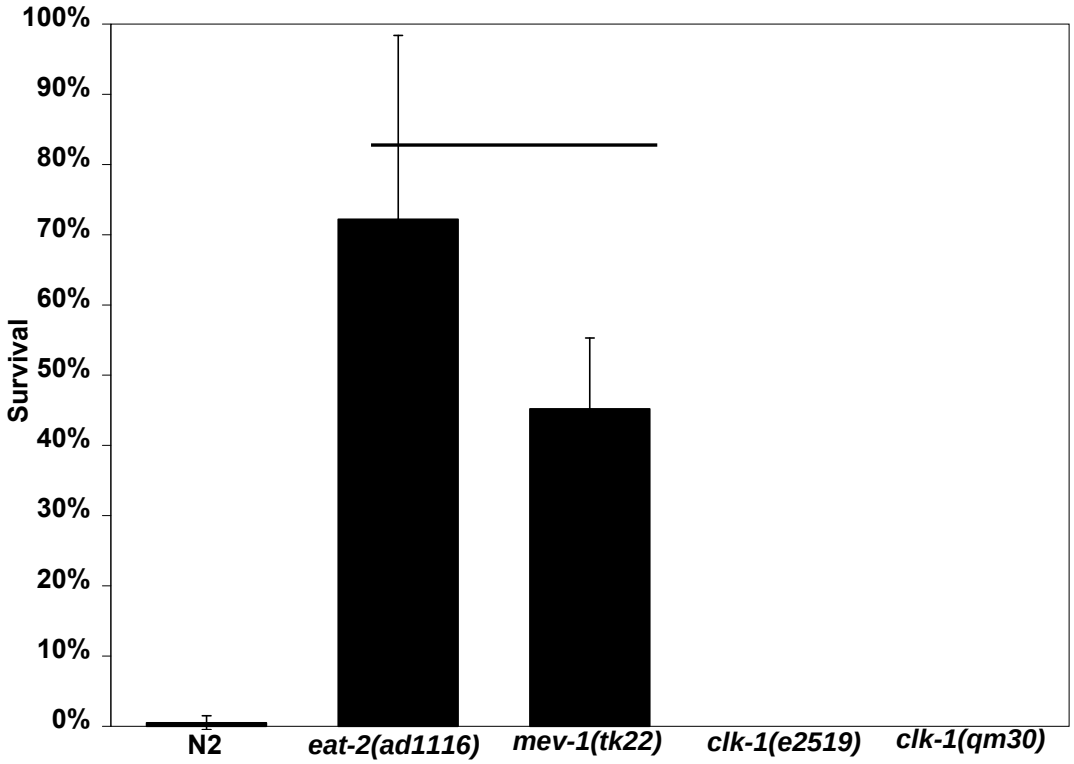


Figure 7. Long-term anoxia survival (3 days of anoxia at 20°C) of dietary restriction and mitochondrial function animals that are known to increase lifespan compared to N2 controls. Line indicates groups with a statistically significant increase in mean survival rate over wild type (n >150 over at least 3 independent trials, p< .05 Student's paired one-

tailed t-test. Error bars indicate standard deviation).

Effects of Developmental Temperature on Anoxia Survival

Another environmental factor known to influence lifespan is heat stress. As mentioned earlier, others have shown that cycling through 10 minute periods of lower temperatures and higher temperatures, it was possible to increase the lifespan of wild type animals[41]. Additionally, several of the genes upregulated in *daf-2(e1370)* mutant animals are heat-shock proteins.[65] I hypothesized that animals living at an elevated temperature during development (25^{o}C), or thermal preconditioning as we refer to it, would be better able to survive long-term 20^{o}C anoxia.

To investigate whether thermal preconditioning could be used to enhance anoxia survival in wild type animals, we maintained N2 animals at 25^{o}C during development compared to 20^{o}C for controls. We then exposed them to 72 hours of anoxia at 20^{o}C. The population maintatined at 25^{o}C survived at a significantly higher rate than those continually maintained at 20^{o}C (Figure 8).

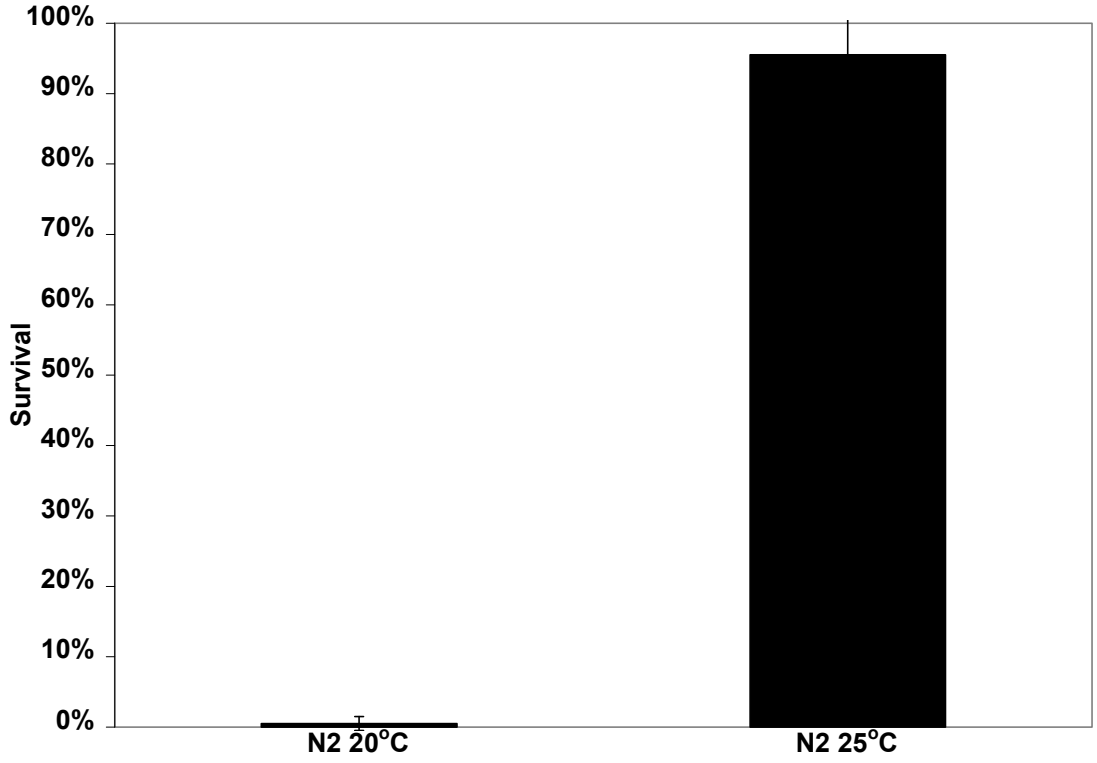


Figure 8. Mean long-term anoxia survival rates of wild type animals raised at 20°C versus animals raised at 25°C. ($p < .001$, Student's paired one-tailed t-test; n >190 over four independent trials; Error bars represent standard deviation).

Differences in Anoxia Survival Mediated by Food Type

One of the major contributors of metabolic disorders is the diet associated with western cultures. These "rich" high fat, high sugar diets cause global changes in tissue and cell morphologies in vertabrates,[97] and contribute to many conditions associated with metabolic syndrome.[98] It is possible that different food types that are considered to be "normal" for an organism could affect the ability of an organism to survive stressful environments. *C. elegans* utilizes bacteria as a

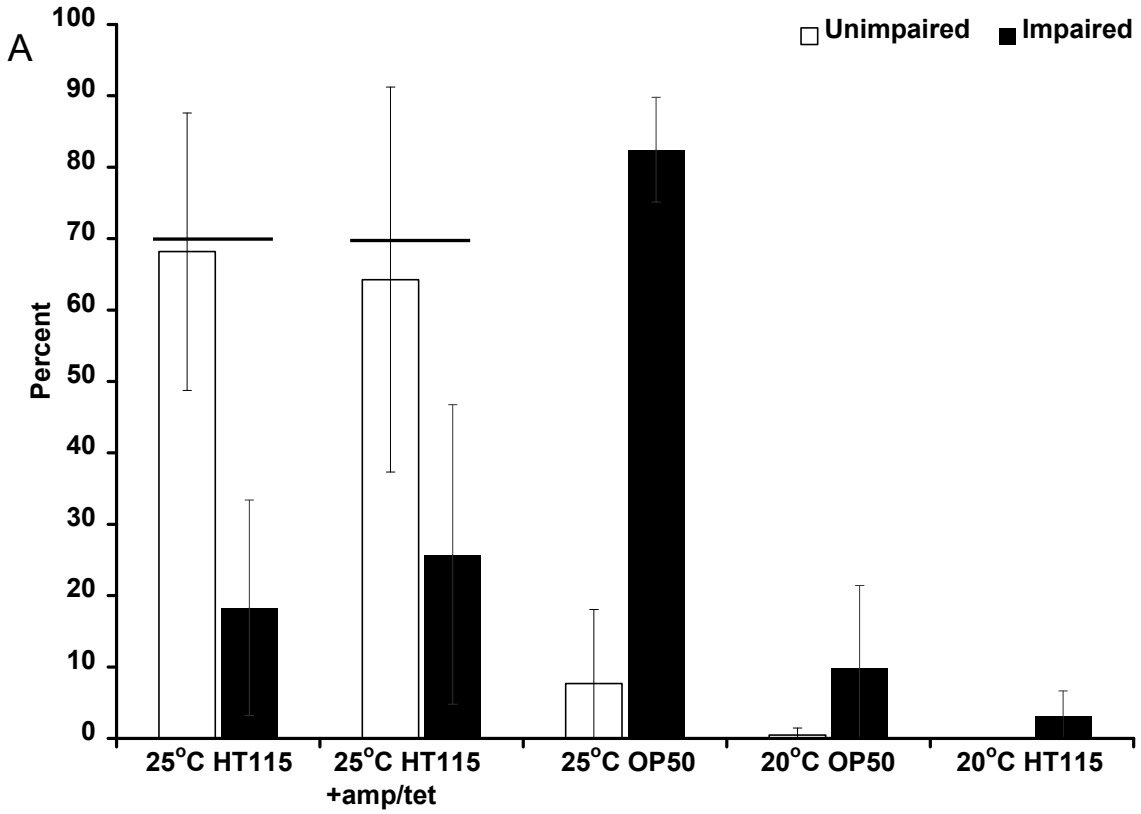
food source, and is typically grown on various strains of uracil auxotroph *E.Coli* bacteria.[31] For standard nematode growth media plates, *E.Coli* strain OP50 is used, and the majority of work done outside of reverse genetics and liquid cultures (used for growing large masses of worms for biochemical assays) is done using this strain. The typical transgenic strain used for RNAi by feeding is the *E.coli* strain HT115, which contains the high-copy L440 plasmid, containing a gene for β-lactamase, and multiple cloning site under the control of a *lac* promoter. The bacteria itself also has a tetracycline resistance gene inserted into its RNAase III gene, which, when selected for by tetracycline, hinders the degradation of the double stranded RNA produced by the L440 plasmid in the presence of IPTG.[99] Until recently, OP50 and HT115 strain bacteria were thought of as interchangeable food sources for the worm.

While in the process of further characterizing the genes involved in temperature mediated anoxia survival, it was discovered that by growing the animals on control RNAi plates (HT115 bacteria with the L440 vector on nematode growth media with ampicillin, tetracycline, and IPTG added) at 25^{o}C for development, that led to an increase in the quality of survivorship (defined below). While N2's grown on standard OP50 plates were able to survive, the quality of survivorship was noticeably less than organisms such as *daf-2(e1370)* (Figure 9). To describe the difference in the quality of survivorship observed between nematodes fed the two food sources used during 25^{o}C development, a binary quantification system was developed. In the classification system

"unimpaired" is described as a nematode with no visible defects in motility or morphology using a standard low-power bench top stereoscope (25X magnification). Anything that does not fit into the "unimpaired" category, but is not dead is described as "impaired".

As described in the previous paragraph, RNAi control food plates have ampicillin, tetracycline, and IPTG added in addition to the plates that are used to grow *C. elegans* . To determine which of the components of the RNAi media were responsible for the anoxia viability phenotype, NGM media for the assay either containing antibiotics or no antibiotics, but seeded with *E. coli* HT115 bacteria as a food source for the nematodes were used. NGM media without antibiotic supplementation and seeded with *E. coli* OP50 as a food source were used as controls. (Figure 9; Table 2). It is also important to note that all of these experiments involve animals that are raised at 25^{o}C and shifted to 20^{o}C for the anoxia treatment. RNAi control media does not produce an observable benefit to anoxia survival when temperature is maintained at 20^{o}C.

