AGING IS A DETERMINANT IN ANOXIA STRESS TOLERANCE IN *Caenorhabditis elegans*

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Oxygen availability is critical for survival for most organisms. The nematode, *C. elegans,* has been useful for studying genetic regulation of anoxia tolerance due to the oxygen deprivation response mechanisms shared with other metazoans. Studies examining long-term anoxia (72h, LTA) tolerance have only been conducted at adult day 1. To investigate the effect of aging on anoxia tolerance wild-type and mutant strains were exposed to LTA between adult day 1 and day 9. Wild-type isolates and *daf-16(mu86)* (FOXO transcription factor regulated by insulin-signaling) and *aak-2(gt33)* (catalytic subunit of AMP-activated protein kinase) strains were anoxia sensitive at day 1 and displayed increased LTA tolerance with aging correlated with reproductive senescence followed by a decline in survivorhsip through day 9. The *daf-2(e1370)* (insulin receptor homologue of *C. elegans*), *glp-1(e2141)* (a lin-12/Notch receptor) and *fog-2(q71)* (required for spermatogenesis) strains were LTA-tolerant through day 5. I conclude that aging influences LTA-tolerance in a strain- and age-dependent manner. In addition to being LTA-tolerant the *daf-2(e1370)* and *glp-1(e2141)* strains have a longevity phenotype that is suppressed by loss of *kri-1* or *daf-12*. While loss of *kri-1* did not suppress the LTA-tolerant phenotype of *glp-1(e2141)* at day 1 the portion of impaired survivors increased at day 3 and by day 5 tolerance was suppressed. Similarly, when exposed to 4 days of anoxia the *glp-1(e2141)*;*daf-12(rh41rh611)* double mutant had a reduced survivor rate at all ages analyzed compared to *glp-1(e2141)* controls. To better understand formation of an anoxia-tolerant physiology I exposed adults to one or more 24h bouts. Recurrent bouts increased LTA tolerance in wild-type hermaphrodites in a dose-dependent manner. Bout-treated *daf-16(mu86)* animals

had increased survival rate compared to controls yet maximum survival remained below age-matched wild-type. Anoxia bouts decreased LTA-tolerance in *aak-2(gt33)* mutants, indicating the requirement for ATP regulation in establishing an LTA-tolerant phenotype. These data support the idea that anoxia tolerance is multi-factorial and influenced by environment, metabolism, food, reproduction, sex phenotype and likely additional factors.

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CHAPTER 1

THE BIOLOGICAL FRAMEWORK FOR STUDYING ANOXIA TOLERANCE

Oxygen Deprivation and Metazoans

Oxygen availability is a critical factor for the survival for most organisms and relatively few species can survive extended periods of hypoxia or anoxia. Global oxygen depletion has the potential to impact organisms on many levels - from niche selection to species viability. Declines in global ocean oxygen content creating "oxygen minimum zones," regions where O_2 levels are too low to support biogeochemical cycling, are expected to reduce the volume of the ocean inhabitable by commercial species such as tuna resulting in population decline (Keeling *et al.* 2010; Rabalais *et al.* 2010). Commercially important plant crops are susceptible to catastrophic loss when exposed to flooding-induced anoxia for extended periods. At the individual level, oxygen deprivation contributes to long-term impairment in humans following acute ischemic events such as stroke, myocardial infarction, near drowning and physical traumas.

While oxygen deprived environments are encountered by all phyla of organisms the physiologic and biochemical impact of oxygen deprivation varies among taxa. An example of extreme anoxia tolerance is the recently discovered species of *Loriciferous* that live in the sediment of the Mediterranean seafloor and are the first documented multicellular organisms capable of surviving in an entirely oxygen-free environment(Danovaro *et al.* 2010). In contrast, most organisms, even those considered anoxia tolerant, survive only a finite period ofexposure after which their survival and recovery rates plummet. The response of anoxia-tolerant animals to absence of oxygen is usually a drastic reduction in overall activity and entry into a form of

stupor, diapause or suspended animation. When exposed to anoxia shrimp (Clegg *et al.* 1997), *Drosophila* (Foe & Alberts 1985), killifish (Podrabsky *et al.* 2007) and zebrafish (Padilla & Roth 2001) embryos enter a phase of total developmental arrest that is relieved only by reoxygenation. The duration of anoxia tolerance for embryos of these species is varied (Table 1).

Anoxia tolerance by adults is also known. Adult *Drosophila melanogaster* can survive 3-4h of anoxia without showing evidence of cell injury (Haddad *et al.* 1997). Adult nematodes, *Caenorhabditis elegans*, survive 24h of anoxia and recover normal movement and reproductive capabilities after reoxygenation (Padilla *et al.* 2002). Freshwater turtles (*Trachemys scripta elegans* and *Chrysemys picta*) can survive in an anoxic aquatic environment up to 60 day with no evidence of neural degradation (Jackson 2004) and as long as 3-4mo of anoxia (at 0-5˚C) with virtually zero blood oxygen content. In contrast, mammals exposed to minutes of anoxia often experience irreversible neuronal cell trauma either rapidly via necrosis or in a delayed manner through the activation of apoptotic pathways.

Adaptations for Surviving Anoxia

As studies examining the physiological adaptations of anoxia-tolerant species accumulate, it is becoming apparent that oxygen deprivation tolerance strategies utilized by various species share common features. Metazoans, including *C. elegans*, possess complex biochemical mechanisms that operate at the cellular level to promote oxygen deprivation tolerance (O'Farrell 2001). These adaptations allow anaerobiosis in severe hypoxia and anoxia through a range of physiological responses that operate via three general strategies, increase

the rate of flux through glycolytic pathways, decrease overall energy demand by rapid reduction in metabolic rate, or activation of

Table 1.1 Anoxia duration maximums for quiescent embryos.

Organism	Duration of Anoxia Survival
Brine shrimp, *Artemia franciscana*	4 years
Fruit fly, *Drosophila melanogaster*	~36 hours
Killifish embryo, *Austrofundulus limnaeus*	30-60 days

Adaptations for Surviving Anoxia

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production (*Carassius carassius*, crucian carp) as well as raised levels of the inhibitory neurotransmitter γ-aminobutyrate (GABA) in neural tissue (Lutz *et al.* 1996). Accordingly, animals switch-off energy source molecules during oxygen-deprivation from fat that is primarily utilized for aerobic energy metabolism to glycogen/glucose stores. During a 24 hour anoxia exposure as much as two-thirds of the animals carbohydrate reserve may be utilized as an energy source; this usage nearly accounts for the mass of glycosyl units of metabolites produced during the oxygen deprivation period (Foll *et al.* 1999).

Perhaps the most critical shared feature of oxygen deprivation tolerance is ability to severely downregulate metabolism, thereby reducing both production and demand for ATP (Hand & Hardewig 1996; Jackson 2000; Krumschnabel 2000; Lutz & Nilsson 2004). This strategy forms the basis of a well-accepted model of the survival tactics used to survive extended periods of hypoxia exposure (Hochochka & Somero 2002).

Hochochka and Somero's model has provided a useful framework within which to investigate molecular and cellular response to oxygen-deprivation. Studies of oxygen deprivation-tolerant organisms show the response to occur in two primary phases. First, during the "very early acute phases" of hypoxia three profound physiologic alterations occur.

1. Global decline in protein biosynthesis possibly due to translational arrest
2. Decline in membrane permeability (generalized across the tissues) and firing frequency (in nervous tissue)
3. Alteration in metabolic processes allowing the demand and supply of ATP to remain in a steady state of flux

A fourth adaptive strategy (Brooks & Storey 1989) addresses the tactic of compensatory ion changes that buffer acidosis, along with having an overall high tolerance to metabolic acidosis

that occurs during glycolytic fermentation. Early phase alterations are followed by a "rescue" phase in which only a subset of genes are expressed (both transcribed and translated) in an orchestrated manner such that proteins required for anaerobic metabolism, cell stabilization and hypoxia survival are available (such as *h*ypoxia-*i*nducing *f*actor 1, HIF-1, encoded by *hif-1*). This model emphasizes the importance of greatly reduced yet sustained steady state energy balance, as well as, maintenance of the genome and proteome, and stabilization of cell structure and function.

C. elegans as a Model Organism for Anoxia Investigation

C. elegans adult animals have been useful for understanding the genetic and physiological responses to oxygen deprivation particularly because of the mechanistic overlap in oxygen deprivation responses between *C. elegans* and other metazoans including humans (Powell-Coffman 2010). Several unique characteristics of adult *C. elegans* make it a valuable model to study responses to oxygen deprivation. First, the adult animal has a relatively simple anatomy, easily observable somatic tissues and meiotic cells. These tissues amenable to analysis include muscle, neurons, intestinal cells and a very well studied germline that contains meiotic cells that give rise to oocytes and sperm in the hermaphrodite. Second, *C. elegans* has been used by many within the community to study genes involved with stress responses and lifespan; these studies allow investigators to identify overlapping and distinct mechanisms between stress responses and lifespan. Finally, *C. elegans*, as a soil nematode, has adapted to changing oxygen levels in the environment. Taken together, the anatomical, genetic and environmental niche characteristics of *C. elegans* provide a unique opportunity to identify the

ways in which this simple model responds to and survives oxygen deprivation. Such information can aid in our understanding of why species do or do not have limitations in oxygen deprivation response and survival.