Animals raised at 25^{o}C on media seeded with HT115 bacteria had an increased percentage of unimpaired survivors compared to nematodes raised where OP50 bacteria was used as a food source when exposed to three days of 20^{o}C anoxia (Figure 9 A). In four days of 20^{o}C anoxia any of the organism raised at 25^{o}C on HT115 cultures were statistically similar in terms of overall survivorship, and percentage of unimpaired survivors. All other groups were not different statistically from animals raised at 20^{o}C on (Figure 9 B; Table 2).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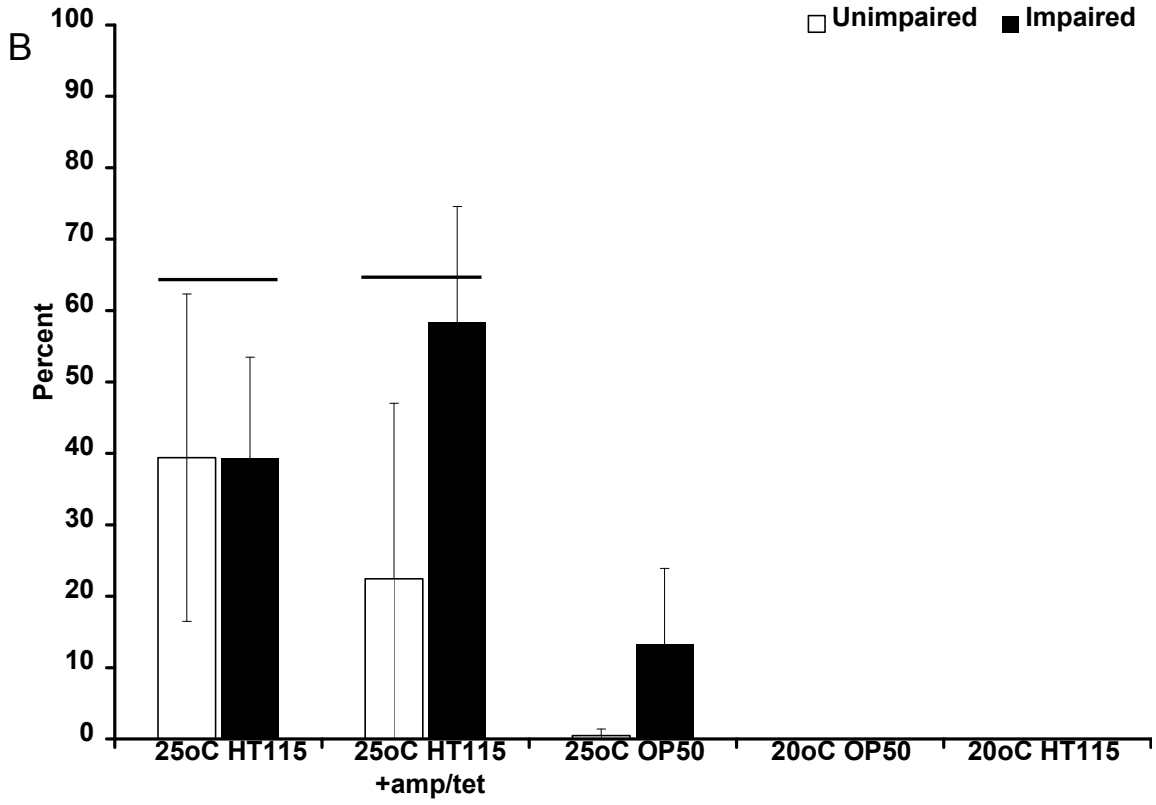
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20oC OP50
20oC HT115

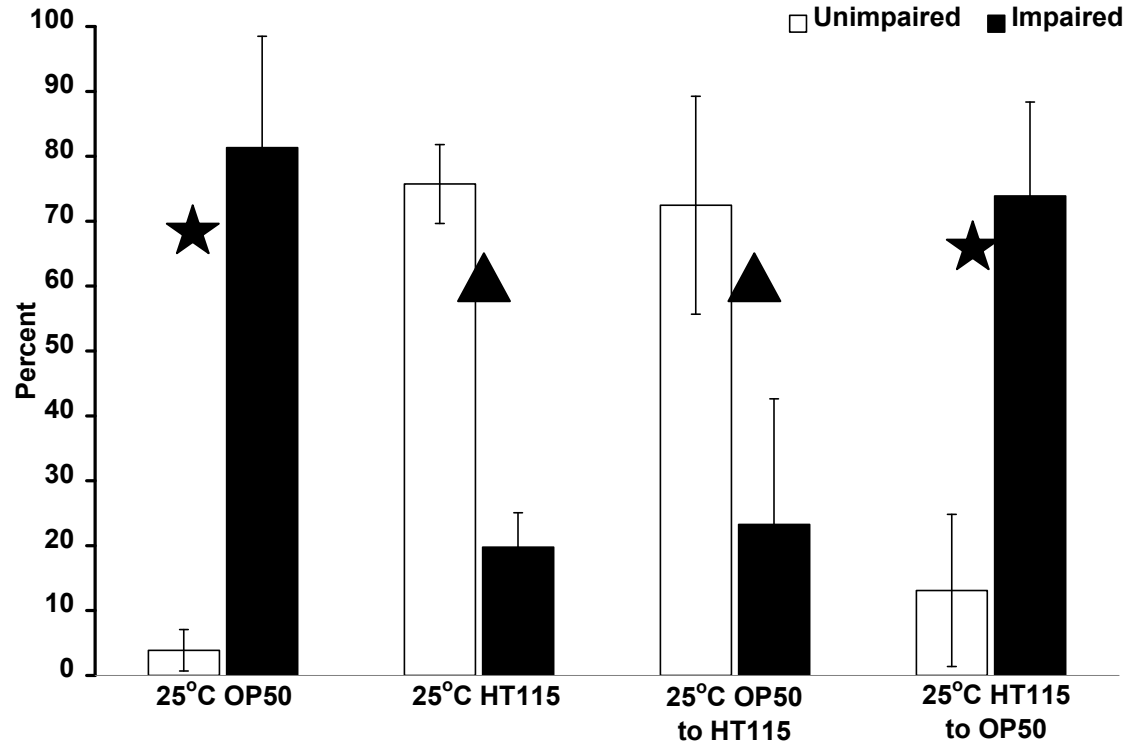
Figure 9. Long-term 20°C anoxia survival rates of *C. elegans* grown on HT115 vs. OP50 *E. coli* at 25°C and 20°C. A) 3 day 20°C anoxia survival rates of wild type animals under a variety of 25°C preconditioning regimens. Impaired is defined as any visible behavioral or morphological defect from normoxia; stars and diamonds denote statistically significant groups (α=.05 SNK multiple range test), and triangle denotes significance from 20°C control ($p < .05$ Student's t-test; $n > 180$ over four independent trials for all treatments. Error bars indicate standard deviation). B) 4 day 20°C anoxia survival rates of wild type animals under a variety of 25°C preconditioning regimens; lines denote statistically significant groups (α=.05 SNK multiple range test; $n > 175$ over four independent trials for all treatments. Error bars indicate standard deviation).

After evaluating these results, it seemed to us that the bacterial strain used for a food source is important for an improved survival rate and improved quality of survivorship. To determine if it was a pre-conditioning advantage, or if it was an effect the bacteria were having while in long-term anoxia, I designed an experiment to raise animals on one food source and then transfer the animals to the other food source prior to long-term anoxia exposure. Animals maintained on either HT115 and OP50 were placed into anoxia on their original food source were used as controls. Animals maintained at 25°C on OP50 and switched to HT115 prior to exposure to three days of 20°C anoxia had a similar percentage of unimpaired survivors compared to nematodes maintained on HT115 bacteria throughout the experiment (HT115 controls). Conversely, animals placed into 3 days of 20°C anoxia on OP50 that had been raised at 25°C on HT115 had a reduced percentage of unimpaired survivors compared to animals that were on HT115 bacteria while they were in anoxia and were more similar in their

percentage of unimpaired survivors to animals maintained on OP50 bacteria for the entire experiment (Figure 10 A).

When exposed to four days of 20°C anoxia, the percentage of unimpaired survivors of both of the two "food switching" regimens were significantly different from animals maintained on HT115 for the entire experiment. All three of the groups that were exposed to HT115 at any point in the experiment had an increase in overall survival rate compared to those maintained only on OP50 (Figure 10 B).

A



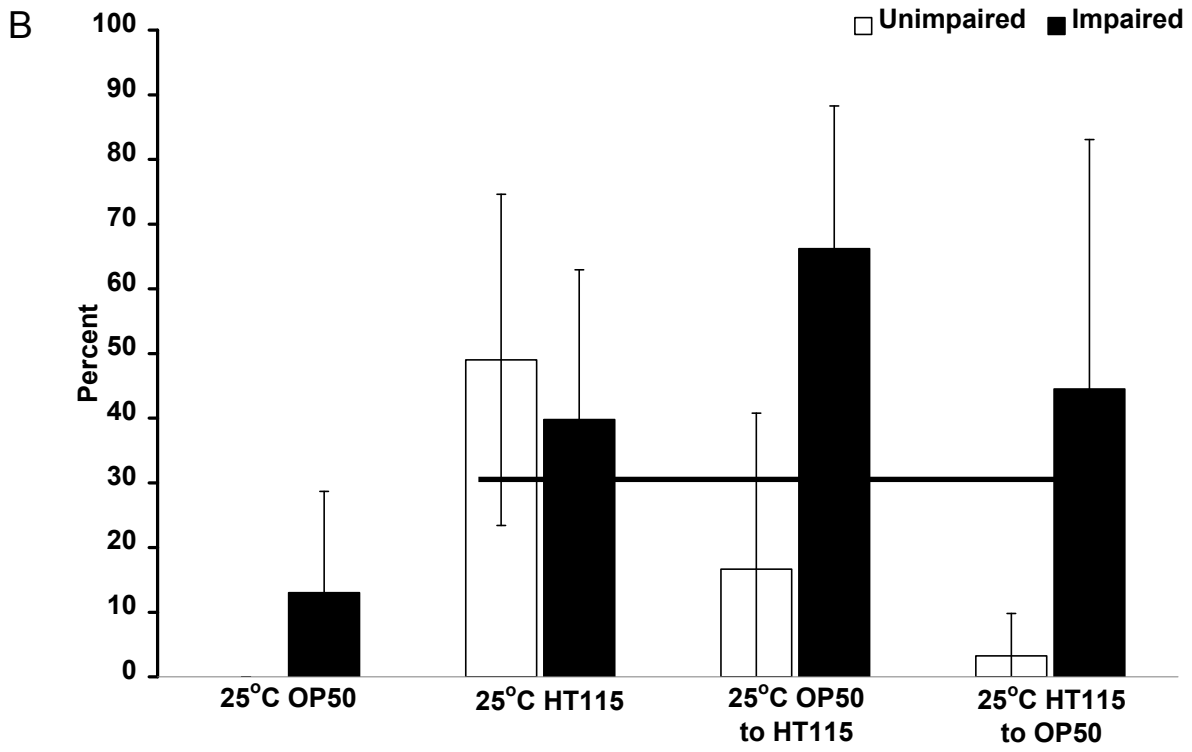


Figure 10. Effects of altering food type of nematodes raised at 25°C and exposed to 20°C long-term anoxia. A) 3 day 20°C anoxia survival rates of wild type animals that were switched from either OP50 to HT115 bacteria or from HT115 to OP50 just prior to anoxia treatments. Siblings placed into anoxia on their original bacterial strain were used as controls. Impaired is defined as any visible behavioral or morphological defect from normoxia. Stars and triangles denote statistically significant groups (α=.05 SNK multiple range test; n > 180 over four independent trials for all treatments. Error bars indicate standard deviation). B) 4 day 20°C anoxia survival rates of wild type animals that were preconditioned in the same manner as in A); line denotes statistically significant groups in total survivorship (α=.05 SNK multiple range test; n > 180 over four independent trials for all treatments. Error bars indicate standard deviation).

Table 2. Survival rate of environmentally preconditioned wild type animals exposed to anoxia

Pre-Anoxia Exposure Environment			
Temperature (°C)	*E. coli* Strain (antibiotics)	Anoxia Exposure (days)	Survival Rate ± SD
20	OP50	3	10.4 ±12.0
25	OP50	3	90.1 ±8.6 [a]
20	HT115	3	3.1 ± 3.6
25	HT115	3	86.5 ±6.8 [b]
25	HT115 (Amp, Tet)	3	90.0 ±6.3 [b]
20	OP50	4	0.0 ±0.0
25	OP50	4	13.7 ±10.0
20	HT115	4	0.0 ±0.0
25	HT115	4	78.8 ±20.1 [b]
25	HT115 (Amp, Tet)	4	80.8 ±8.1 [b]
25	OP50	3	85.2 ±17.7
25	HT115	3	95.5 ±1.1
25	OP50 to HT115	3	95.7 ±4.1
25	HT115 to OP50	3	87.0 ±11.4
25	OP50	4	13.0 ±15.4
25	HT115	4	88.8 ±9.9 [c]
25	OP50 to HT115	4	82.8 ±11.5 [c]
25	HT115 to OP50	4	47.8 ±41.1

Survival rates for data presented in Figures 9 and 10
For all experiments N2 strain was used
[a] P<.05 in comparison to animals grown on OP50 at 20°C; identical anoxia exposure.
[b] P<.05 in comparison to animals grown on HT115 at 20°C; identical anoxia exposure.
[c] P<.05 in comparison to animals grown only on OP50 at 25°C; identical anoxia exposure.

CHAPTER 4

GENETIC SUPRESSORS OF ENVIRONMENTAL AND GENETIC ENHANCERS OF ANOXIA SURVIVAL IN *Caenorhabditis elegans* [34]

Initial Observations of the Suppression of the Enhanced Anoxia Phenotype in *daf-2(e1370)* Animals

In chapter 2, I discussed a collaborative work that I was involved with regarding the enhanced anoxia survival of the *daf-2(e1370)* mutant. In addition to characterizing the ability of *daf-2(e1370)* to survive prolonged anoxia, we also investigated ways to suppress the enhanced anoxia phenotype. Once again this material was included in our 2006 publication in genetics. [90]

DAF-16 is the known and well characterized transcription factor that increases stress resistance and lifespan in situations where I/IGF-like signaling is altered. It is known to upregulate genes involved with glucose transport, heat-shock proteins, detoxification enzymes, and glycolysis to name a few. We started looking for suppressors of *daf-2(e1370)* mediated anoxia survival by looking at *daf-2(e1370);daf-16(m26)* double mutant animals. *daf-2(e1370); daf-16(m26)* animals survived four day 20^{o}C anoxia at a reduced rate (26.7%

[3] Portions of this chapter have been published with permission of the Genetics Society of America.
[90] Mendenhall AR, Larue B, Padilla PA. Glyceraldehyde-3-phosphate dehydrogenase mediates anoxia response and survival in Caenorhabditis elegans. Genetics 2006(Journal Article).
[4] Portions of this chapter are in review

±27.2%; n=208 animals, 4 independent experiments; p< .05 Student's independent t-test) compared to *daf-2(e1370)* controls (91.6±9.4%). [90] We attributed the high variability of suppression to the weak loss of function allele daf-16(m26) (L.Osherovich personal communication).