C. elegans frequently encounters oxygen-deprived microenvironments in its natural habitat and adult animals have adapted to tolerate these exposures. Wild-type hermaphrodites that are 1-day old (1 day after the L4 larval to adult molt) survive 24 hours of anoxia at ≥90% (20˚C) when assayed on solid NGM medium (Padilla *et al.* 2002; Van Voorhies & Ward 2000), however, survival rate plummets as the duration of anoxia is lengthened (Mendenhall *et al.* 2006; Mendenhall *et al.* 2009; Padilla *et al.* 2002). The 1-day old adult has a markedly decreased survival rate (4.7%) in long-term anoxia, defined as a 72 hour or more anoxia exposure at 20˚C, demonstrating that there is an anoxia survival limitation (Mendenhall *et al.* 2006). This fact has been useful for identifying genetic mutations that lead to long-term anoxia (LTA) sensitivity (inability to survive 24 hours of anoxia) and LTA tolerance (able to survive LTA, 3 or more days of anoxia).

The adult anoxia-tolerance strategies include the worm entering a reversible state of suspended animation. In this state adults do not feed, do not lay eggs and cease to be motile. The process of crawling has been reported to carry a relatively low metabolic cost to the worm compared to the high cost of reproduction and tissue maintenance and this assessment is supported by the observation that animals whose metabolic rate has been reduced by greater than 90% do not show abnormal motility (Van Voorhies & Ward 2000; Vanfleteren & De Vreese 1996). Among the adaptations *C. elegans* posses is the ability to sustain a steady metabolic rate even when exposed to a range of decreasing oxygen tensions, and not until ambient oxygen

tension falls to 3.6 kPa will metabolic rates begin to drop for young adults (Anderson & Dusenbery 1977; Suda *et al.* 2005; Van Voorhies & Ward 2000). However, once the environment becomes anoxic metabolic rates drop to as low as 5% of that in normoxic conditions and recover in a slow linear fashion only after removal from anoxia.

The length-of-time animals remain active after the onset of anoxia varies among *C. elegans* strains. The majority of wild-type adults cease movement within 8 hours of the onset of anoxia (Mendenhall *et al.* 2006). However, the *daf-2(e1370*) animal, which is an LTA tolerant mutant strain and carries a reduction-of-function mutation in the insulin-like receptor will delay entering into suspended animation as demonstrated by observable movement after 24 hours of anoxia. Although movement is observed in the *daf-2(e1370*) animal exposed to anoxia it is slower than normoxic controls (Mendenhall *et al.* 2006). To date no mutation has been isolated that prevents the worm from entering into a state of suspended animation.

The cylindrical body and simple gut design of the worm favors rapid diffusion of gases across both the gut lumen and cuticle into the metabolically active intestine*. C. elegans* is an oxygen regulator and seems to be insensitive to hyperoxia (Van Voorhies & Ward 2000). However, when confronted with oxygen deprivation the worm must respond by either remaining animated or entering into suspended animation; the determining factor often being oxygen tension and perhaps metabolic state (Nystul and Roth, 2004). Which factors are critical in the molecular decision to suspend or continue processes such as movement and how these factors are regulated at the cellular and tissue level remains unclear. Nevertheless, valuable information regarding genes required for both hypoxia and anoxia survival has been gleaned (Jiang *et al.* 2001; Padilla *et al.* 2002; Scott *et al.* 2002).

Environmental Factors Affect Anoxia Tolerance

There is evidence that the environment in which the animal is exposed will precondition for an enhanced anoxia survival phenotype by altering the physiology of the animal (LaRue & Padilla 2010). Animals grown at varying temperatures likely have different cellular milieus (varying levels of heat-shock proteins for example). In the laboratory wild-type *C. elegans* grown at 20°C and fed a diet of *E. coli*-OP50 are sensitive to long-term anoxia in that the majority of the animals die and among those that survive most have an impaired phenotype. However, animals grown at 25°C and fed the *E. coli*-HT115 throughout larval development have a significantly increased LTA survival rate and high levels of unimpaired phenotype. The animals grown at 25°C and fed *E. coli*-OP50 also survive long-term anoxia yet many have an impaired phenotype in that they display visible defects in motility and tissue morphology. These data suggest that growth at 25°C and a diet of *E. coli* HT115 during development may synergistically enhance anoxia survival for *C. elegans*. It is possible that the 25°C temperature induces stress response genes (Ex: heat shock proteins) that prepare the animal to survive long-term anoxia. Alternatively, the preconditioning environment could alter energy stores leading to an increase in anoxia survival. Metabolic stores are altered in *C. elegans* raised at 25°C and fed the *E. coli* HT115 diet during development. The *E. coli* HT115 strain has higher carbohydrate levels in comparison to the OP50 strain; this may influence the metabolism of the worm (Brooks *et al.* 2009). Staining with carminic acid indicates that intestinal carbohydrate levels are increased of animals grown at 25°C and fed the *E. coli* HT115 diet compared to those raised at 20°C (LaRue & Padilla 2011). Animals grown at 25°C or higher likely have a different cellular milieu than those grown at 15-20°C (varying levels of heat-shock proteins for example). Further analysis is needed

to determine mechanistically how preconditioned metabolic and physiological changes within the nematode contribute to the enhanced long-term anoxia phenotype.

The Anatomical and Physiological Effects of Anoxia Exposure

C elegans move in a rhythmic pattern that resembles a sine wave (Fig. 1.1A). While in a state of suspended animation the immobile *C. elegans* often adopt linearly extended bodies or a bent or curved-sickle shape (Fig. 1.1C). Upon re-oxygenation survivors will resume movement and the overwhelming majority exposed to 24 hours of anoxia will move normally within several hours after reoxygenation (Fig. 1.1B).

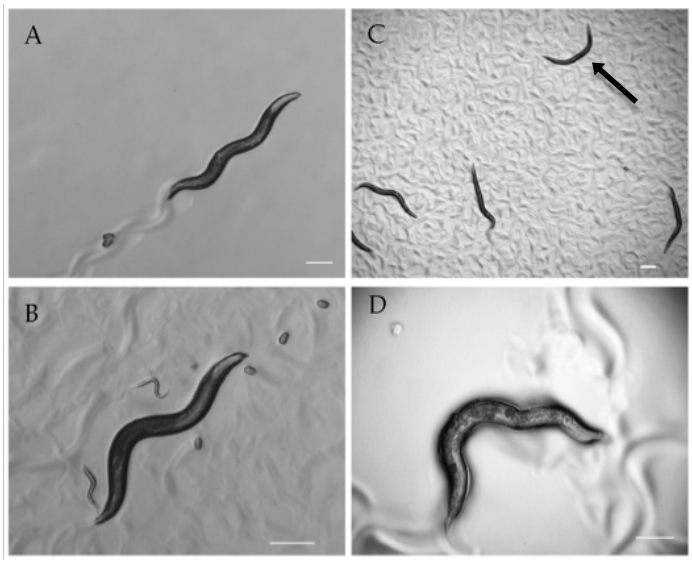

Fig. 1.1 Wild-type C. elegans adults survive and fully recover motility after 24 hours of anoxia whereas animals exposed to 72 hours of anoxia have a reduced survival rate and impaired motility. Wild-type animals were raised to 1-day old adults then exposed to anoxia for 24 hours or 72 hours. A) One-day old adult hermaphrodite prior to anoxia exposure. B) The same adult following 24 hours of anoxia and given 1-hour of recovery in normoxia. Animal recovered normal pattern of movement and resumed egg-laying within an hour of re-oxygenation. C) One-day old adult hermaphrodites in suspended animation immediately following 72 hours of anoxia. Note the slightly curved body posture (arrow). D) Example of an impaired survivor following 72 hours of anoxia and 24 hours recovery in normoxia. Note the impaired animal has consumed the bacterial food in a fan-shaped halo surrounding the anterior head region. All anoxia exposures were conducted at 20˚C. Scale bar = 100 µm (A, B, D); scale bar = 20 µm (C).