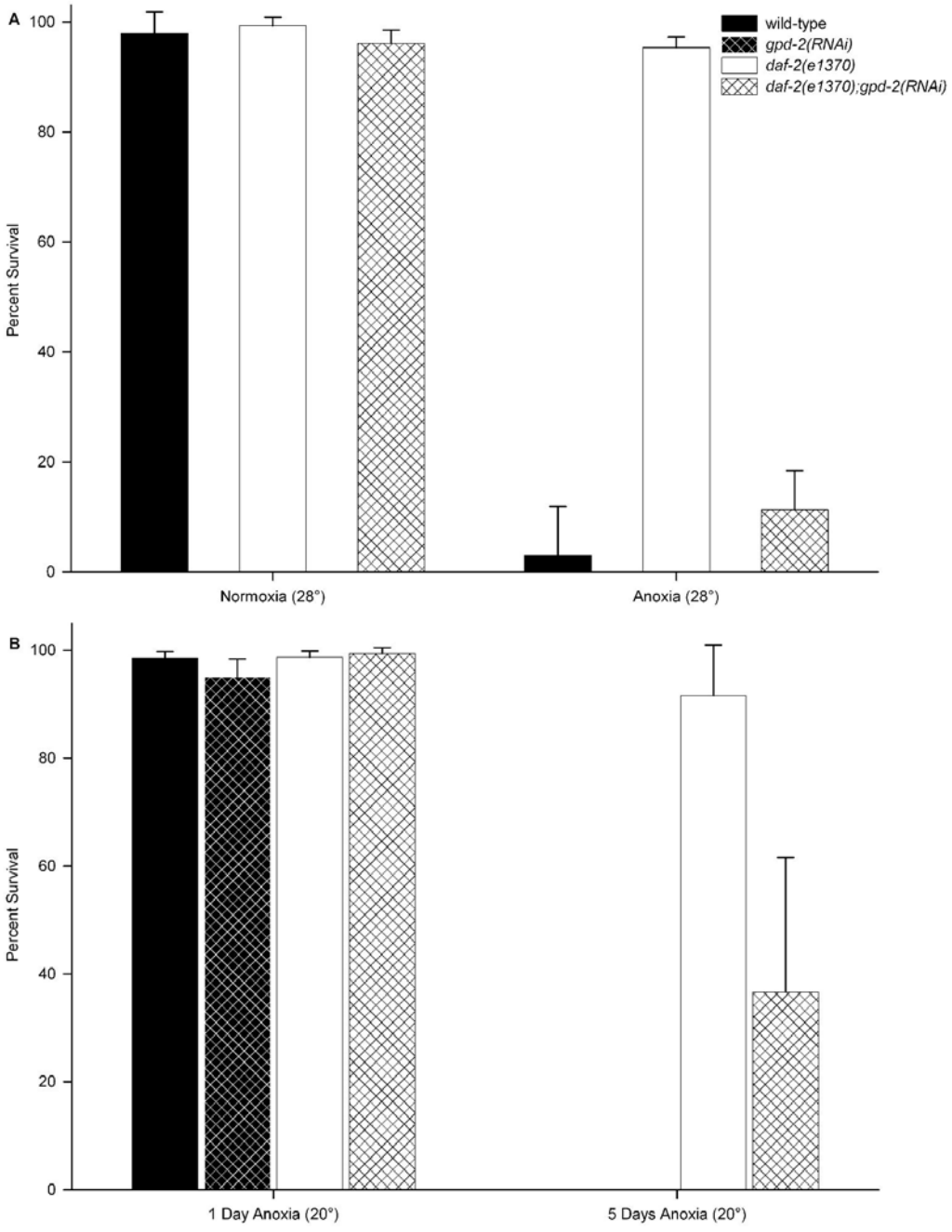


Figure 11. The survival rate of *gpd-2/3*(RNAi) and *daf-2(e1370);gpd-2/3*(RNAi) adult animals exposed to anoxia. (A) Wild type, *daf-2(e1370)* and *daf-2(e1370)*;*gpd-2/3*(RNAi) adult hermaphrodites were exposed to 1 day of normoxia or anoxia at 28°C. (B) Wild type, *daf-2(e1370), gpd-2/3(RNAi)* and *daf-2(e1370);gpd-2/3*(RNAi) adult hermaphrodites were exposed to 1 or 5 days of anoxia at 20°C. There is a significant difference between *daf-2(e1370)* and *daf-2(e1370);gpd-2/3*(RNAi) adult hermaphrodites exposed to 5 days of anoxia ($p < .001$). For A and B the data shown are representative of at least five independent experiments with a total of approximately 50 animals for each independent experiment. Error bars represent standard deviation. [90]

To determine if specific genes regulated by DAF-16 are required for the aforementioned long-term anoxia and high-temperature (28^{o}C) anoxia phenotype observed in *daf-2(e1370)* animals, we used RNAi to knockdown the function of the 40 genes that have been shown to be upregulated in *daf-2(e1370)* animals by DAF-16. Out of those 40 genes only a *C. elegans* isoform of GAPDH, *gpd-2/3*, was significantly effective at suppressing the anoxia survival phenotypes of *daf-2(e1370)* mutant animals. *daf-2(e1370);gpd-2/3(RNAi)* animals had a reduced ability to survive long-term 20^{o}C anoxia or 28^{o}C anoxia compared to *daf-2(e1370)* animals (Figure 11 A and B). [90]

In our initial observations of wild type worms exposed to anoxia stress, we observed tissue abnormalities and aberrations of pharyngeal structure upon recovery in survivors of both long-term and high-temperature (28^{o}C) anoxia. We wanted to determine if *gpd-2/3* function was necessary for the preserved tissue structure of *daf-2(e1370)* mutant animals. We examined surviving *daf-2(e1370);gpd-2/3(RNAi)* animals following long-term 20^{o}C anoxia and 28^{o}C anoxia. The tissue abnormalities seen in wild type worms exposed to anoxia and

absent from *daf-2(e1370)* animals were present in *daf-2(e1370)*;*gpd-2/3(RNAi)* animals. Additionally, wild type animals were unable to recover tissue morphology following shorter bouts (24 hours) of anoxia when *gpd-2/3* function was reduced via RNAi (Figure 12).[90]

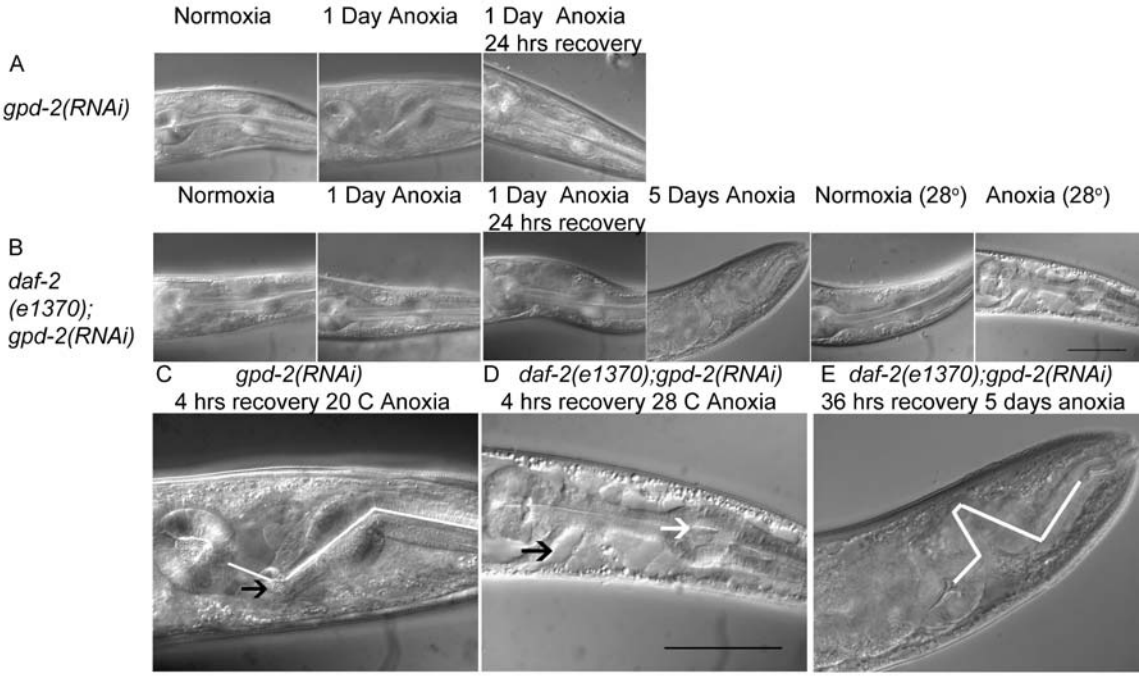


Figure 12. The tissue morphology of the *gpd-2/3*(RNAi) and *daf-2(e1370);gpd-2/3*(RNAi) adult animals exposed to long-term anoxia and high-temperature anoxia. The anterior head region of *gpd-2/3*(RNAi) and *daf-2(e1370);gpd-2/3*(RNAi) adult animals were examined using DIC microscopy. (A) *gpd-2/3*(RNAi) animals were exposed to normoxia or 1 day of anoxia at 20°C and allowed to recover in air for 4 hrs or 24 hrs (as indicated). (B) *daf-2(e1370);gpd-2/3*(RNAi) animals were exposed to normoxia (20°C), 1 day of anoxia (20°C), 5 days of anoxia (20°C), 1 day of normoxia (28°C) or 1 day of anoxia (28°C). Animals exposed to anoxia were allowed to recover in air for 4 hrs or 24 hrs of anoxia (as indicated) before analysis. (C) An enlarged image of *gpd-2/3*(RNAi) animals exposed to 1 day of anoxia at 20°C is shown. White line is drawn along the lumen and an arrow points to a region in the isthmus that is abnormal. (D) An enlarged image of *daf-2(e1370);gpd-2/3*(RNAi) animals exposed to 1 day of anoxia at 28°C is shown. (E) An enlarged image of *daf-2(e1370);gpd-2/3*(RNAi) animals exposed to 5 day

of anoxia at 20°C then allowed to recover in normoxia for 36 hrs. For C and D the black arrows point to cavities surrounding the pharynx and the white arrow points to cavities within the pharynx tissue. For C and E the white line is drawn along the lumen. The scale bar equals 50μm.[90]

gpd-2/3 RNAi knocks down the levels of two nearly identical genes that are produced in an operon with each other, along with the mitochondrial ATPase inhibitor *mai-1*. RNAi of either *gpd-2* or *gpd-3* would result in a knockdown of the other due to sequence homology. The other two isoforms of GAPDH, *gpd-1/4* are not targeted by the dsRNA used for RNAi of *gpd-2/3*, so the knockdown is specific for *gpd-2/3*. *gpd-1/4* transcripts are detectable, and *gpd-2/3* transcripts are not detectable via RTQ-PCR in *gpd-2/3(RNAi)* animals, so all glycolytic function is not ablated. Also *gpd-2/3(RNAi)* animals are able to survive 24 hours of 20^{o}C anoxia and 28^{o}C normoxia. [90]

Since *gpd-2/3* functions in glycolysis, we were concerned that we were simply reducing glycolytic flow creating “sickly” animals that were energy starved going into anoxia. To investigate this potential problem, we knocked down the levels of glycolytic enzymes for which there was only one predicted isoform in *C. elegans* , which were glucose phosphate isomerase (*gpi-1*), triose phosphate isomerase (*tpi-1*), phosphoglycerate kinase (*pgk-1*), enolase (*enol-1*), and the other isoforms of GAPDH, *gpd-1/4*. Once again, only the knockdown of *gpd-2/3* could reduce the enhanced long-term anoxia phenotype of *daf-2(e1370)*.[90]

As mentioned earlier, *daf-2(e1370)* is a class 2 allele, which means that it has other known phenotypes associated with the allele other than abnormal

dauer formation. So to determine if elevated gpd-2/3 transcript levels correlated with anoxia survival we re-examined all of the *daf-2* alleles from Table 1. We assayed *gpd-2/3* transcript levels via RTQ-PCR. *daf-2(m579)* animals, which had a slightly reduced anoxia survival rate compared to *daf-2(e1370)* animals, had a slightly less increase of transcript gpd-2/3 levels over wild type animals compared to *daf-2(e1370)* animals. As could be expected, the animals carrying either of the two alleles that had no enhanced anoxia phenotype had *gpd-2/3* transcript levels not statistically different from that of wild type animals (Table 3). Thus the evidence supports that there is a correlation between long-term anoxia survival and gpd-2/3 transcript levels.90survival and gpd-2/3 transcript levels.[90]

Our data suggests that there might be an additional role for these particular isoforms of GAPDH. However, it is also possible that *gpd-2/3* is an anoxia/stress specific isoform of GAPDH with *gpd-1/4* being able to compensate for the loss of function via RNAi under normoxic conditions. In that scenario, *gpd-2/3* transcript levels could be attributed to the strength of each allele to localize DAF-16 to the nucleus and thus upregulate all genes under the control of DAF-16.

Table 3. Transcript level of *gpd-2/3* in *daf-2* alleles relative to control. (n=total number of animals for 3 independent trials.)[90]

Strain	Allele Class	28°C 24 hour anoxia survival	20°C 72 hour anoxia survival	Transcript fold increase*
daf-2(e1370)	2	95.2 ± 3.3 (n = 150)	91.6 ± 9.4 (n = 150)	3.36
daf-2(e1371)	1	4.5 ± 5.3 (n = 200)	0.0 ± 0.0 (n = 200)	1.09
daf-2(m596)	2	0.0 ± 0.0 (n = 200)	0.0 ± 0.0 (n = 200)	1.05
daf-2(m579)	2	77.6 ± 17.7 (n = 300)	75.3 ± 32.1 (n = 300)	1.91

Enhanced Anoxia Phenotype of *glp-1(e2141)* and the Role of AMPK in Anoxia Survival

As mentioned earlier germline mutations that extend life span have been shown to function in a DAF-16 dependent manner. Colleagues in my lab have demonstrated that a reduction in oocyte maturation and ovulation will increase anoxia survival [100] and to some extent this is also dependent on *daf-16*. To better understand the mechanisms by which this increased anoxia tolerance occurs, we decided to do a specific suppressor screen using known modulators of dauer formation, longevity and stress resistance to abate this ability in *glp-1* and *daf-2(e1370)* animals.

glp-1 stands for germ-line proliferation mutant, mutations in the loci cause primordial germ cells that would normally undergo mitosis to prematurely enter meiosis and differentiate into sperm cells rather than the distal tip cells that give

rise to the normal germline. [101] Thus, glp-1 mutant animals have a non-functioning germline with only a few sperm cells present. *glp-1* is a member of the notch signaling family and functions through the *let-60 ras* pathway. [102] Loss of *glp-1* function leads to sterility, so to maintain mutant lines one method is to use temperature sensitive alleles, such as *glp-1(e2141).* Also, use of a balanced strain in which a working copy of the gene must be maintained via a an extra chromosomal duplication; for example *glp-1(q158)* is maintained as a duplication in a *dpy-19* background (Dumpy body phenotype). Animals that have lost the duplication, will have a dumpy body shape in addition to being sterile due to the *dpy-19* and *glp-1* mutation.