Initial movement begins with slight side-to-side movement of the anterior head region then slowly spreads to include the entire head region. As recovery progresses the worm regains the ability to move the mid-body and tail regions in the classic sinusoidal motion and resumes foraging and egg laying. Recovery from LTA takes longer and not all physiological processes appear to resume at the same rate. For example, in the few wild-type animals that survive long-term anoxia, contraction of the somatic gonad sheath and ovulation has been observed within 12 hours of post-anoxia, which is often before full body motility has resumed. The asynchronous nature of recovery of anatomical and organ function may act to compromise the viability of the animal. For example, if resumption of ovulation or embryonic development precedes recovery of the hermaphrodite's vulva muscles and ability to extrude eggs the accumulated eggs may hatch within the uterus (the bagging phenotype) and impede recovery of function in organs such as neurons, muscles or the intestine. It may be that long-term anoxia survivors are able to resume anatomical and physiological processes faster than nonsurvivors. By monitoring rates of recovery one can assess how well a population of animals tolerates anoxia. Often impaired worms will consume the bacteria nearby leaving a fan-shaped area emptied of food (Fig. 1.1D). The underlying cause of anoxia-induced impairment, such as a compromise of muscle and/or neuronal function, is yet to be determined. It is possible that LTA-sensitive strains are unable to execute the processes required for tissue maintenance or needed to maintain cellular integrity (Mendenhall *et al.* 2006).

Wild-type animals recovering from long-term anoxia have an overall loss of tissue structure in the head region that contains both neuron and muscle. Along with distortions in the muscle isthmuses of the pharynx, relatively large vacuoles or cavities also appear

throughout the soma (Fig. 1.2). However, long-term anoxia tolerant strains do not show the same tissue disorganization and appear to be better able to maintain tissue structure.

Fig. 1.2 Long-term anoxia exposure results in tissue abnormalities. One-day old adult wild-type hermaphrodites were exposed to 72 hours of either normoxia or anoxia followed by 24 hours of recovery in normoxia at 20°C. Anoxia treated animals, in comparison to controls, have an overall more grainy appearance, cavities/vacuoles (white arrows) in the psuedocoloem and head region. In addition, the anoxia-exposed animal shows accumulated fluid (black arrows) around the gut, intestine and pharynx. The unexposed animal has a normally structured intestinal lumen, which forms a large atrium-like cavity (A) at the anterior end of the gut at the pharynx-lumen juncture. In contrast, the anoxia survivor has bends in the pharynx and an intestinal lumen that is constricted and distorted forming irregular jagged kinks. The white line traces the lumen of the gut from the anterior bulb of the pharynx through the first intestinal cell. The intestinal cells of the anoxia-exposed worm are also packed with many very small intracellular globules (white arrowhead). The germline morphology is also affected by the anoxia stress. Asterisks mark the nucleus of some of the oocytes which are abnormally stacked well beyond the gonad bend in the anoxia treated animal compared to presence of syncytial germ cells (labeled GCs) visible in the distal gonad of the control animal. Images are both composites of three individual frames reassembled using Adobe PhotoShop CS. Scale bar = 20 µm.

This is presumably accomplished by either sustaining a homeostatic physiology during the anoxic period or by activating tissue maintenance and repair pathways post-reoxygenation. Tissue maintenace is likely to be a critical factor in stress tolerance and is an as yet unsaturated research area.

Genetic Factors are Associated with Anoxia Tolerance in Adults

Arguably the most well known pathway in the study of anoxia tolerance in *C. elegans* is the insulin/IGF-1-mediated signaling (IIS) pathway. Identification and characterization of the molecular nature of the genes functioning in the insulin-like signaling pathway has revealed the pathway regulates metabolism, lifespan, stress responses and the development of the dauer state, which is a stress-resistant larva in diapause (Gottlieb & Ruvkun 1994; Kenyon *et al.* 1993; Kimura *et al.* 1997; Riddle *et al.* 1981; Tissenbaum & Ruvkun 1998). Much is known about the genes that function in *da*uer *f*ormation respectively designated as the *daf* pathway. The dauer regulatory pathway involves the *daf-2* and *daf-16* genes, which encode the insulin/IGF-1 receptor-like protein and a fork-head transcription factor, respectively (Kimura *et al.* 1997; Larsen *et al.* 1995; Riddle *et al.* 1981). It is thought that DAF-2 interacts with a variety of insulin-like ligands and sends a signal via the AGE-1/PI3/AKT signaling pathway to repress the translocation of the transcription factor DAF-16 into the nucleus (Kenyon 2010). Loss of DAF-2 or reduction in signaling through the IIS pathway allows DAF-16 to be translocated into the nucleus of the intestinal cells where it is thought to link with other nuclear factors to induce expression of a variety of genes in a coordinated manner to promote dauer formation, longevity, fat metabolism, stress response, innate immunity and anoxia tolerance (Kenyon *et al.* 1993; McElwee *et al.* 2003; Mendenhall *et al.* 2006; Murphy *et al.* 2003; Oliveira *et al.* 2009).

The reduction in function *daf-2(e1370)* allele confers a greatly extended lifespan (from 18 to 42 days) for worms are grown early in development at a permissive temperature (functional DAF-2 is present) then shifted to the non-permissive temperature (non-functional DAF-2) at the L4 stage of development or when grown continually through development at

20°C (Kenyon *et al.* 1993). In addition to modulating lifespan, the *daf-2(e1370)* allele also confers various stress responses including long-term anoxia tolerance (Mendenhall *et al.* 2006). Like the longevity phenotype, the anoxia tolerance of the *daf-2(e1370)* mutant is DAF-16-dependent. It is worth noting that factors influencing stress response and lifespan have both common and distinct genetic signals. Further investigation of the overlap in these pathways is of interest to the study of anoxia response and tolerance.

Metabolic Regulation is Linked to Anoxia Tolerance

Several *daf-2* alleles induce a long-term anoxia or high-temperature anoxia/hypoxia survival phenotype; these phenotypes are suppressed by mutations in *daf-16* (Mendenhall *et al.* 2006; Scott *et al.* 2002). An RNAi screen of genes known to be up-regulated by DAF-16 led to the identification of the *gpd-2* and *gpd-3* genes; these genes are nearly identical at the amino acid level and encode two of four glycolytic enzyme isoforms of glyceraldehyde-3 phosphate dehydrogenase (GAPDH; GPD-2/3) (Mendenhall *et al.* 2006). The *daf-2(e1370);gpd-2/3(RNAi)* animal exposed to 1 day of anoxia (28°C) or long-term anoxia (20°C) has a significantly reduced viability in comparison to *daf-2(e1370)* animals. The *gpd-2/3(RNAi)* animals survive short-term anoxia exposure yet are often impaired. These observations demonstrate that the physiological state generated by the *daf-2(e1370)* mutation is capable of protecting somatic tissue during anoxic stress and that *gpd-2* and *gpd-3* suppresses the *daf-2(e1370)* anoxia tolerance phenotype. Other genes involved with glycolysis were subjected to RNAi but did not result in an anoxia sensitivity phenotype suggesting that the anoxia sensitive phenotype due to knockdown of *gpd-2/3* may be due to something other than changes in glycolysis (Mendenhall *et al.* 2006).

The ability to survive periods of environmental stress such as anoxia involves integration of signals emanating from many sources. Transcription and translation are modulated to decrease production of unnecessary gene products while ensuring proper levels of immediately necessary ones. Execution of the appropriate pathways and processes require adequate accessibility to energy, specifically ATP. 5'-AMP-activated protein kinase (AMPK) is one of the energy sensors that monitors cellular AMP/ATP ratios and is conserved between humans and nematodes (Beale 2008). In even small decreases in cellular energy status AMPK will operate on substrates such that anabolic pathways are stimulated and catabolic ones inhibited. Stresses triggering AMPK activation include glucose deprivation, ischemia, oxygen deprivation, exercise and skeletal muscle contraction. However, the key-activating trigger for AMPK is probably starvation, making its primary role to function as a whole body energy balancer (Hardie *et al.* 2006). LaRue and Padilla (2011) evaluated the role of AMPK in anoxia tolerance. While the overall survival rate of wild-type hermaphrodites and *daf-2(e1370)* were not affected by knockdown of *aak*-2 compared to untreated controls there was a significant decrease in the number of animals surviving in an unimpaired condition. However, after 4 days of anoxia *aak-2*(RNAi) suppressed the survival rate and unimpaired phenotype in both wild-type animals grown at 25°C and *daf-2(e1370)* animal (LaRue & Padilla 2010). These observations implicate AMPK as a player in anoxia tolerance and necessary for preventing loss of coordination during anoxia treatment. Interestingly, AMPK activity has also been implicated as a metabolic regulator of lifespan extension in *C. elegans*, particularly under starvation conditions.

Through work with other metazoan species it has been shown that during periods of anoxia a significant rise in the activities of enzymes responsible for glycogen degradation occurs

in liver (Mehrani & Storey 1995). *C. elegans'* simple body design localizes many of the functions accomplished by a variety of organs in higher eukaryotes almost exclusively to the intestine, including carbohydrate storage (McGhee 2007). In the LTA tolerant mutant strain *daf-2(e1370)* metabolism favors production of fat and glycogen in the intestine and hypodermal cells (Kimura *et al.* 1997). LaRue and Padilla (2011) used carminic acid to investigate the effect of anoxia on levels of stored carbohydrates in wild-type and *daf-2(e1370)* individuals. Carminic acid is a fluorescent derivative of glucose that binds to glycogen and trehalose. As expected, animals exposed to LTA showed a decrease in carminic acid staining post anoxia supporting the assumption that carbohydrates stores are utilized as an energy fuel during anoxic stress. They determined that wild-type adults grown at 25°C had higher levels of carminic staining in the intestine than control animals grown at 20°C and significantly elevated survival rates when exposed to 3 or 4 days of anoxia relative to 20°C controls. The long-term anoxia tolerant strains *daf-2(e1370)* and *glp-1(e2141)* both had high levels of carminic acid staining prior to anoxia exposure. RNAi knockdown of *aak-2* suppressed this high level of staining indicating a reduction in stored carbohydrate levels and suppressed the *daf-2(e1370)* LTA tolerant phenotype during exposure to extended anoxic stress (4 days). Together these observations suggest that the level of carbohydrate available to the worm for use as fuel at the time it encounters anoxia can influence its ability to tolerate the stress, and that culture at 25˚C may have preconditioned the worms at least in part by increasing the amount of stored carbohydrate available during anaerobiosis. Interestingly, AMPK activity has also been implicated as the master metabolic regulator of lifespan extension in *C. elegans*, particularly under starvation conditions. There is evidence that aak-2 promotes lifespan extension in the insulin-like signaling (ILS) pathway

mutants such as daf-2 in a daf-16-independent manner (Apfeld et al. 2004). Under conditions of relatively high ATP concentrations, AMPK is inactive and complexes such as mTOR act to pomote cell proliferation, protein translation and actions promoting cell growth (Fig. 1.3).

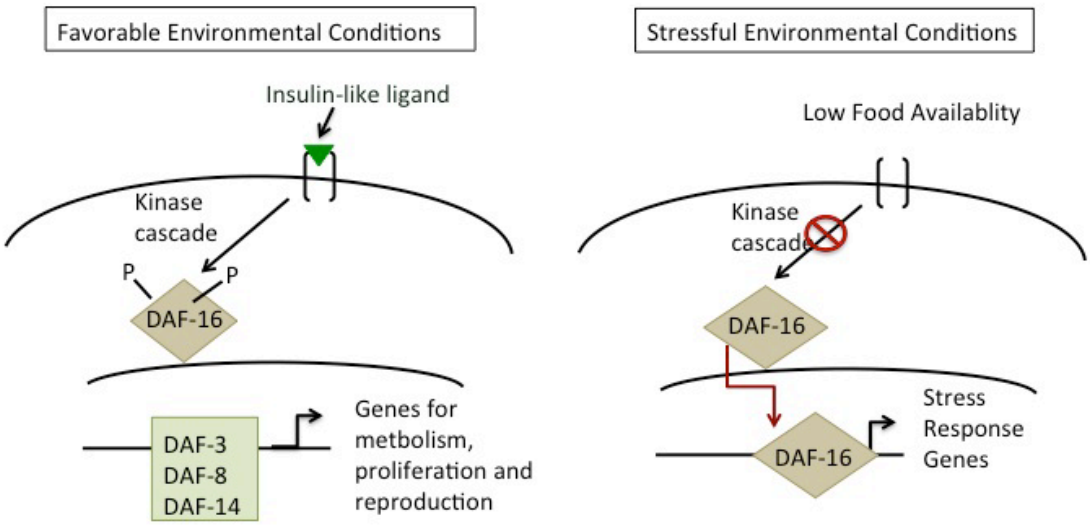

Fig. 1.3 Model of the environmental regulation of DAF-16-induced gene expression. Under favorable environmental conditions such as abundant food resources, space, and oxygen insulin-like ligands bind to intestinal cell DAF-2 receptors triggering flux through a kinase cascade resulting in phosphorylation of DAF-16 and it being sequestered in the cytoplasm. When stressful conditions are encountered, such as low food availability, flux through the insulin-like signaling pathway is reduced and dephosphorylated-DAF-16 relocates into the nucleus where it likely cooperates with other transcription factors to upregulate expression of genes whose products promote stress tolerance.

Under stress conditions that lead to reduced ATP availability and increased AMP concentrations, AMP allosterically activates AMPK which indirectly blocks the action of the complex resulting in activation of cellular processes that promote stress resistance. AMP/ATP ratios do not differ between wild-type and daf-2, suggesting that two nucleotide molecules. Instead, *aak-2* influences lifespan via an the longevity phenotype of *daf-2* mutants is not simply due to an altered ratio of the alternative mechanism. Individuals of the *daf-16(mu86);aak-2(ok524)* genotype have a reduced lifespan compared to wild-type or animals carrying only one

of the mutations. These observations suggest that while both of these genes are required for lifespan extension they are operating in parallel pathways. Again linking longevity and anoxia-tolerance phenotypes, *daf-16* and *aak-2* are required for both lifespan extension and long-term anoxia tolerance. However, loss of *daf-16* completely suppresses the long-term anoxia phenotype of *daf-2(e1370)* while loss of *aak-2* does not reduce overall survival rate following 3 days of anoxia, but significantly affected post-anoxia health. This discrepancy in phenotypes suggests that the two genes operate in different pathways to influence anoxia-tolerance. It will be of interest to determine how the double mutant, *daf-16(mu86);aak-2(ok524)* fairs in long-term anoxia.

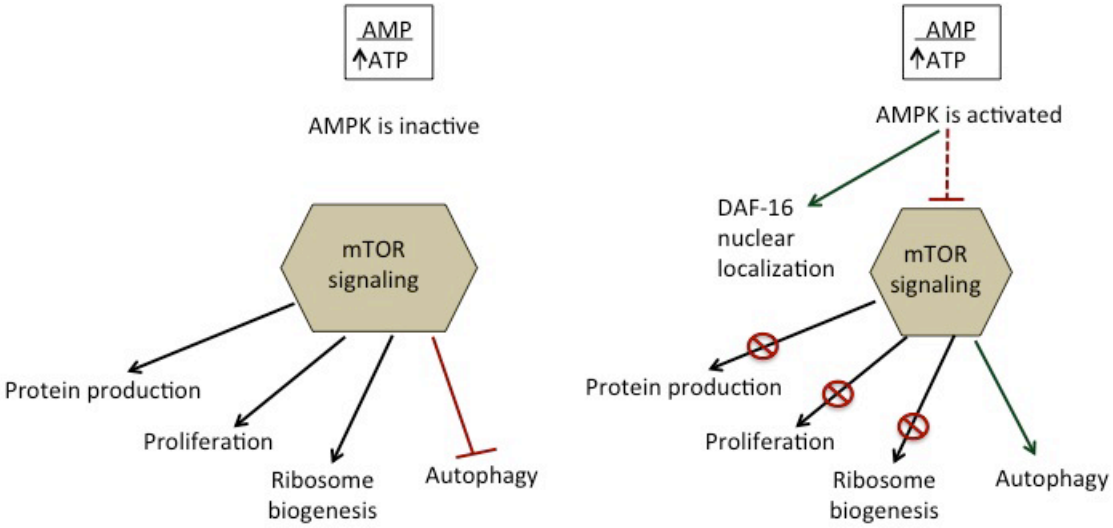

Fig. 1.4 Model of the regulation of stress pathway activation through the action of AMPK. Under conditions of relatively high ATP concentrations AMPK is inactive and the mTOR complex promotes cellular activities that enable cell growth and proliferation. When exposed to stressful conditions AMP levels fall increase and AMP allosterically activates AMPK, which blocks the action of mTOR resulting in activation of cellular activities that promote stress resistance.

Long-Term Anoxia Tolerance is Influenced by Reproductive State

As 1-day old adults, wild-type hermaphrodites actively reproduce via self-fertilization. Ovulation is initiated by binding of MSP (major sperm protein) to surface receptors on the proximal oocyte (Greenstein 2005; Miller *et al.* 2001). Adult hermaphrodites undergoing oocyte maturation, fertilization and ovulation do not survive long-term anoxia (Mendenhall *et al.* 2009). In contrast, sterile animals that do not undergo oocyte maturation or ovulation (ex: *glp-1(e2141)*, *fog-2(q71)* and *fem-3(q20)*) and animals with reduced progeny due to a reduced rate of ovulation (ex: *ksr-1(ku68)*) display long-term anoxia tolerant phenotype that is *daf-16* independent.