As previously mentioned, *dpy-19* mutants have a phenotype that consists of a dumpy body type, and have an overall altered morphology. I wanted to be able to compare our current studies of long-term anoxia suppression to wild type worms, so I chose to utilize a temperature sensitive allele *glp-1(e2141)*. *glp-1(e2141)* is mutant animals develop as wild type organisms at 15^{o}C, but are germline deficient when raised or shifted to 25^{o}C. First, however, it was necessary to characterize the ability of this allele to survive 20^{o}C anoxia.

I chose to characterize the survival rates of both *glp-1(e2141)* animals, and *glp-1(e2141);daf-16(mu86).* The *glp-1(e2141);daf-16(mu86)* double mutant animals will allow us to understand if DAF-16 modulates a long-term anoxia survival phenotype displayed by *glp-1(e2141)* animals. Additionally, any subsequent RNAi targets screened for suppression could be screened in the

presence of a *glp-1* mutant background, or for synthetic suppression in a *glp-1;daf-16* mutant background. Due to the temperature sensitive nature of the mutation, it was necessary to raise the animals from L1 larval stage at 15^{o}C for 24 hours, and then shift them to 25^{o}C for approximately 48 hours to reach adulthood. Both *glp-1(e2141)* and *glp-1(e2141);daf-16(mu86)* survive three and four days of 20^{o}C anoxia at very high overall rates and are unimpaired at a higher percentage compared to wild type animals (Figure 13). *glp-1(e2141);daf-16(mu86)* animals show a modest but statistically significant decrease in quality of survivorship at four days of 20^{o}C anoxia compared to *glp-1(e2141)* animals. This is indicative of a partial suppression of the very robust *glp-1(e2141)* unimpaired phenotype following 4 days of 20^{o}C anoxia.

Reduction of DAF-16 function is able to partially suppress the enhanced anoxia survival phenotype of both *glp-1(e2141)* mutant animals and *daf-2(e1370)* animals (pg. 47). As mentioned in the background information, other pathways are known to modulate and be modulated by classic I/IGF signaling. For example germline signaling, members of the TOR pathway, TGF-β pathway, and interactions with other signaling pathways are well described in the literature in both *C. elegans* as well as other model systems. By compiling a list of potential targets (Appendix Table 1)from known genetic modulators of DAF-16/FOXO signaling, I designed a screen to find known suppressors of the long-term anoxia phenotypes of *daf-2(e1370)*, *glp-1(e2141)*and *glp-1(e2141);daf-16(mu86)* animals.

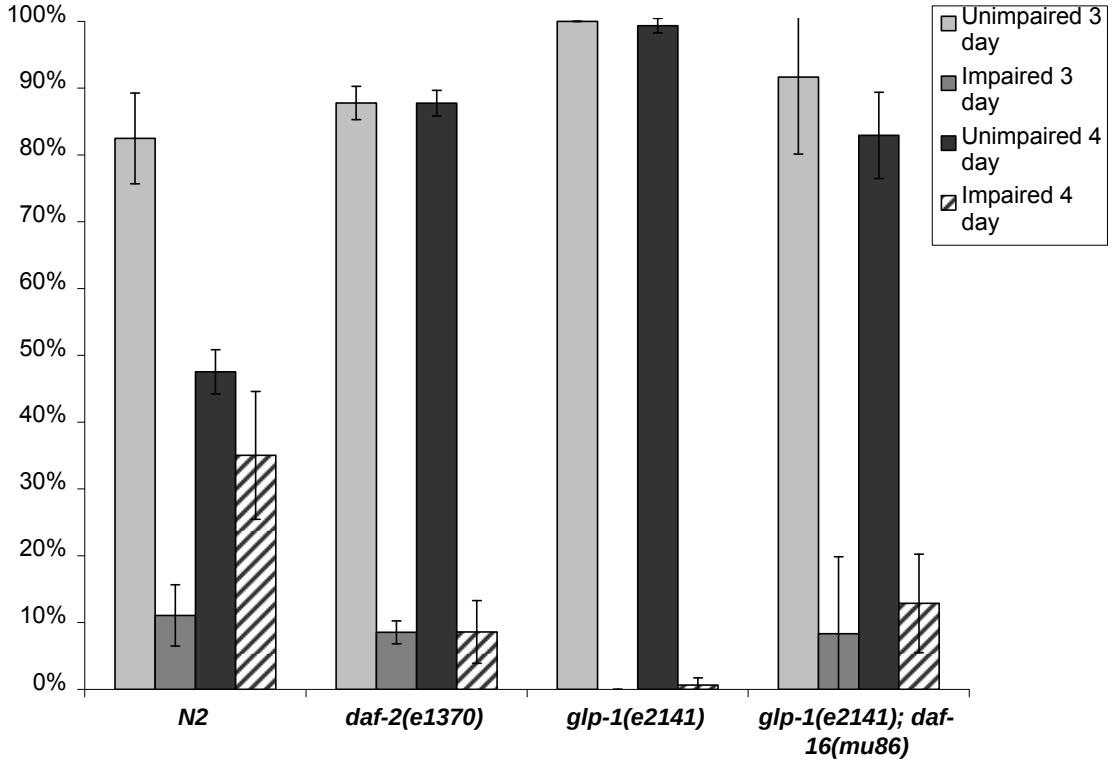


Figure 13. 3 and 4 day 20°C anoxia survival rates of *glp-1(e2141)* mutant animals and *glp-1(e2141);daf-16(mu86)* double mutant animals on *E. coli* OP50 plates. Impaired is defined as any visible behavioral or morphological defect from normoxia; Line denotes statistical significance between *glp-1(e2141)* and *glp-1(e2141);daf-16(mu86)* in 3 and 4 days of 25°C anoxia ($p < .05$ Student's paired one-tailed t-test ; $n > 180$ over four independent trials for all treatments. Error bars indicate standard deviation).

Overall survival rates, and the previously mentioned binary quality of survivorship system (unimpaired versus impaired) will be used to describe any visible defects noted in survivors as described in Chapter 3.

Out of the targets screened, one gene, *aak-2,* was identified as a suppressor of the long-term anoxia phenotype of *daf-2(e1370)*, *glp-1(e2141)*

animals, and environmentally pre-conditioned wild type worms that were used as controls for animals containing the *glp-1(e2141)* mutation. As previously mentioned, AAK-2 is the catalytic subunit of AMPK, and is one of two homologs of this gene in *C. elegans* . Also, as discussed in chapter 1 the role of AAK-2 includes modulation of DAF-16 mediated effects associated with longevity and stress resistance. [75-79]

Using *aak-2* as a lead, I then screened through the genes that encode for the predicted AMPK subunit isoforms that were available in our RNAi library to determine the role of AMPK in the survival of long-term anoxia. I was able to screen through eight of the nine identified AMPK subunit isoforms in the *C. elegans* genome. I used either animals with mutant alleles, or RNAi of the various subunits to screen for the suppression of the enhanced long-term anoxia survival phenotype of environmentally preconditioned, *daf-2(e1370)* mutant, and *glp-1(e2141)* mutant animals.

As mentioned in chapter 2, animals that were raised on HT115 *E. coli* bacteria at 25^{o}C have an decrease in mortality and morbidity associated with long-term anoxia at 20^{o}C. When AMPK function was reduced either by the alleles *aak-2(rr48)*, or *aak-2(gt33)*, or RNAi knockdown of *aak-2*, the increase in the amount of “unimpaired” survivors was suppressed compared to wild type animals grown on HT115 bacteria with an empty RNAi (L440) plasmid as a food source when exposed to three days of 20^{o}C anoxia. Inanimals exposed to four days of anoxia the quality of survivorship and overall survivorship was reduced

compared to wild type animals on HT115 food. (Figure 14 and Table 4).

Reduction of function of one of the gamma subunits of AMPK, *aakg-2*, via RNAi caused a suppression of the enhanced anoxia survival phenotype of environmentally preconditioned animals exposed to four days of 20^{o}C anoxia, with a decrease in overall survival, as well as the number of impaired survivors. RNAi knockdown of the other subunits did not have any effect on mortality or morbidity that was significantly different from animals grown on HT115 control media (Figure 14 and Table 4).

RNAi knockdown of either of the two beta subunits of AMPK, *aakb-1* and *aakb-2*, individually did not cause a loss of the enhanced long-term anoxia phenotype associated with environmentally preconditioned animals. However, in a double-RNAi knockdown of both *aakb-1* and *aakb-2*, there was a significant reduction in the amount of unimpaired survivors of three day 20^{o}C anoxia (Figure 14 A). Environmentally preconditioned *aakb-1(RNAi);aakb-2(RNAi)* animals also had a decrease in the number of overall and unimpaired survivors when exposed to four days of 20^{o}C anoxia (Figure 14 B, Table 4).

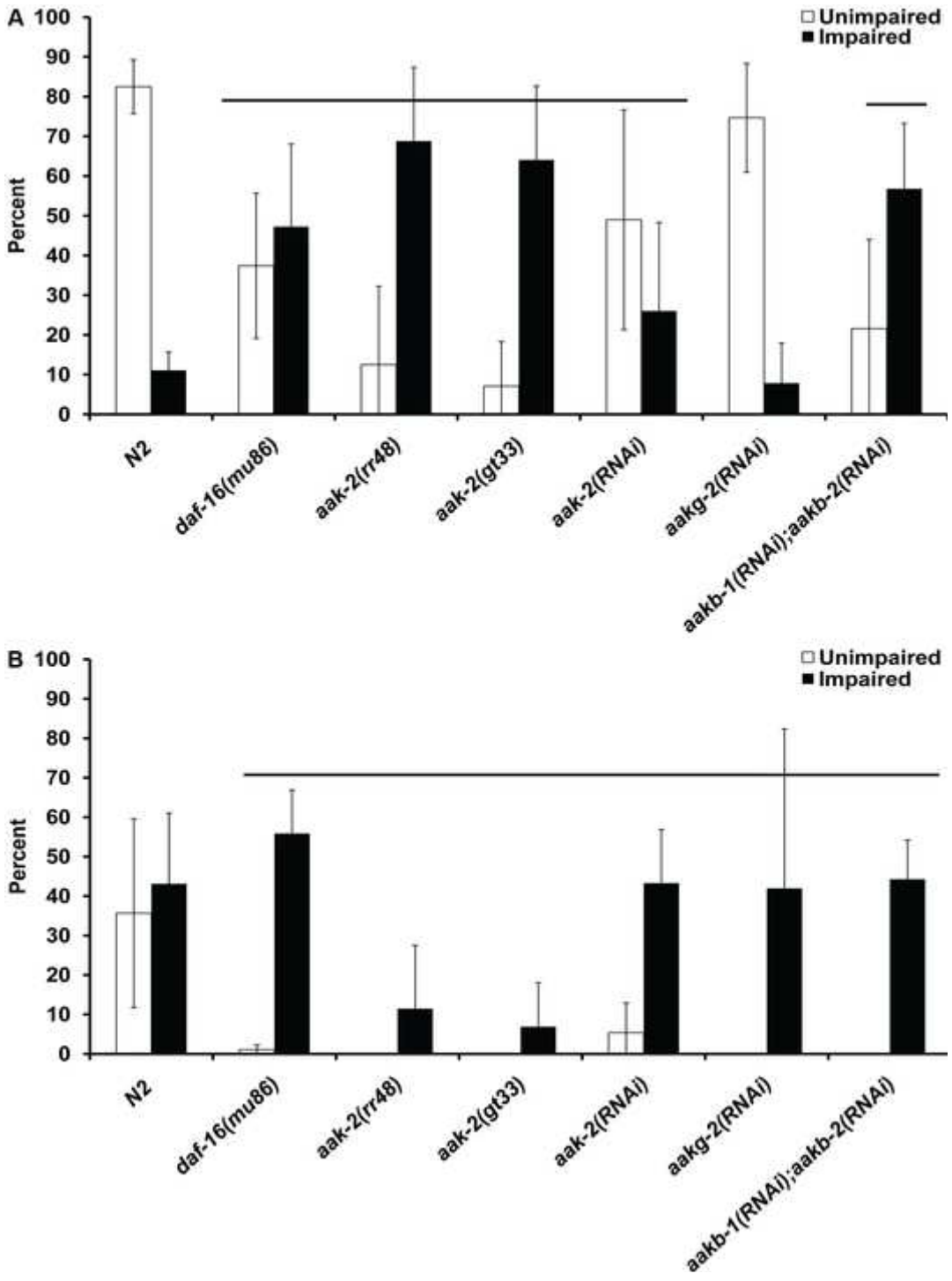

A
B
Percent
Unimpaired
Impaired
0
10
20
30
40
50
60
70
80
90
100
N2
daf-16(mu86)
aak-2(rr48)
aak-2(gt33)
aak-2(RNAi)
aakg-2(RNAi)
aakb-1(RNAi);aakb-2(RNAi)

Figure 14. Reduction in AMPK or daf-16 function suppresses the long-term anoxia survival preconditioning induced by temperature and *E. coli* food source. Adult hermaphrodites, of specified genotype or RNAi experiment, were raised at 25°C (HT115 *E. coli* food source) and exposed to either three days (A) or four days (B) of anoxia; survivors were examined for an unimpaired or impaired phenotype. Line denotes groups with a significant decrease in the number of animals with an unimpaired phenotype in comparison to N2 control (p<. 05, Dunnett's Multiple Range test) (A). The AMPK mutants exposed to four days of anoxia had a significant decrease in survival rate in comparison to control (B). Line denotes groups with a significant decrease in the number of animals with an unimpaired phenotype in comparison to N2 control (p<. 05, Dunnett's Multiple Range test). For all experiments, the total number of animals assayed is N>180 from four independent experiments; error bar represents standard deviation.

daf-2(e1370) animals as previously described both earlier in this chapter and in chapter 1 as having a very robust enhanced anoxia survival phenotype. This has been shown to be dependent on *gpd-2/3* [90]and *daf-16*. I screened through the available known AMPK subunit isoforms to see if they were capable of suppressing the enhanced anoxia survival phenotype of *daf-2(e1370)* animals.