The *glp-1* gene encodes an N-glycosylated transmembrane receptor that is one of two members of the LIN-12/Notch family of receptors present in *C. elegans.* Loss-of-function *glp-1* alleles cause germ cells to prematurely enter meiosis preventing formation of self-renewing germ cells. While *glp-1(e2141)* sterile mutants have a somatic gonad they are incapable of producing oocytes and sperm (Crittenden *et al.* 1994; Mendenhall *et al.* 2009). Anoxia survival analysis of 1-day old adult *glp-1(e2141)* hermaphrodites showed them to be highly LTA tolerant with a survival rate of approximately 98% (Mendenhall *et al.* 2009). LaRue and Padilla (2011) were able to partially suppress this LTA tolerant phenotype via RNAi reduction of *aak*-2 expression in the double mutant *glp-1(e2141);daf-16(mu86)*. It is worth noting that in addition to having an LTA tolerant phenotype, sterile *glp-1(e2141)* mutants also have an increased lifespan relative to wild-type animals (Arantes-Oliveira *et al.* 2002). In contrast to *glp-1* the gonochoristic mutant *fog-2*(*q71*) strain produces females incapable of producing self-sperm and fertile males. The LTA tolerant phenotype of the unmated *fog-2(q71)* is suppressed by mating

with a fertile male, supporting the theory that the maternal soma is under the regulatory control of the germline. Exceptions to the observation that sterility induces LTA tolerance are known. The sterile strains *spe-9(hc52ts*) and *fer-15*(*hc15*) are capable of completing the initial steps of oocyte maturation but produce no viable offspring, yet both strains are LTA sensitive (survival rate= 23.4% and 2.6%, respectively). The sensitivity of these strains is presumably due to an altered physiology triggered in the somatic tissues in response to signals originating in the germline.

Long-Term Anoxia Tolerance correlates With Longevity

C. elegans has been used as a model system to examine the relationship between stress tolerance and longevity (Antebi 2011). Genetic studies have identified several processes that regulate stress response and lifespan including caloric intake, mitochondrial respiration, insulin-IGF-1 (IIS), and Jun N-terminal kinase (Kaeberlein *et al.* 2006). Mutations in the insulin-like signaling pathway and TOR signaling networks are known to play a role in resistance to metals, oxidative stress and longevity (Barsyte *et al.* 2001). While pathways involved in these phenotypes have been identified in *C. elegans*, few down-stream effector molecules have been identified. Interestingly, while the mutant strains *fog-2(q71)*, *daf-2(e1370)* and *glp-1(e2141)* are all long-term anoxia tolerant, only the latter two are also long-lived (Arantes-Oliveira *et al.* 2002)

It is of interest to note here that a relationship between diet, longevity and hypoxia (5% O_2) tolerance has been detected in *Drosophila* (Vigne & Frelin 2010). Flies fed high protein diets showed reduced longevity under chronic hypoxia. These effects were mimicked by individual

amino acids and polyamines and abrogated by inhibitors of polyamine synthesis. In a supporting subsequent study (Vigne *et al.* 2009) strong dietary restrictions were found to protect flies against anoxia and reoxygenation stresses. Mutations in the *eat* genes of *C. elegans* are thought to result in restricted caloric intake and have been shown to increase lifespan (Lakowski & Hekimi 1998). While the aforementioned observations link dietary restriction to increased lifespan in worms and flies, and increased lifespan to anoxia survival in worms, it remains unclear how the three are interlinked in establishing the physiological state of *C. elegans*. Some insight into this question may be found by examining the signaling mechanism by which germline depletion regulates longevity. In an RNAi screen for suppressors of lifespan extension in *glp-1(e2141),* loss of *kri-1* gene expression was found to dramatically shorten *glp-1* longevity while having no effect on wild-type life span (Berman & Kenyon 2006). Loss of *kri-1* did not shorten the lifespan of the insulin-signaling mutant *daf-2(e1370)* and instead resulted in a further lifespan extension and Berman and Kenyon concluded that its role is likely restricted to the mechanism by which germline depletion influences lifespan extension. In wild-type and germline depleted animals *kri-1* gene is expressed in the intestine and pharynx. Absence of KRI-1 was associated with reduced nuclear localization of DAF-16 in germline depleted animals. Berman and Kenyon have found that mutations in genes involved in lipophilic-hormone signaling, specifically *daf-9* and *daf-12*, also resulted in shortening of the extended lifespan of germline depleted animals. The authors proposed that along with KRI-1, DAF-9/450 and DAF-12/NHR operate to promote DAF-16 localization to the nucleus in sterile g*lp-1(e2141)* individuals. Identification of these proteins as either required for or contributing to the longevity phenotype of the *glp-1(e2141)* strain brings to bear the question of whether

these proteins are may also acting to regulate cellular responses to germline depletion thus promoting the strain's high LTA-tolerance phenotype. However, how this pathway links sterility, longevity and anoxia tolerance is not fully understood.

Anoxia Tolerance is Sex Influenced

The overwhelming majority of stress response studies, at the genetic and cellular level, have been conducted in adult hermaphrodites. This is likely due to the ease in obtaining and maintaining hermaphrodite animals in comparison to males. Yet, analysis of males and their response to stress may provide insight into the understanding of mechanistic responses to and survival of anoxia. The wild-type male and hermaphrodite differ in several respects aside from the obvious sex-differentiated phenotypes such as different germline structure and function. For example, the lifespan of males is shorter than that of hermaphrodites and is dependent upon whether they are grown in isolation or in groups of other males (Gems & Riddle 2000). Males grown in solitary have a longer lifespans than males cultured as groups, indicating that male-male interactions reduce lifespan.

LTA survival also differs between wild-type adult hermaphrodites and males. At 1-day-old wild-type and *daf-16(mu86)* mutant hermaphrodites survive LTA exposure at approximately 10% and are considered LTA sensitive. In contrast, wild-type and *daf-16(mu86)* males survive LTA with a viability >98% (Mendenhall *et al.* 2009). The males maintain normal motility and demonstrate an unimpaired phenotype after long-term anoxia exposure. Males raised in the presence of hermaphrodites and likely to have had an opportunity to mate maintained a higher LTA tolerance relative to age matched hermaphrodites indicating that mating does not

compromise the LTA phenotype of males. The *tra-2(q276)* mutant was used to show that the long-term anoxia survival phenotype observed in males is dependent on male phenotype rather than male genotype. The *tra-2(q276)* mutant is phenotypically male but instead of having the male genotype (X0) is genotypically hermaphroditic (XX). The *tra-2(q276)* animals survived LTA similarly to wild-type males indicating that something inherent about the male phenotype confers anoxia tolerance.

Research Focus

The central hypothesis of the work related in this document is that when in a proper physiologic condition adult hermaphrodites are primed to tolerate severe oxygen deprivation at a high rate and in an unimpaired condition (LTA-tolerant-state). The LTA-tolerant state is characterized by ability to survive the period of oxygen deprivation, recover normal movement and function upon reoxygenation, and execute the processes required to preserve critical tissue morphology during the anoxic stress. To better understand when during their lifespan adult worms are in an anoxia-tolerant state, I evaluated the effect of aging on long-term anoxia viability and post-anoxia motility in wild-type adults and mutant strains identified as either anoxia tolerant or anoxia sensitive (Chapter 2). In order to determine if the anoxia-tolerant physiological state is promoted in adult hermaphrodites by previous exposure to anoxic stress, I compared viability and motility of aged hermaphrodites to that of individuals preconditioned with 1 or more short exposures of anoxia. Finally, to investigate whether adult age is a factor in the genetic requirements for LTA-tolerance I assessed the effect of loss of *kri-1* and *daf-12* on the post-recovery viability and motility of sterile aging *glp-1(e2141)*.

CHAPTER 2

THE EFFECT OF AGING ON LONG-TERM ANOXIA TOLERANCE

Introduction

C. elegans *and Anoxia Tolerance*

After remaining fairly constant for most of human history, average life expectancy has nearly doubled in the past century. The portion of America's population considered to be older will double in the next three decades. Consequently, research targeting a better understanding of the aging process is among the funding priorities of the US federal government. Through the use of model genetic organisms progress has been made in understanding the genetic underpinnings of the anatomical and physical changes associated with an aging soma. Oxygen deprivation is a common feature of diseases that affect the elderly. By understanding the genetic requirements and molecular mechanisms utilized by organisms that naturally encounter low oxygen environments we may development treatments that prevent or delay the effects of oxygen deprivation associated with human diseases. Currently, it remains unclear how aging affects the cellular mechanisms that regulate response to oxygen deprivation. *C. elegans* is well suited for investigating such molecular mechanisms by having a short generation time, a transparent body, and availability of a wide range of genetic tools (mutant strains, techniques, etc.). *C. elegans* hermaphrodites and males (1 day old) survive 24h of anoxia at high rates (>90% survival rate) with no visible loss of body movement (Padilla *et al.* 2002). However, hermaphrodites exposed to LTA have a low survivorship, and survivors are noticeably impaired (Mendenhall *et al.* 2006). Mutant alleles resulting in high survival rates and unimpaired phenotype following 72h of anoxia have allowed us to identify specific physiologic processes

that influence anoxia tolerance (Mendenhall *et al.* 2006, Mendenhall *et al.* 2009, LaRue & Padilla, 2011). For example, reduction in embryo production and reduced signaling through the insulin/IGF pathway both confer LTA tolerance in 1-day old adults. Some of the mutations that confer LTA-tolerance also operate to extend lifespan (Kleemann & Murphy 2009; Panowski & Dillin 2009; Tatar *et al.* 2003). Within these pathways mutant alleles have been identified that either inhibit or increase anoxia survival. Until now work with strains carrying these mutant alleles has focused on examining embryos, larvae and 1-day old adults (Mendenhall *et al.* 2006; Mendenhall *et al.* 2009; Padilla & Roth 2002; LaRue & Padilla 2011). Consequently, whether these alleles confer anoxia tolerance at later stages of adulthood is unknown. To better understand the role of signaling pathways in anoxia tolerance I examined aging adults carrying alleles (described below) previously identified as either conferring LTA-tolerance or LTA-sensitivity in young adults (Table 2.1). Oxygen deprivation is a common characteristic of diseases that affect the elderly. An understanding of how signaling pathways in model organisms regulate anoxia tolerance has potentially broad medical implications through development of preventative treatments and therapies for human diseases associated with oxygen deprivation.