Table 4. Suppression analysis of environmentally preconditioned animals

Genotype	Anoxia Exposure (days)	Survival Rate ± SD
N2	3	93.6 ±3.9
daf-16(mu86)	3	84.6 ±39.1
aak-2(rr48)	3	81.3 ±38.4
aak-2(gt33)	3	71.2 ±29.8
aak-2(RNAi)	3	75.0 ±13.4
aak-1(RNAi)	3	95.5 ±5.2
aakb-1(RNAi)	3	97.3 ±1.4
aakb-2(RNAi)	3	98.6 ±1.2
aakg-1(RNAi)	3	89.6 ±9.8
aakg-2(RNAi)	3	82.5 ±4.9
aakg-4(RNAi)	3	93.5 ±8.1
aakg-5(RNAi)	3	98.7 ±1.1
aakb-1(RNAi);aakb-2(RNAi)	3	78.4 ±22.4
daf-16(mu86);aak-2(RNAi)	3	48.9 ±31.7
N2	4	78.8 ±10.6
daf-16(mu86)	4	56.9 ±12.2
aak-2(rr48)	4	11.5 ±16.1 [a]
aak-2(gt33)	4	6.9 ±11.2 [a]
aak-2(RNAi)	4	48.7 ±16.2
aak-1(RNAi)	4	90.1 ±8.6
aakb-1(RNAi)	4	95.2 ±1.0
aakb-2(RNAi)	4	90. ±6.4
aakg-1(RNAi)	4	88.1 ±6.3
aakg-2(RNAi)	4	41.9 ±40.4 [a]
aakg-4(RNAi)	4	94.8 ±1.6
aakg-5(RNAi)	4	98.1 ±.13
aakb-1(RNAi);aakb-2(RNAi)	4	44.3 ±10.0 [a]
daf-16(mu86);aak-2(RNAi)	4	16.5 ±16.5 [a]

Survival rates for data presented in Figure 14
For all experiments the *E. coli* food source was HT115 and NGM was supplemented with ampicillin and tetracycline
[a] P<.05 in comparison to wild type animals and identical anoxia exposure.

In a fashion similar to environmentally preconditioned animals, *daf-2(e1370);aak-2(RNAi)* animals exposed to three days of 20°C anoxia had a significant reduction in the number of unimpaired survivors compared to *daf-2(e1370)* animals grown on HT115 food. *daf-2(e1370)*;*aak-2(RNAi)*, and *daf-2(e1370);aakb-1(RNAi);aakb-2(RNAi)* animals had a reduction in the number of unimpaired survivors when exposed to four days of 20°C anoxia compared to *daf-2(e1370)* animals grown on HT115 control media (Figure 15). *daf-2(e1370)*;*aak-2(RNAi)* animals exposed to four days of 20°C anoxia also had a reduction in overall survivorship compared to *daf-2(e1370)* on control media (Table 5)

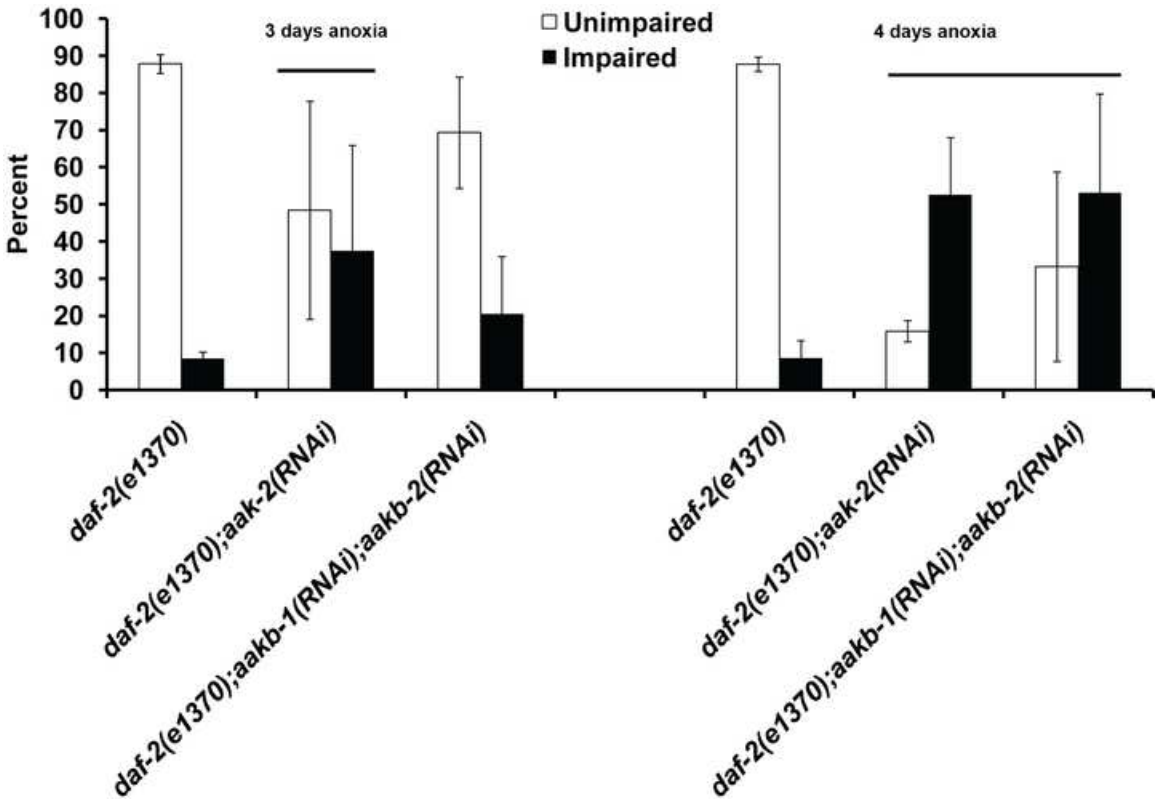


Figure 15. Suppression of *daf-2(e1370)* enhanced long-term anoxia phenotype. Adult hermaphrodites of the given genotype were fed HT115 *E. coli* strain or specified RNAi food and exposed to either three days (A) or four days of anoxia (B). The survivors were examined for an unimpaired or impaired phenotype. Line denotes groups with a significant decrease in the number of animals with an unimpaired phenotype in comparison to *daf-2(e1370)* ($p<.05$ Student's paired one tailed-t-test). For all experiments, the total number of animals assayed is $N>180$ from four independent experiments; error bar represents standard deviation.

Suppression of the Enhanced Long-Term Anoxia Phenotype of Germ-Line Deficient Mutant Animals by Loss of AMPK Function in a DAF-16 Dependent Fashion

It has been demonstrated by others in our lab,[100] and discussed earlier in this chapter that germ-line mutations can enhance long-term anoxia survival. I wanted to determine if AMPK played a role in the enhanced long-term anoxia phenotype of the *glp-1(e2141)* germ-line mutant animals. To determine the nature of any role that AMPK could play, I knocked down the function of the AMPK subunit isoforms available via RNAi in *glp-1(e2141)* animals, and exposed them to long-term anoxia. RNAi knockdown in the *glp-1(e2141)* background had no significant effect on the morbidity or mortality of *glp-1(e2141)* animals compared to animals raised on HT115 controls in three days of 20^{o}C anoxia. In four days of 20^{o}C anoxia, there was a modest, but significant, decrease in the number of unimpaired survivors compared to *glp-1(e2141)* animals grown on control media (Figure 16 and Table 6).

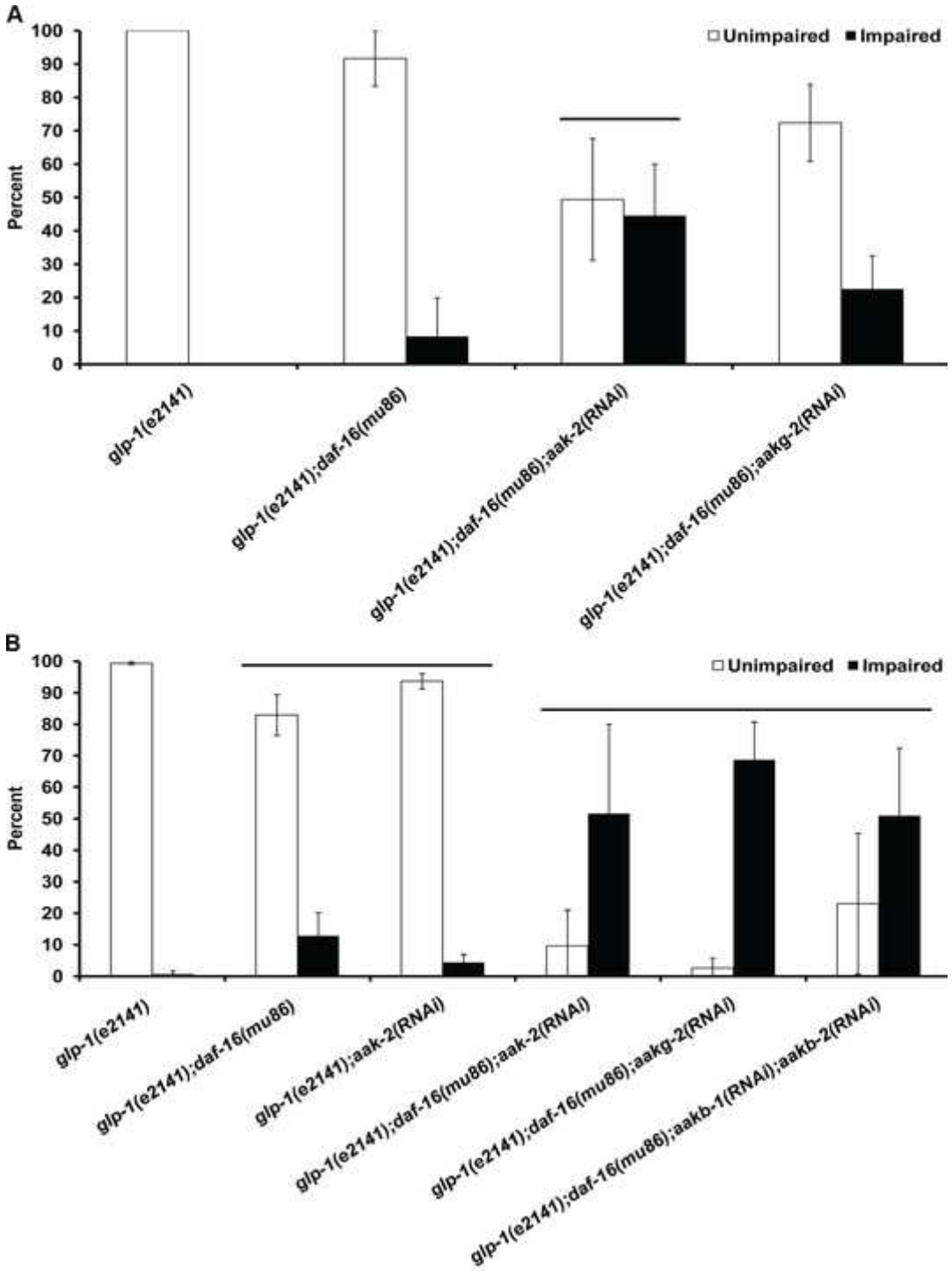

A
Unimpaired
Impaired
Percent
100
90
80
70
60
50
40
30
20
10
0
glp-1(e2141)
glp-1(e2141);daf-16(mu86)
glp-1(e2141);daf-16(mu86);aak-2(RNAi)
glp-1(e2141);daf-16(mu86);aakg-2(RNAi)
B
Unimpaired
Impaired
Percent
100
90
80
70
60
50
40
30
20
10
0
glp-1(e2141)
glp-1(e2141);daf-16(mu86)
glp-1(e2141);aak-2(RNAi)
glp-1(e2141);daf-16(mu86);aak-2(RNAi)
glp-1(e2141);daf-16(mu86);aakg-2(RNAi)
glp-1(e2141);daf-16(mu86);aakb-1(RNAi);aakb-2(RNAi)

Figure 16.
Suppression of *glp-1(e2141)* enhanced long-term anoxia phenotype. Adult hermaphrodites of the given genotype were fed HT115 *E. coli* strain or specified RNAi food and exposed to either three days (A) or four days of anoxia (B). The survivors were examined for an unimpaired or impaired phenotype. Line denotes groups with a significant decrease in the number of animals with an unimpaired phenotype in comparison to *glp-1(e2141)* ($p<.05$ Student's paired one tailed-t-test). For all experiments, the total number of animals assayed is $N>180$ from four independent experiments; error bar represents standard deviation

Table 5. Suppression analysis of long-term anoxia survival in *daf-2(e1370)* animals

Genotype	Anoxia Exposure (days)	Survival Rate ± SD
daf-2(e1370)	3	96.3 ±2.4
daf-2(e1370);aak-1(RNAi)	3	96.1 ±3.1
daf-2(e1370);aak-2(RNAi)	3	85.9 ±10.6
daf-2(e1370);aakb-1(RNAi)	3	97.9 ±2.9
daf-2(e1370);aakb-2(RNAi)	3	98.4 ±1.1
daf-2(e1370);aakg-1(RNAi)	3	98.9 ±2.1
daf-2(e1370);aakg-2(RNAi)	3	97.7 ±3.4
daf-2(e1370);aakg-4(RNAi)	3	98.4 ±1.1
daf-2(e1370);aakg-5(RNAi)	3	100.0 ±0.0
daf-2(e1370);aakb-1(RNAi);aakb-2(RNAi)	3	89.9 ±4.3
daf-2(e1370)	4	96.4 ±3.9
daf-2(e1370),aak-1(RNAi)	4	94.8 ±4.7
daf-2(e1370);aak-2(RNAi)	4	68.5 ±15.5 [a]
daf-2(e1370);aakb-1(RNAi)	4	99.3 ±1.3
daf-2(e1370);aakb-2(RNAi)	4	100.0 ±0.0
daf-2(e1370);aakg-1(RNAi)	4	95.0 ±5.3
daf-2(e1370),aakg-2(RNAi)	4	100.0 ±0.0
daf-2(e1370);aakg-4(RNAi)	4	97.9 ±2.0
daf-2(e1370);aakg-5(RNAi)	4	99.3 ±1.1
daf-2(e1370),aakb-1(RNAi);aakb-2(RNAi)	4	86.3 ±10.3

Survival rates for data presented in Figure 15
For all experiments the *E. coli* food source was HT115 and NGM was supplemented with ampicillin and tetracycline
Animals were grown at 20°C due to *daf-2(e1370)* dauer constitutive phenotype.
[a] P<.05 in comparison to *daf-2(e1370)* animals exposed to identical anoxic conditions.

In addition to screening for suppressors in a *glp-1(e2141)* background which has a very robust long-term anoxia survival phenotype, I wanted to determine if there were any DAF-16 independent components of the *glp-1(e2141)* long-term anoxia phenotype that could be suppressed by altering AMPK function. To do this, I also performed our AMPK subunit suppressor screen in a *glp-1(e2141);daf-16(mu86)* double mutant background.
When exposed to three days of 20^{o}C anoxia, *glp-1(e2141);daf-16(mu86)*;*aak-2(RNAi)* animals had a reduction in the number of unimpaired animals compared to *glp-1(e2141);daf-16(mu86)* animals grown on control media (Figure 16 A). However in four days of 20^{o}C anoxia knockdown via RNAi of either *aak-2, aakg-2, or aakb-1;aakb-2* double RNAi knockdown caused an significant decrease in overall survivorship as well as in the number of unimpaired survivors when compared to (Figure 16 B, Table 7).

Table 6. Suppression analysis of long-term anoxia survival in *glp-1(e2141)* animals.

Genotype	Anoxia Exposure (days)	Survival Rate ± SD
glp-1(e2141)	3	100.0 ±0.0
glp-1(e2141);aak-1(RNAi)	3	99.5 ±.98
glp-1(e2141) aak-2(RNAi)	3	99.0 ±1.9
glp-1(e2141);aakb-1(RNAi)	3	100.0 ±0.0
glp-1(e2141);aakb-2(RNAi)	3	100.0 ±0.0
glp-1(e2141);aakg-1(RNAi)	3	98.7 ±1.2
glp-1(e2141);aakg-2(RNAi)	3	98.5 ±1.8
glp-1(e2141) ;aakg-4(RNAi)	3	100.0 ±0.0
glp-1(e2141);aakg-5(RNAi)	3	100.0 ±0.0
glp-1(e2141);aakb-1(RNAi);aakb-2(RNAi)	3	98.9 ±2.3
glp-1(e2141)	4	100.0 ±0.0
glp-1(e2141);aak-1(RNAi)	4	100.0 ±0.0
glp-1(e2141) aak-2(RNAi)	4	98.1 ±1.5
glp-1(e2141);aakb-1(RNAi)	4	99.3 ±1.2
glp-1(e2141);aakb-2(RNAi)	4	97.6 ±2.1
glp-1(e2141);aakg-1(RNAi)	4	100.0 ±0.0
glp-1(e2141);aakg-2(RNAi)	4	96.4 ±5.7
glp-1(e2141) ;aakg-4(RNAi)	4	99.2 ±1.4
glp-1(e2141);aakg-5(RNAi)	4	99.7 ±0.7
glp-1(e2141);aakb-1(RNAi);aakb-2(RNAi)	4	98.2 ±3.7

For all experiments the *E. coli* food source was HT115 and NGM was supplemented with ampicillin and tetracycline
Due to the *glp-1* sterile phenotype L1 larvae were grown at 15°C for 24 hours and then transferred to 25°C and allowed to develop to young adults; all controls were grown in an identical manner.

Table 7. Suppression analysis of long-term anoxia survival in *glp-1(e2141);daf-16(mu86)* animals.

Genotype	Anoxia Exposure (days)	Survival Rate ± SD
glp-1(e2141);daf-16(mu86)	3	100.0 ±0.0
glp-1(e2141); daf-16(mu86);aak-1(RNAi)	3	100.0 ±0.0
glp-1(e2141);daf-16(mu86);aak-2(RNAi)	3	93.3 ±3.8
glp-1(e2141);daf-16(mu86);aakb-1(RNAi)	3	97.5 ±.89
glp-1(e2141);daf-16(mu86);aakb-2(RNAi)	3	96.7 ±1.1
glp-1(e2141);daf-16(mu86);aakg-1(RNAi)	3	97.2 ±3.0
glp-1(e2141);daf-16(mu86);aakg-2(RNAi)	3	94.9 ±2.5
glp-1(e2141);daf-16(mu86);aakg-4(RNAi)	3	95.5 ±4.9
glp-1(e2141); daf-16(mu86);aakg-5(RNAi)	3	98.8 ±2.0
glp-1(e2141),daf-16(mu86);aakb-1(RNAi);aakb-2(RNAi)	3	99.5 ±1.1
glp-1(e2141),daf-16(mu86)	4	95.8 ±3.0
glp-1(e2141); daf-16(mu86);aak-1(RNAi)	4	95.0 ±6.4
glp-1(e2141),daf-16(mu86);aak-2(RNAi)	4	61.3 ±9.7 [a]
glp-1(e2141);daf-16(mu86);aakb-1(RNAi)	4	97.1 ±5.0
glp-1(e2141);daf-16(mu86);aakb-2(RNAi)	4	97.4 ±1.0
glp-1(e2141);daf-16(mu86);aakg-1(RNAi)	4	94.3 ±3.7
glp-1(e2141),daf-16(mu86);aakg-2(RNAi)	4	71.3 ±11.2 [a]
glp-1(e2141);daf-16(mu86);aakg-4(RNAi)	4	97.4 ±2.8
glp-1(e2141); daf-16(mu86);aakg-5(RNAi)	4	98.2 ±1.5
glp-1(e2141),daf-16(mu86);aakb-1(RNAi);aakb-2(RNAi)	4	74.0 ±29.9 [a]

Survival rates for data presented in Figure 4
For all experiments the *E. coli* food source was HT115 and NGM was supplemented with ampicillin and tetracycline
Due to the *glp-1* sterile phenotype L1 larvae were grown at 15°C for 24 hours and then transferred to 25°C and allowed to develop to young adults; all controls were grown in an identical manner.
[a]P<.05 in comparison to *glp-1(e2141)* animals exposed to identical anoxic conditions.

Metformin Preconditions for Long-Term Anoxia Survival

In light of the apparent necessity of various AMPK subunits for the enhanced long-term anoxia survival phenotype of environmentally preconditioned, *daf-2(e1370)*, and (in a DAF-16 dependent fashion) *glp-1(e2141)* animals, I hypothesized that activation of AMPK by pharmaceutical means would be sufficient to enhance the long-term anoxia survival phenotype of non-preconditioned wild type animals. Metformin has been shown to indirectly increase AMPK activity in rat hepatocytes,[103] skeletal muscle,[104] and CHO cell culture.[105] It has also been shown to increase lifespan in *C. elegans* in an *aak-2* and *skn-1* dependent fashion.[79]

I exposed animals to 50mM metformin from L1 larval stage to adulthood[79] to determine if metformin had the ability to enhance long-term anoxia survival. Wild type animals raised on OP50 *E. coli* plates that included 50mM metformin at 20^{o}C survived three days of 20^{o}C anoxia at a significantly increased level compared to animals raised on OP50 control plates that did not contain Metformin (Figure 17 and Table 8).

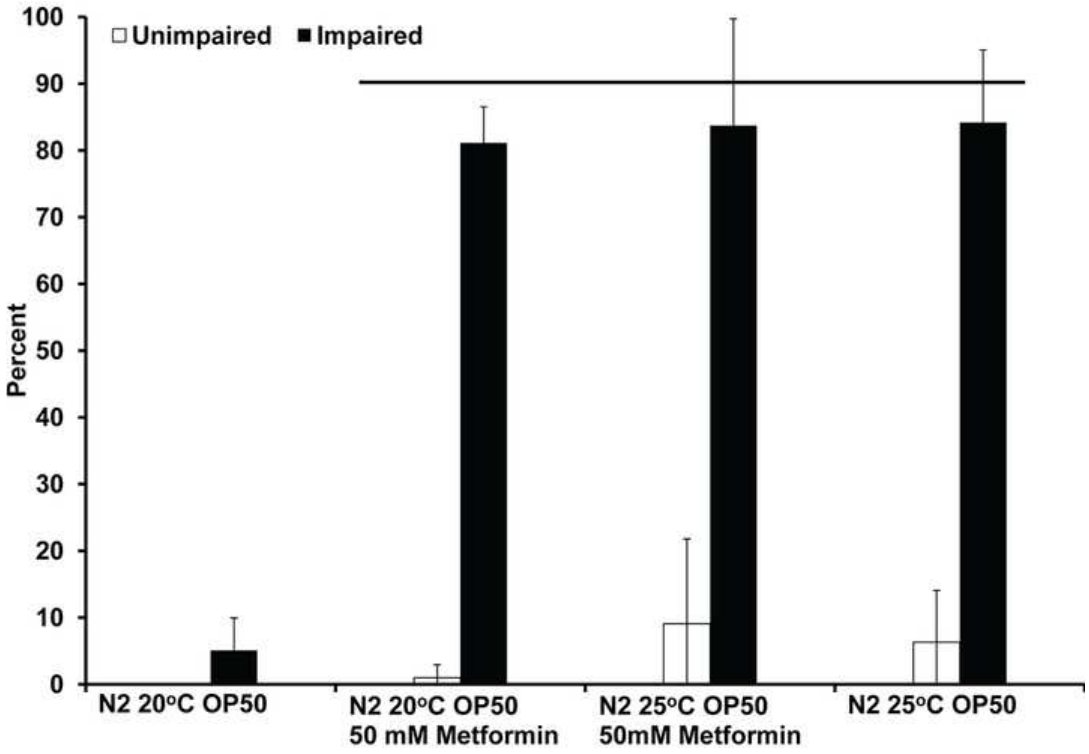


Figure 17. Wild type animals fed metformin survive long-term anoxia. N2 adult hermaphrodites were raised in the specified conditions from L1 larvae to one-day old adult and then exposed to three days of anoxia. The survivors were examined for an unimpaired or impaired phenotype. Line indicates a statistically significant increase in overall survival compared to animals grown at 20°C on OP50 bacteria in the absence of 50mM Metformin ($p<.05$ Student's paired one-tailed t-test). For all experiments the total number of animals assays is N>180 from four independent experiments; error bars indicate standard deviation.

Table 8. Metformin increases long-term anoxia survival rate of wild type animals

Media	Development Temperature (^{o}C)	Survival Rate ± SD
OP50 control	20	5.1 ±4.9
OP50	25	90.5 ±5.2
OP50 + 50mM Metformin	20	82.0 ±4.0[a]
OP50 + 50mM Metformin	25	92.8 ±7.8

Survival rates for data presented in Figure 5
[a] P<.05 in comparison to appropriate control

Metformin treated animals raised at 25^{o}C on OP50 did not have an additional increase in the number of unimpaired compared to non-treated animals also raised at the thermal preconditioning temperature of 25^{o}C on *E. coli* OP50 bacteria (Figure 17).

High Levels of Carbohydrate Correlate with Conditions that Enhance Long-Term Anoxia Survival

Among the physiological concerns in low oxygen conditions, as described in Chapter 1, are the limitations placed on which energy stores are available for metabolism to maintain concentration gradients and critical cellular structures that allow cells to recover following hypoxic/anoxic conditions. Glycogen, or more generally carbohydrates, is readily available for metabolism in low oxygen tensions compared to lipids which are too reduced for catabolic processes.

Carmine is a derivative of insects that has been used as a histological stain for glycogen at least as far back as 1878.[106] A purified form of this extract that is now produced synthetically is carminic acid, and has recently been shown to not only bind to carbohydrates[107], but to have fluorescent properties.[108] It was used in *C. elegans* to stain glycogen levels of animals, and these differences in glycogen levels were verified via hplc.[109-110]

I used the protocol from Hanover *et al*.[109] to assay the carbohydrate levels of adult hermaphrodite *C. elegans* that had been exposed to various environmental pre-conditioning regimens that correlated with varying levels of long-term anoxia survival. Animals raised at 20^{o}C either on HT115 or OP50 *E. coli* bacteria had some visible carbohydrate granules that were stained with carminic acid in merged images (Figure 18 A). Animals raised at 25^{o}C on OP50 bacteria had an increase in intensity compared to animals raised on either of the two bacteria strains at 20^{o}C (Figure 18 A). Animals raised on HT115 *E. coli* bacteria at 25^{o}C had a much greater intensity of fluorescence from carminic acid staining compared to animals raised at either 20^{o}C on either food source, or on OP50 *E. coli* at 25^{o}C (Figure 18 A).