Notch/GLP-1 Signaling

The *glp-1* gene encodes a member of the LIN-12/Notch family of receptors that is required for cell fate specification in germline and somatic tissues and is essential for maintaining mitotic proliferation of nuclei and germ cells renewal in the distal gonad (Austin & Kimble 1987; Priess 2005).

Table 2.1. Categorization of strains by anoxia-tolerance at day 1 of adulthood.

Anoxia Sensitive: *Long-term anoxia survival rate <10%*	
N2	Wild-type isolate, Bristol, England
CB4856	Wild-type isolate, Hawaii, USA
daf-16(mu86)	Transcription factor required for *daf-2* longenvity and LTA tolerance
aak-2(gt33)	Catalytic subunit of AMPK; activated by AMP; required for *daf-2* longevity and dauer formation
Anoxia Tolerant: *Long-term anoxia survival rate >90%*	
daf-2(e1370)	Insulin receptor homologue; regulates lifespan, dauer formation and stress resistance; signals through *daf-16*
glp-1(e2141)	LIN-12/Notch family receptor; required for germline stem cell renewal
fog-2(q71)	Regulates germline specific translation; required for spermatogenesis in hermaphrodites

When grown at the nonpermissive temperature (25˚C) animals carrying the temperature-sensitive *glp-1(e2141)* allele develop a somatic gonad but do not develop a functional germline due to germ cells prematurely entering meiosis and eventual exhaustion of stem cell nuclei (Austin & Kimble 1987). Sterility in the *glp-1(e2141)* strain confers both increased longevity, enhanced innate immunity and long-term anoxia tolerance (Alper *et al.* 2010; Mendenhall *et al.* 2009; LaRue & Padilla 2011). In contrast to the absence of a germline in *glp-1(e2141)* animals, the sterile *fog-2(q71)* strain develops a germline containing viable oocytes, although incomplete spermatogenesis results in no production of viable sperm, thus oocytes are not self-fertilized (Schedl & Kimble, 1988). FOG-2 is active during the L1 to L4 larval stages of development promoting spermatogenesis (Clifford *et al.* 2000). Hermaphrodites carrying the *fog-2(q71)* null allele are morphologically female but do not ovulate and are sterile unless mated to a fertile male. In the unmated state *fog-2(q71)* females are LTA-tolerant (Mendenhall *et al.* 2009).

The *daf-2(e1370)* and *glp-1(e2141)* strains have elevated carbohydrate stores as detected by carminic acid staining (LaRue & Padilla 2011). The authors reported a correlation between LTA tolerance and elevated levels of fuel source carbohydrates. Mutations reducing the catalytic activity of AMPK (AMP-activated protein kinase, an energy sensor) along with the *daf-16(mu86)* null allele suppressed the high carbohydrate stores of *glp-1(e2141)* and resulted in a decrease in the percentage of LTA survivors with an unimpaired phenotype. Furthermore, the drug metformin, which induces a dietary-restriction like state in animals, is known to activate AMPK in mammalian cell cultures. Worms fed metformin had an enhanced survival rate when exposed to LTA. The influence of *aak-2* on anoxia tolerance is linked to the activation of cellular stress responses that are under the control of the transcription factor *daf-16*. LaRue and Padilla (2011) concluded that DAF-16 and specific components of AMPK (*aak-2*, *aakg-2*, *aakb-1*, *aakb-2*) function in parallel to enhance anoxia survival in *glp-1* mutant animals.

Insulin-like Signaling

The ability to survive extended periods of anoxia is arguably un-adaptive if the animal is unable to resume normal activity such as foraging and reproduction after reoxygenation. It is reasonable to expect that adaptive mechanisms have evolved that protect or repair tissues when damage is incurred during stress. Specific genes have been recognized as required for post-anoxia health and they function in diverse biological processes. For example, *gpd-2* and *gpd*-3 are necessary for tissue maintenance during anoxic stress and function in the glycolytic pathway while *hyl-2*, a ceremide synthase required for short-term anoxia survival, functions in a

seemingly unrelated manner to provide proper length fatty acyl chains which serve as the precursors of membrane sphingolipids and cell signaling molecules.

The *daf-2* gene encodes a receptor tyrosine kinase that is the *C. elegans* insulin/IGF receptor ortholog (Gems *et al.* 1998). DAF-2 activity is required for a number of processes including development, dauer formation, adult longevity, reproduction, and stress resistance (Kenyon *et al.* 1993; Larsen *et al.* 1995). The *e1370* allele of *daf-2* confers robust anoxia tolerance including high survival rates and low impairment following 5 days of anoxia (Mendenhall *et al*. 2006). In contrast to the sterile *glp-1(e2141)* and *fog-2(q71)* strains, *daf-2(e1370)* when cultured at a permissive temperature (20˚C) has a brood size nearly equal to wild-type (Tissenbaum & Ruvkun, 1998). The DAF-2 receptor signals through a pathway that regulates the single *C. elegans* forkhead box O (FOXO) homologue encoded by the *daf-16* gene. DAF-16 is a transcription factor whose activity regulates multiple processes including dauer formation, longevity, fat metabolism, innate immunity and stress response (Ogg *et al.* 1997). DAF-16 is kept inactive by phosphorylation resulting from flux through the insulin/IGF-1-mediated signaling (IIS) pathway. The physiological differences between these two strains are numerous, for example *daf-2* mutants accumulate fat and glycogen while *glp-1* mutants do not, *daf-2* mutants are dauer-constitutive at 25°C but *glp-1* mutants are not. When flux through the insulin-signaling pathway is reduced (as in *daf-2(e1370)*) the dephosphorylated form of DAF-16 is allowed to localize to the nucleus and upregulate stress response specific gene expression. For example, *daf*-2 mutant animals have a significantly higher LC_{50} concentrations of cadmium and copper than wild-type controls and increased mRNA leveles of metallothionein, a major player in metal detoxification (Barsyte *et al*. 2001). These observations are consistent with a

model in which the insulin-signaling pathway determines life span through regulation of stress protein genes.

Presence of the *daf-16(mu86)* null allele in a *daf-2(e1370)* genetic background effectively suppresses LTA-tolerance (Mendenhall *et al.* 2009). In addition to *daf-16*, the *aak*-2 gene is required for the robust anoxia tolerant phenotype seen in the *daf-2(e1370)* strain (Larue & Padilla 2011). In *C. elegans*, the *aak-2* gene encodes 1 of 2 catalytic alpha subunits of AMP-activated protein kinases (AMPKs). AAK-2 functions in a variety of cellular processes; as a sensor of environmental stress helping to negatively regulate germline proliferation during dauer formation, regulator of energy level signals (AMP:ATP ratio), and likely works in parallel with DAF-16 to regulate lifespan through *daf-2*-mediated insulin signaling (Apfeld 2004). Reduction in *aak-2* expression via RNAi in a *daf-2(e1370)* mutant genetic background resulted in a significant increase in the proportion of survivors with an impaired phenotype (LaRue & Padilla 2011). These observations led me to question how loss of *aak-2* would affect LTA-tolerance in aging wild-type adults.

Influence of Growth Temperature

The benefit of therapeutic hypothermia for traumatic brain injury in humans has already been demonstrated in national studies (Clifton *et al.* 2009). A reduced culture temperature has been shown to have a beneficial effect on longevity and to delay onset of senescence in invertebrates (*Drosophila melanogaster* and *C. elegans)* and in a vertebrate fish (*Cynolebias adloffi*) (Liu & Walford 1966). Reduced core body temperature was also hypothesized to be an influential factor in the extended longevity and delayed aging seen in response to calorie

restriction in poikilotherms (Conti 2008). In light of these observations I asked if a modest reduction in culture temperature (from 20˚C to 15˚C) could reduce the detrimental effects of anoxia exposure and increase LTA-tolerance in aging wild-type adults. I found that worms grown from embryos at 15˚C were significantly less LTA-tolerant than 20˚C controls. Indicating that reduced culture temperature fails to precondition *C. elegans* for LTA stress tolerance.