I next hypothesized that metformin treatment would increase carbohydrate levels in animals that were exposed compared to untreated animals. Treated animals raised at 20^{o}C on OP50 *E. coli* bacteria, did have an

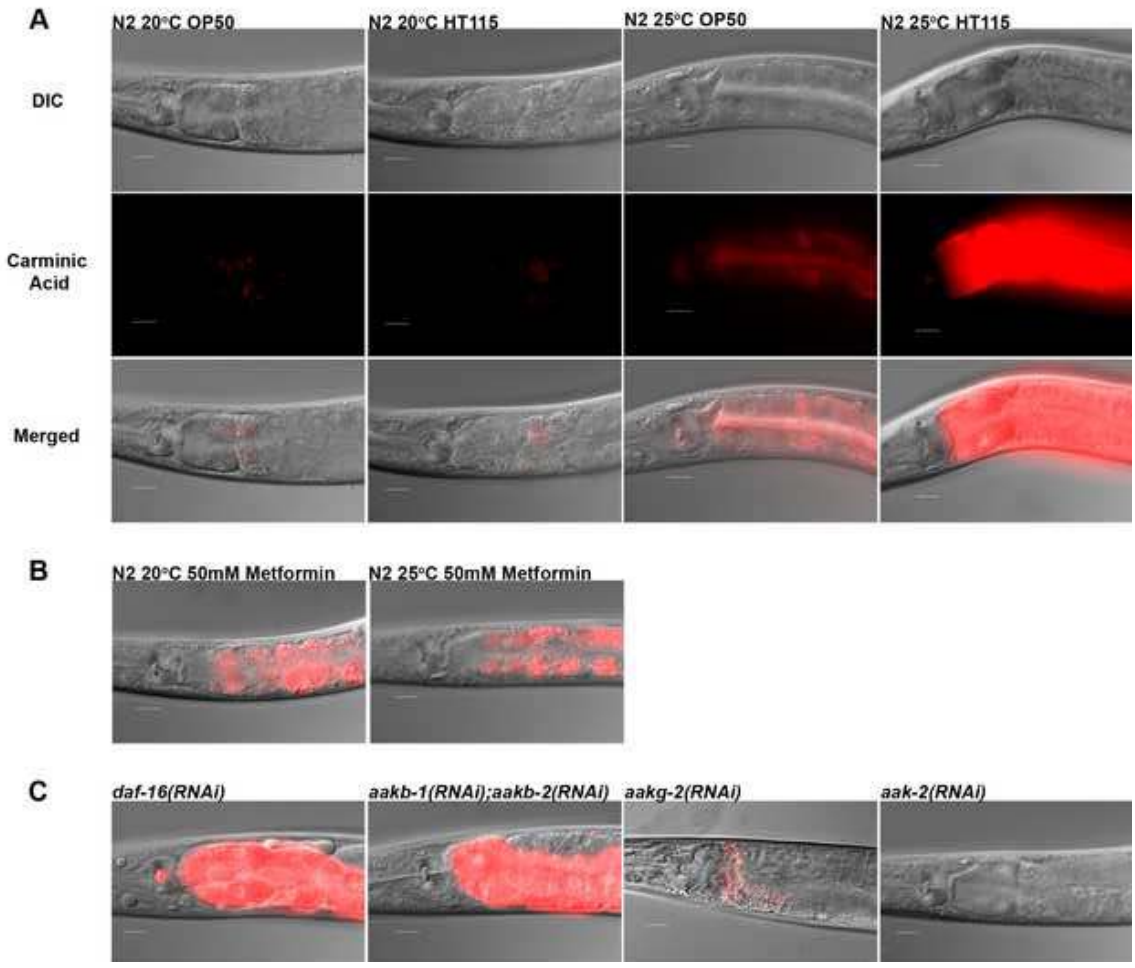


Figure 18. Animals with an enhanced anoxia survival phenotype have increased levels of carminic acid staining. Animals were fed carminic acid, a fluorescent derivative of glucose that incorporates into glycogen and trehalose, to detect carbohydrates in the intestine of *C. elegans* . Images shown include DIC, fluorescent and merged image (A); merged images are show for (B, C). Animals grown at 25°C unless otherwise noted.

increase in fluorescent intensity compared to untreated animals raised in a similar fashion, but as correlated with our viability data, I did not see a marked difference in intensity between treated animals raised on OP50 bacteria at 20^{o}C or 25^{o}C, and between either and untreated animals raised on OP50 at 25^{o}C

(Figure 18 A and B, Figure 17).

Next I wanted to determine if the genetic suppression of the environmental preconditioning phenotype correlated with a reduction in carbohydrate levels as evidenced by fluorescent intensity from carminic acid staining. Knockdown of *aak-2* and *aakg-2* by RNAi diminished the carminic acid fluorescent intensity in animals compared to those animals grown on HT115 control media (25^{o}C). *daf-16(RNAi)* or *aakb-1(RNAi);aakb-2(RNAi)* animals raised at 25^{o}C did not have a decrease in carminic acid fluorescence (Figure 18 C).

I wanted to determine if change in carbohydrate levels correlated with the enhanced long-term anoxia phenotypes that *daf-2(e1370)* and *glp-1(e2141)* animals display. In the case of *daf-2(e1370)* animals, there is a large increase in fluorescent intensity compared to wild type animals grown in similar conditions (20^{o}C due to a temperature sensitive mutation). *glp-1(e2141)* animals, grown at 25^{o}C, had elevated levels of carminic acid fluorescence compared to 20^{o}C wild type animals, this strain did not display an increase in carminic acid staining compared to wild type animals grown at 25^{o}C (Figure 19). The increase in carminic acid staining for *daf-2(e1370)* and *glp-1(e2141)* animals correlates with the animals increased percentage of unimpaired survivors.

RNAi knockdown of aak-2 suppresses the increase in carminic acid fluorescence of *daf-2(e1370)* animals. The reduction in fluorescence correlates with the reduction in the percentage of unimpaired survivors after three days of

20°C anoxia and of overall survival rates following four days of 20°C anoxia in *daf-2(e1370)* animals (Figure 19).

The suppression of carminic acid fluorescence in the *glp-1(e2141)* background may be more complex. The 25°C temperature required for the allele and the loss of germ-line function may both contribute to the carminic acid fluorescence increase in *glp-1(e2141)* animals. The *daf-16(mu86)* mutation, *aakg-2(RNAi)* or *aak-2(RNAi)* suppresses the higher level of carminic acid staining in *glp-1(e2141)*. Double RNAi of *aakb-1* and *aakb-2* did not suppress the high levels of carminic acid in *glp-1(e2141)* animals except in the *daf-16(mu86)* background.

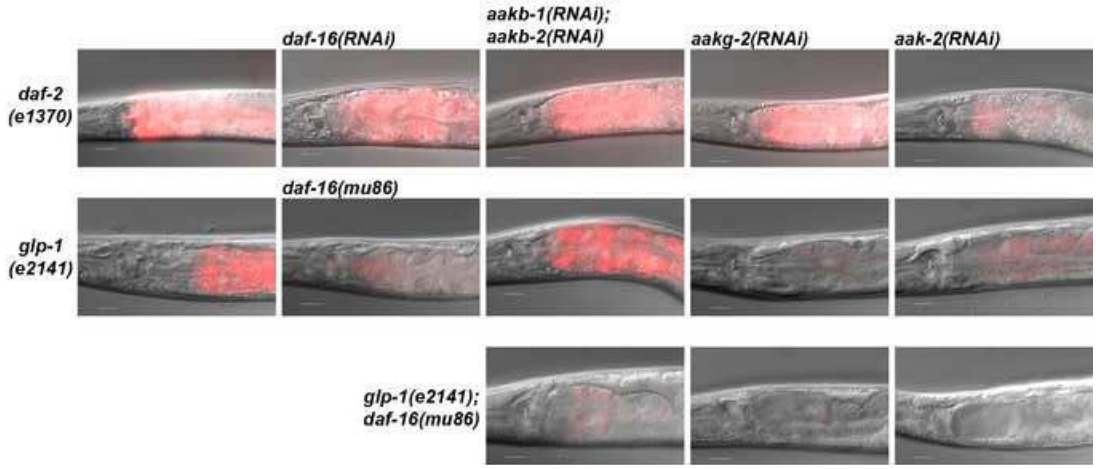


Figure 19. The *daf-2(e1370)* and *glp-1(e2141)* animals have increased levels of carminic acid staining that can be suppressed by specific components of AMPK. Animals of specified genotype or RNAi experiment were fed carminic acid to detect carbohydrate levels in the intestine. Merged images (DIC and fluorescence) of anterior region of the animal are shown. Images shown are representative of >25 number of animals assayed for each experiment. Scale bar, 20μm.

CHAPTER 5

DISCUSSION

Oxygen deprivation in the form of ischemia is an important issue with regards to human health. The increase in ATP yield afforded by oxygen metabolism allows a greater level of organismal complexity to exist. The downside of an increased ATP yield is that the organism has a reliance on oxygen to continue the higher level of ATP output facilitated by oxidative phosphorylation. Organisms vary in their tolerance to oxygen deprivation based on their ability to modulate a conserved set of biochemical and cell-signaling pathways, or by their ability to down regulate energy intensive processes.

Tissue Specific Modulators of Long-Term Anoxia Survival

Neuronal function is an energetically expensive process, and reduction of neuronal function via glutamate receptors has been shown to moderate hypoxia-induced neuronal cell death[93]. Or initial findings that unc-32(f131) animals had an enhanced long-term anoxia phenotype suggested that an alteration of neuronal function in *C. elegans* was protective in anoxia, but it has been published in the literature that three V-H^+ATPase mutants (*unc-32(e189), vha-12(ok821)*, and *spe-5(hc93)*) demonstrate an ability to survive exposure to sodium azide, an inhibitor of cytochrome c oxidase of the electron transport chain complex II that is sometimes used as a chemical surrogate of hypoxia[111]. It was interpreted that this phenotype was mediated by a lack of lysosomal acidification.

The argument is that the proteolytic enzymes and low pH conditions that usually arise with the rupture of these organelles under traumatic conditions or during the initial cascades of programmed death pathways are absent, and this prevents necrosis [111]. Our results suggest that while this could be part of the reason that *unc-32(f131)* has an enhanced anoxia survival phenotype, the altered synaptic function is critical for *unc-32(f131)* and *unc-32(e189)* mutant animal's survival of long-term anoxia and this phenotype differs from the long-term anoxia phenotype of animals with the two other V-H^+ATPase mutations described in Syntichaki *et al*. Our hypothesis is also supported by the localization studies that show that UNC-32 localizes only to neurons, [95] and the classic altered neuronal function phenotype of *unc-32* mutant animals. Localization studies have shown that VHA-12 is localized to the gut, the body wall muscles, and the excretory pore and SPE-5 is involved in sperm function.[112] Just because the gene products have similar ontologies, doesn't imply that they function in a similar fashion in all cell types. For example, SPE-5 plays a role in germline function. Germline function is known to influence anoxia survival, [100] among other stresses, and as mentioned in the introduction can even play a role in increased lifespan. [66]

Environmental Preconditioning can Enhance Anoxia Survival in *C. elegans*

The observed phenotype of an animal is the combination of the animal's genetic background, or genotype, and the interaction that the animal's particular genotype has had with the surrounding environment during development. A

major factor to take into consideration when studying the physiology of ectotherms is temperature. The developmental time of *C. elegans* hermaphrodites from embryo to gravid adult varies by several days between animals raised at 15^{o}C versus animals raised at 25^{o}C. [31]

The results of our experiments suggest that not only is the developmental timescale increased at 25^{o}C, but that tissue metabolism, and macromolecule levels might be altered at elevated temperatures. Animals raised at 25^{o}C, a temperature not considered stressful for the organism, have an enhanced ability to survive long-term anoxia. Animals raised at 25^{o}C also have elevated levels of carbohydrates compared to animals raised at 20^{o}C. Why this is occurring is not understood, but it is possible that it involves HSF-1. HSF-1 is a transcription factor that is responsible for upregulating protective genes during heat stress [89]. And it has been shown than the long lifespan phenotype of I/IGF-like mutants can be suppressed by suppression of *hsf-1.* [113] It is also possible that the bacteria when maintained at a slightly elevated temperature change their macromolecule composition, and induce a protective effect as a food source.

In any case, I found that there is a synergistic relationship between thermal preconditioning and food type. I found in *C. elegans* that an elevated temperature and HT115 *E. coli* diet precondition for an enhanced long-term anoxia phenotype. This enhanced long-term anoxia phenotype includes a significantly higher rate of unimpaired survivors; the survivors have normal movement after long-term anoxia treatment indicating that tissue and organ

maintenance occurs. This is also not fully characterized, but could involve a number of mechanisms. It was shown that sphingosines were protective in anoxia, and that hyl-2 was involved with this process in *C. elegans.* [114] It is possible that HT115 is secreting a protective molecule while in anoxia conditions, and this evidence could be interpreted from our results. Alternatively it is possible that in anoxic conditions, that OP50 bacteria changes its metabolism to the point of being mildly pathogenic. When *C. elegans* encounters pathogenic bacteria, the response in the gut is a generation of free radicals in the lumen of the intestine. [115] If the OP50 strain of bacteria is slightly pathogenic in anoxic conditions, then the worm could be generating additional free radicals to keep the growth of the food bacteria in check in the lumen of the gut. Under the extreme low energy conditions encountered in long-term anoxia, the added free radicals could be the proverbial “straw that breaks the camel’s back” during anoxia/reperfusion. An alternate hypothesis, as to why on an OP50 diet prior to long-term anoxia , includes the idea that an increase in osmolarity in the gut in response to bacterial defense occurs in *C. elegans* exposed to anoxia.[116] It is known that to maintain osmotic balance, the animal must alternate provisioning of sugars from glycogen which is readily catabolized in low oxygen conditions to glycerol[117] which is not catabolized in low oxygen conditions. This alternate sugar provisioning has been shown to be important for the anoxia survival of *C. elegans* embryos exposed to shorter periods of anoxia.[118]

Regardless of the mechanism, animals raised on an HT115 diet at 25^{o}C

have an increase in carbohydrate stores compared to either animals raise at 25°C on an OP50 diet or animals on an HT115 diet at 20°C based on the results of our carminic acid staining assays. The increase in carbohydrate stores of 25°C animals on an HT115 diet appears to be uniform throughout the organism, representing a global metabolism change for the animal. The animals raised on an HT115 diet at 25°C go into anoxia with a larger store of easily catabolized energy stores, and these levels correlate with an increase in overall survivorship and quality of survivorship in long-term anoxia.

Role of Insulin/IGF-Like Signaling in Long-Term Anoxia

Insulin/IGF-like signaling is know to be a regulator of energy homeostasis[2] and is known to be involved in longevity and stress resistance in *C. elegans.* [37,88] Mutations in I/IGF-like signaling in *C. elegans* can increase survival of long-term anoxia, and functions at least partially through an upregulation of genes via DAF-16/FOXO. Knockdown of the transcription factor DAF-16 and one of its targets, the glycolytic enzyme GPD-2/3 were sufficient to suppress this phenotype. This provides some support for the idea that possibly the GPD-2/3 isoform is a stress specific paralog of GAPDH, and that GPD-1/4 is sufficient for survival in normoxia, but not in anoxia. Increased expression of *gpd-2* and *gpd-3* in the daf-2(e1370) animal has a role in the ability to survive anoxia. Theoretically, an increase in gpd-2/3 expression could result in increased ratios of 1,3-bisphosphoglycerate to glyceraldehyde-3-phosphate; ATP

to ADP, and/or a NADH to $NAD^{+.}$ Perhaps an increase in ATP/ADP ratios provides the necessary energy for survival of long-term anoxia. Alternatively, It is possible that the *gpd-2/3* isoforms have optimal activity is low oxygen conditions compared to the *gpd-1/4* isoforms.

RNAi knockdown of the other genes upregulated by DAF-16 did not significantly suppress the long-term anoxia phenotype of *daf-2(e1370)* mutant animals. Surprisingly, thermally preconditioned *daf-16(mu86)* animals on an HT115 diet did not have a significant decrease in survival in three days of anoxia compared to wild type. The lack of full suppression sheds doubt on the possibility that canonical insulin/IGF-like signaling pathway plays the lone role in the previously mentioned environmentally preconditioned phenotype. It is possible that the individual targets contribute to small increments in increased survival. If that were the case, then knockdown of each of the other genes individually might not have a resolvable effect on long-term anoxia survival, but combined they become significant.

Although the mechanism is not known, *daf-2(e1370)* animals, in a fashion similar to 25^{o}C wild type worms on an HT115 diet, had an increase in carbohydrate levels as indicated by carminic acid staining. Once again the pattern shows a correlation between enhanced long-term anoxia survival and increased levels of carbohydrate stores.

Role of Germline Signaling in Long-Term Anoxia Survival

Studies with *glp-1(e2141)* reinforced work that was previously done in our lab that showed altered germ-line function was enhanced long-term anoxia. The enhanced long-term anoxia phenotype of *glp-1(e2141)* animals was not suppressed in three day anoxia, and was only moderately suppressed in four days of 20°C anoxia in the *glp-1(e2141);daf-16(mu86)* double mutant. The suppression of long-term anoxia survival in *glp-1(e2141)* may be more complex to interpret because due to the nature of the allele these animals are raised at 25°C thus the temperature and allele may both contribute to increased carminic acid levels. As was noted with animals raised at 25°C on an HT115 diet and *daf-2(e1370)* animals, *glp-1(e2141)* animals had an increase in carbohydrate levels visualized by carminic acid staining, once again highlighting the role of energy stores easily catabolized in low oxygen conditions.

Role of AMPK in Enhanced Anoxia Survival

Given the role of insulin/IGF-like signaling and the correlation of increased carbohydrate levels with enhanced anoxia survival in *C. elegans* , energy homeostasis seems to be a central theme in surviving long-term anoxia survival. AMPK is a known modulator of energy homeostasis, and is involved with longevity and stress resistance. As was covered in Chapter 1, it is a heterotrimeric protein made consisting of an α catalytic subunit, a β polysaccharide binding subunit, and γ AMP ligand binding subunit. In *C. elegans* , multiple isoforms exist of each subunit, which allows for different forms of AMPK

proteins to exist depending on which subunits are incorporated. Energy levels inside the cell are detected in a bimodal fashion by AMPK, in both the form of low-energy nucleotides, and by the polysaccharide binding motifs that regulate the kinase domain. Evidence exists to tie AMPK into pathways involved with protein, lipid, and carbohydrate sensing pathways, along with TGF-β, FOXA and I/IGF-like signaling pathways. [75-79]

It is likely that AMPK's role in environmental enhanced and genetically induced anoxia phenotypes probably involves specific isoforms. The beta subunit of AMPK is known to bind polysaccharides, but knockdown of either of the subunits alone did not significantly reduce long-term anoxia survival in animals with an enhanced survival phenotype. In the *aakb-1(RNAi);aakb-2(RNAi)* double mutant background environmentally preconditioned animals, *daf-2(e1370)* animals, and in a DAF-16 dependent fashion *glp-1(e2141)* animals had a reduction in their ability to survive long-term anoxia. So, without the carbohydrate-level mediated moderation of AMPK signaling, the animals didn't seem to do as well in long-term anoxia, but either of the two isoforms could fill this role in oxygen poor environments.

aak-2(RNAi), one of two catalytic isoforms, was able to knockdown the enhanced long-term anoxia phenotypes of *daf-2(e1370)*, pre-conditioned wild type, *glp-1(e2141);daf-16(mu86)*, and to a lesser but significant extent, *glp-1(e2141)* animals. These results suggest that *aak-2* plays a key role in

enhancing long-term anoxia survival. Knockdown of the other catalytic subunit, *aak-1*, had no significant effect on anoxia survival. This again lends to the idea that, for some processes, there may be a stress or anoxia isoform of a common enzyme to deal with the unique challenges of low energy conditions. *aakg-2(RNAi)*, an isoform of the gamma subunit, reduced the enhanced long-term anoxia phenotype of environmentally preconditioned, and *glp-1(e2141);daf-16(mu86)* animals, but did not significantly reduce the ability of *daf-2(e1370)* animals to survive long-term anoxia. RNAi of the other gamma subunits had no significant effect on any of the enhanced long-term anoxia phenotypes. The aakg-2(RNAi) evidence suggests the possibilities for parallel pathways of long-term anoxia survival in the different mutant and environmentally preconditioned backgrounds. For example, it is possible that in environmentally preconditioned animals a specific gamma subunit is required, where in the *daf-2(e1370)* background a specific gamma subunit is not as important for anoxia survival.

The physiological role AMPK plays may involve the correlative data observed between enhanced anoxia survival and an increase in carbohydrate stores. In environmentally preconditioned and *glp-1(e2141);daf-16(m86)* animals the high levels of carbohydrates observed were reduced in an *aak-2(RNAi)* and *aakg-2(RNAi)* background. *aak-2(RNAi)* , but not *aakg-2(RNAi)*, was also able to reduce the carbohydrate levels of *daf-2(e1370)* animals, which once again correlates viability data with increased levels of carbohydrates. In the *glp-1(e2141)* allele however there may be multiple components enhancing the

anoxia survival phenotype. For example the massive reduction in carbohydrate stores in *glp-1(e2141);daf-16(mu86)*;*aak-2(RNAi)* still allows approximately 60% of animals to survive four days of 20^{o}C anoxia as impaired individuals. While this is significantly less overall survivorship than animals not subjected to *aak-2* knockdown via RNAi, it is a considerably higher rate than wild type animals raised at 20^{o}C.

Metformin is an anti-diabetic drug that has been shown in mammalian studies to indirectly activate AMPK,[103] and is known to increase longevity and stress resistance in an aak-2 dependent fashion in *C. elegans.* [79] Is sufficient to increase overall long-term anoxia survival rates, but fails to replicate the robust quality of survivorship of environmentally preconditioned, *daf-2(e1370)*, and *glp-1(e2141)* animals. Based on the data generated in our experiments, it is not additive with thermal preconditioning, suggesting that the two may operate through similar mechanisms.

In the end, it seems that many bifurcated and parallel pathways are involved in stress resistance. The pathways possibly have isoforms of enzymes that are specific for a given stress, and some that are common for dealing with other types of stress. I have presented evidence that enhanced survival of long-term anoxia in *Caenorhabditis elegans* involves two nodes of stress signaling pathways DAF-16/FOXO, and AMPK. These two nodes regulate genes that convey general stress resistance to the whole organism. Also as is the case with tissue specific genes, it might be possible that altering the function of genes

not directly modulated by DAF-16 or AAK-2 might confer an enhanced long-term anoxia survival phenotype (Figure 20).

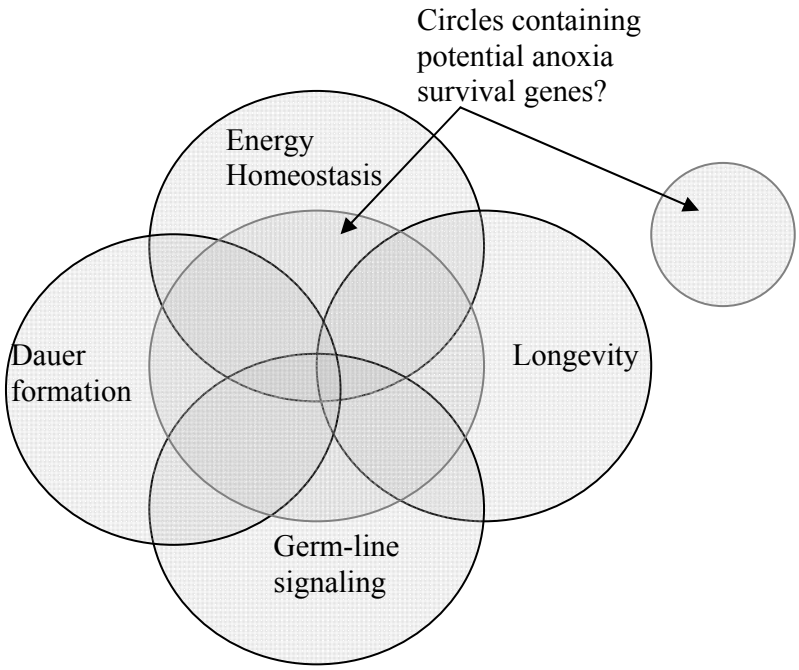


Figure 20. Venn diagram describing genes involved in long-term anoxia survival and other stress and longevity phenotypes.

CHAPTER 6

MATERIALS AND METHODS

Worm Strains Used

The following strains and alleles were obtained from the CGC in St Paul Minnesota and used in the previous experiments; N2 bristol (wild type), *daf-2(e1370)*, *daf-2(e1371), daf-2(m596), daf-2(m579);daf-16(m26), daf-2 (m579), age-1(hx456), unc-32(f131), unc-32(e189), vha-12(ok821)*, *spe-5(hc93),eat-2(ad1116), mev-1(tk22), clk-1(e2519), clk-1(qm30), glp-1(e2141), glp-1(e2141);daf-16(mu86), aak-2(gt33), and aak-2(rr48).*

Culture Conditions

All strains other than strains containing the temperature sensitive *glp-1(e2141)* allele and their controls were maintained as stocks on nematode growth media (NGM) plates at 20^{o}C as previously described[31]. Strains containing the *glp-1(e2141)* allele, and their controls were maintained at 15^{o}C. For assays, synchronized populations were obtained via the alkaline hypochlorite protocol, and F1 progeny were allowed to hatch out in a rocked M9 solution on unseeded bacterial plates for approximately 16 hours. All strains were then raised at 20^{o}C on NGM plates seeded with the appropriate food (either OP50 or HT115 strain *E. coli* bacteria) except for *glp-1(e2141)* containing strains and their controls. *glp-1(e2141)* containing strains were raised for 24 hours at 15^{o}C on

seeded NGM plates following synchronization, and then transferred to 25^{o}C for an additional 48 hours to develop to young adults.

Under normal conditions most strains raised at 20^{o}C develop to become gravid young adults in about 72 hours following introduction to food. Animals with the mutant alleles *age-1(hx456), clk-1(e2519), clk-1(qm30) mev-1(tk22), unc-32(f131), unc-32(e189)*, and *eat-2(ad1116)* take approximately an additional twelve hours to reach the same point developmentally. Animals containing the *daf-2(e1370)*,and *daf-2(m579)* take about an extra 24 hours to reach the same point developmentally.

RNAi Experiments

The protocols used were adopted from Kamath et al. [25] RNAi libraries were obtained in 384 well glycerol stocks from MRC geneservice, and maintained at -80^{o}C. RNAi food bacteria was streaked onto LB plates enriched with ampicillin and tetracycline, and incubated for about 18 hours at 37^{o}C. Individual colonies were then picked and placed into culture tubes in a rotary shaker overnight at 37^{o}C in LB/ampicillin/tetracycline liquid cultures. These cultures were then used to start exponential liquid cultures in fresh LB/ ampicillin/tetracycline liquid cultures for 4-6 hours at 37^{o}C in a rotary shaker. The exponential cultures were then seeded onto nematode growth media plates (NGM) enriched with tetracycline, ampicillin and IPTG.

Anoxia Experiments

Animals were placed into anoxia in BD Biobag type A anaerobic environmental chambers (BD biosciences, Rockville MD) at 20^{o}C except for the 28^{o}C anoxia experiments. Anoxic conditions were verified using Resazurin indicators (<.001kPa of O_2 detection limit). Any trials that failed to go anoxic within one hour were nulled.

Thermal Preconditioning and Growth Conditions

To precondition the animals prior to anoxia treatment animals were synchronized and raised to adulthood at 25°C. To synchronize, L1 larvae were obtained by collecting embryos from hypochlorite-treated adults; embryos were given 16 hours to hatch in M9 solution on unseeded NGM plates at 20°C. The time animals were raised at 25°C varied depending on the genotype. Strains containing the *glp-1(e2141)* temperature sensitive allele and experimentally matched control were maintained at 15°C to produce progeny. The *glp-1(e2141)* L1 larvae were raised at 15°C for 24 hours on seeded NGM plates, and then transferred to 25°C for an additional 48 hours to develop to young adults. Strains containing the *daf-2(e1370)* temperature sensitive allele and control were grown to adulthood at 20°C because of the 25°C constitutive dauer phenotype. The *daf-2(e1370)* animals were assayed 96 hours after synchronization, whereas additional strains were assayed 72 hours after synchronization. Experiments were conducted on young adults; anatomical markers such as gonad morphology

or approximate hours after L4 to adult molt were used to determine the young adult stage of the animal. For all experiments at least four independent experiments were conducted.

Carminic Acid Staining

Carminic acid (Cole-Parmer) staining was done as previously described. [109-110] Briefly, carminic acid was added to sterile LB media for a final concentration of 1mg/ml. LB media was filter sterilized and cultured with the OP50, HT115 or specified RNAi strain used to seed NGM plates. For all assays synchronized L1 larvae were grown on the carminc acid NGM plates and assayed as one-day old adults. Microscopy analysis was conducted as previously described,[109] Briefly, adult animals were placed on a 2% agarose pad containing 0.2mM levamisole in M9. Animals were examined using a Zeiss Axioscope fluorescence microscope; image acquisition was done using Axiovision Zeiss and processed using Adobe Photoshop. For all imaging analysis, exposure was identical (100ms). For each experiment > 25 animals were independently analyzed.

Metformin Exposure

Metformin exposure assays were done as previously described. [79] Briefly, NGM plates containing 50mM of metformin (Sigma) were used for assays. Animals were exposed to metformin as L1 larvae and throughout development to one-day old adults. For all experiments at least four independent experiments were

old adults. For all experiments at least four independent experiments were conducted.

Statistical Analysis

Experimental animal values were compared to control animal values via a Student's one-tailed t-test when comparing two groups. When comparing more than two groups, a one-way ANOVA on ranks was performed. This was followed by an SNK multiple range test or a Dunnett's multiple range test. Alpha levels of .05 were deemed as significant. Analyses were performed in either MS Excel® (Microsoft) or SigmaStat ®software (Systat Software inc.)

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