Aging and LTA Tolerance

Conditions of oxygen deprivation are frequently encounterd by the elderly due to health factors such as cerebral ischemia, hypertension, atherosclerosis, reduced heart function, reduced physical activity and other causes (Strasser & Fischer 1995). To date anoxia tolerance studies utilizing adult *C. elegans* have been conducted at day 1 of adulthood. To better understand the combined genotypic and phenotypic effects of aging on long-term anoxia stress tolerance I exposed adults of strains identifed as either LTA-sensitive or LTA-tolerant at day 1 of adulthood to LTA between day 1 and day 9 of adulthood. I found that age influences LTA-anoxia tolerance in a strain-dependent manner. Strains that had similar levels of anoxia resistance at day 1 of adulthood also shared some common effects on LTA-tolerance as they aged. Indicating a role of genotype in formation of an oxygen deprivation tolerant physiological state.

Wild-type Isolates and LTA tolerance

Wild-type isolates from widely separated geographic locations are genetically divergent. For example, the Hawaiian isolate, CB4856, carries a single nucleotide polymorphism in the *tra-3* which attenuates larger growth when grown at a reduced temperature (Kammenga *et al.*

2007). Of the numerous wild-type isolates only the N2 strain isolated from Bristol, England, has been extensively investigated for anoxia tolerance. To gain insight into the subtle effects of natural genetic variation on LTA-tolerance I compared the survival rates and level of post recovery impairment between the wild-type isolate strains N2 and CB4856. I found that aging affected the strains differently, with the CB4856 isolate tolerating LTA better than N2, suggesting that wild-type isolates from diverse natural in the evolution of stress response. These differences in LTA tolerance provide a natural system in which to study the genetic requirements for oxygen deprivation tolerance.

Results

The effect of aging on LTA tolerance in *C. elegans* has not been previously investigated. To understand the interaction of genotype with age, both LTA-tolerant and LTA-sensitive stains (determined by survival rate at day 1 of adulthood) were assayed for survival rates and impairment following LTA exposure between day 1 and day 11 of adulthood (Table 2.1). Wild-type (N2) hermaphrodites and have a mean lifespan of 11.8 and 11.1 days, respectively. However, the lifespan of males grown in the presence of hermaphrodites is shortened to 8.1 days (Van Voorhies 1992). To establish a reference graph of anoxia tolerance, wild-type N2 hermaphrodites were exposed to LTA at each day of adulthood between day 1 and day 11 (Fig. 2.1). Hermaphrodites and males of other strains were exposed to anoxia every-other day between day 1 and day 9. The effect of aging varied within and between the LTA-tolerant and LTA-sensitive groups supporting the idea that aging affects LTA-tolerance in a gene-dependent manner.

The Effects of Aging on LTA-Survival Rates Vary Among Wild-type Isolates

In accordance with previously published results, N2 hermaphrodites LTA-survival rates were low at day 1 (Fig. 2.1). However, survivorship significantly increased at day 3 and reached an identifiable peak at day 5 followed by a gradual decline through day 11. Survival rates remained high through day 10 raltive to day 1. Percentage of LTA-survivors displaying motility impairment did not have an associated age-specific change and did not differ from day 1 percentage until day 11. To determine how age affects anoxia tolerance in other *C. elegans* wild-type isolates I examined adults of the Hawaiian isolate (strain CB4856). Aging CB4856 hermaphrodites were less tolerant of LTA than N2 hermaphrodites (Fig. 2.2).

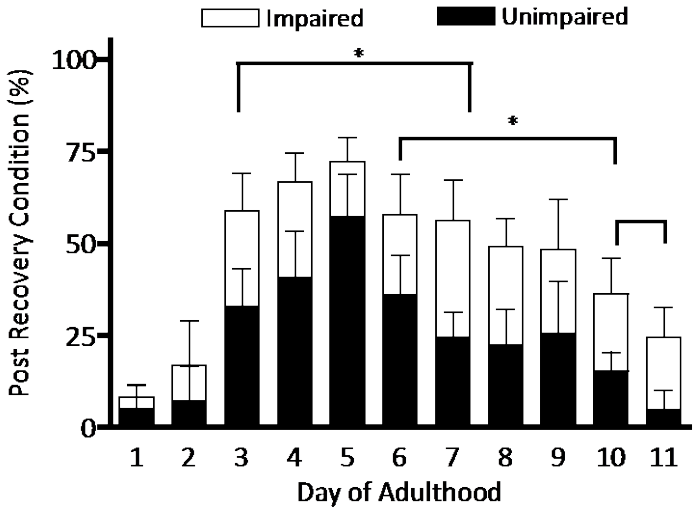

Fig. 2.1 Long-term anoxia tolerance of N2 hermaphrodites is affected by aging. N2 hermaphrodites raised to the specified adult age were exposed to 72h of anoxia (20˚C) followed by a 24h recovery in normoxia and survivors were examined for post treatment condition. Survivorship significantly increased at day 3 compared to day 1 and remained higher through day 10. For all experiments the total number of animals assayed is N>150 from three or more independent experiments; error bars represent standard deviation. Experiment ANOVA ($F_{10,94}$ = 30.16, p<0.0001). Brackets denote groups with statistically similar survival rates, α= 0.05; * denotes significant difference in survival from day 1, Tukey's p<0.05.

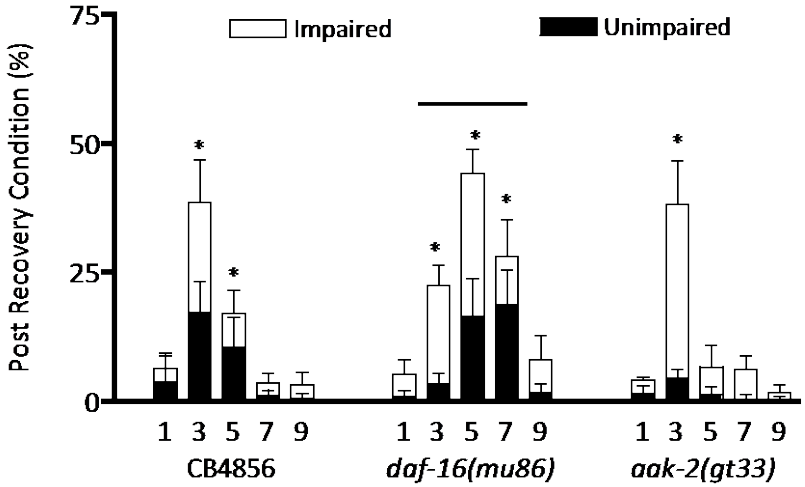

Fig. 2.2 Long-term anoxia tolerance varies between CB4856 and previously identified LTA sensitive strains. CB4856 (wild-type Hawaiian isolate), *daf-16(mu86)*, *aak-2(gt33)* were raised to the specified age and exposed to 72h of anoxia followed by recovery in air. LTA-sensitive strains have similar patterns of LTA-tolerance with increasing age. X-axis values indicate day of adulthood at LTA exposure. For all experiments the total number of animals assayed is N>160 from three independent experiments; error bars represent standard deviation. * indicates significant difference in survival from day 1 within each strain, $p<0.001$, Bar indicates significantly reduced survival rate compared to age-matched CB4856 and *aak-2(gt33)*, $p<0.001$ (One-Way ANOVA and Tukey's Multiple Comparison Test).

Survival rates and levels of impairment for the two wild-type isolates were similar at day 1. Like N2, the CB4856 strain experienced a multi-fold increase in survivorship at adult day 3, however, rates were consistently lower than age-matched N2. The increase in survivorship at day 3 marked the peak for CB4856 (compared to day 5 for N2). By day 5 survival rates for CB4856 had returned to levels equivalent to day 1 and remained low through day 9 (compared to day 11 for N2). Despite survival rates being lower for aged CB4856, the proportion of unimpaired survivors did not differ from age-matched N2, until day 7 when CB4856 displayed an increased impairment level. These data suggest an as yet undescribed geographic variation in anoxia tolerance in wild-type isolates.

The Effect of Aging on LTA tolerance Varies Among LTA sensitive Mutant Strains

Unlike N2, CB4856 or *aak-2(gt33)* strains the highest proportion of *daf-16(mu86)* survivors with an unimpaired phenotype occurred at an older age than the peak survival and after survival rates had begun to decline. While this may be an experimental artifact it may also indicate that survivors have an increased ability to maintain tissue integrity at the older age. The observation that aging *daf-16(mu86)* adults are more LTA tolerant than aging *aak-2(gt33)* adults suggests that management of AMP/ATP ratios may be more critical for LTA tolerance than stress pathway activation. This is a reasonable hypothesis considering that AMPK has been implicated in upregulation of DAF-16 activity. Together with the observations that animals lower their overall energy demand and enter into a steady-state ATP flux, these data emphasize the importance of energy availability and management in anoxia survival.

Studies have documented the need for specific genes to establish a stress-resistant phenotypes. For example, at day 1 of adulthood a functional *daf-16* gene is needed for the anoxia viability of the insulin-like receptor mutant *daf-2(e1370)*. Here I present the first evidence that age of the animal influences the degree to which gene loss impacts LTA viability. In a wild-type genetic background loss of either *daf-16* or *aak-2* is associated with low LTA viability at day 1. However, at various days of adulthood LTA tolerance is increased multiple fold in both *daf-16(mu86)* and *aak-2(gt33)* mutant strains (Fig. 2.2). However, both mutant strains were significantly more sensitive to LTA at all ages analyzed compared to N2 hermaphrodites, indicating that the loss of each gene lowers LTA viability. An interesting observation is that although loss of either *daf-16(mu86)* or *aak-2(gt33)* did not prevent an age associated increase in LTA survivorship, there is still evidence that their loss suppresses the LTA viability of a wild-

type animals. This is most evident by the early decline in survivorship back to day-1-levels in the mutant strains compared to age-matched N2. These observations bring to the forefront the influence aging has on the physiology of an organism, and emphasize the importance of considering age as a variable in genetic studies.

LTA-Tolerant Mutant Strains Maintain High LTA Survival Rates and Unimpaired Motility Through Day 5 of Adulthood

Consistent with previously published observations the *daf-2(e1370)* mutant strain is seemingly unaffected by a long-term anoxia exposure at day 1 of adulthood (Fig. 2.3). Survival rates for aging *daf-2(e1370)* did not decrease until day 7 (Fig. 2.3).

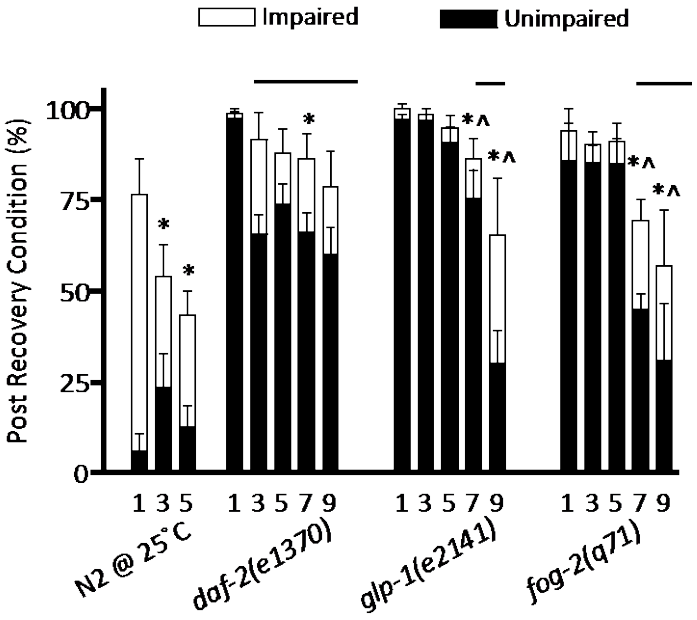

Fig. 2.3 The effect of aging on long-term anoxia tolerance for strains previously identified as LTA-tolerant. Hermaphrodites of *daf-2(e1370)*, *glp-1(e2141)* and *fog-2(q71)* were raised to the specified age and exposed to 72h of anoxia followed by recovery in air. Analyzed strains have similar patterns of LTA-tolerance with aging. X-axis values represent day of adulthood at LTA exposure. For all experiments the total number of animals assayed is N>150 from three independent experiments; error bars represent standard deviation. * indicates significant difference in survival from day 1 within each strain, $p<0.001$; ^ indicates survival rates not significantly different from age-matched N2 hermaphrodites (see Figure 2.1); Bars indicate

significant reduction in percent of survivors with an unimpaired motility phenotype, $p<0.05$ (One-Way ANOVA and Tukey's Multiple Comparison Test).

However, the percentage of survivors with an unimpaired phenotype decreased at day 3 and remained level through day 9, indicating that the stress-tolerant physiological state due to loss of *daf-2(e1370)* is affected by aging. The sterile *glp-1(e2141)* and *fog-2(q71)* animals displayed delayed onset of the anoxia-induced impairment compared to *daf-2(e2141)*. Reproductive characteristics of the *daf-2(e1370)* strain has been extensively studied (Larsen *et al.* 1995). When grown at 15˚C the *daf-2(e1370)* mutant produces a brood that is 14% smaller than N2 controls grown at the same temperature. In addition, *daf-2(e1370)* animals produce approximately 40% fewer embryos at day 1 and day 2 of adulthood compared to age-matched N2. While the number of embryos laid per day by *daf-2(e1370)* animals increased at day 2, the maximal number laid was seen at day 3. The increase in embryo production for the *daf-2(e1370)* adults corresponds to the significant increase in impairment of LTA survivors. This suggests that reduced fecundity of *daf-2(e1370)* animals is likely a contributing factor to the the strain's high LTA tolerance and that the strain's ability to tolerate extended periods of anoxia is affected by reproductive effort.

Although fertile, the physiological state of *daf-2(e1370)* animals is LTA-tolerant. At day 9 survival rates for *daf-2(e1370)* remained higher than either *glp-1(e2141)* or *fog-2(q71)*, suggesting the physiological state generated by reduced insulin-signaling is less sensitive to the effects of aging than the physiological state generated by sterility.

Mating Suppresses Anoxia Tolerance at Day 3 of Adulthood

It is possible that 3 and 5-day old hermaphrodites survive anoxia better than younger adults due to reduced fecundity, and therefore reduced ovulation. To test this hypothesis I placed 2-day old N2 or *fog-2(q71)* hermaphrodites with 1-day old same-strain males and allowed them to mate over a 24h period. I then exposed the mated hermaphrodites, confirmed by presence of eggs on the plate, along with unmated 3-day old control animals to long-term anoxia. Mated animals of both strains that were producing progeny had reduced survival rates and increased levels of impairment compared to unmated controls (Fig. 2.4). These data support the idea that 3 and 5-day old adult hermaphrodites survive anoxia better than younger adults at least in part due to reduction in progeny production.

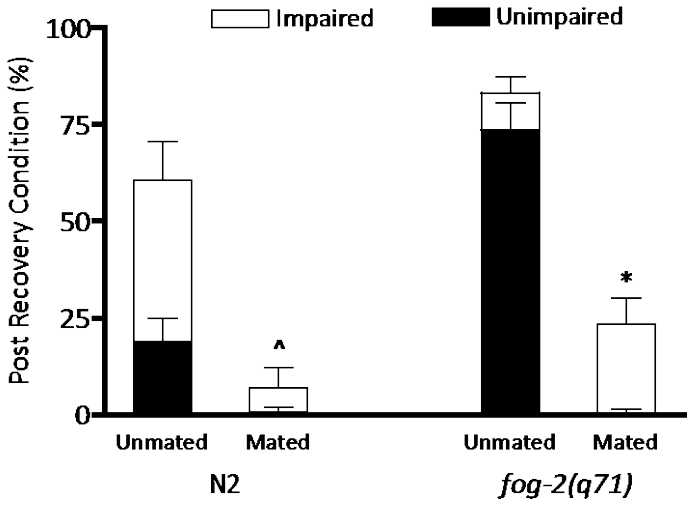

Fig. 2.4 Mating suppresses LTA tolerance in reproductively senescent wild-type adults. At day 2 of adulthood wild-type and sterile *fog-2 (q71)* hermaphrodites were placed either individually (Unmated) or with 5 males of the same strain (Mated) for 24h followed by exposed to 72h of anoxia at day 3 of adulthood and allowed to recover in air. Mating suppressed the increase in survival rate seen at day 3 for N2 hermaphrodites (see text) and the anoxia resistant phenotype of sterile *fog-2(q71)*. For all experiments total number of animals tested is N>100 from three independent experiments; error bars represent standard deviation. ^ and * indicate significant difference in survival compared to unmated controls within each strain, $p<0.05$, $p<0.001$, respectively (One-Way ANOVA and Tukey's Multiple Comparison Test).

Reduced Temperature Culture Conditions Do Not Enhance LTA-Tolerance

C. elegans reared at lower temperatures displayed an inverse relationship between metabolic rate and lifespan and live significantly longer than worms cultured at 20˚ or 25˚C (Van Voorhies & Ward 1999). To investigate the effect of reduced culture temperature on anoxia tolerance wild-type hermaphrodites were maintained for several generations at 15˚C and exposed to long-term anoxia between day 1 and day 5 of adulthood then assayed for post-recovery survivorship and motility. For all ages examined, reduced culture temperatures did not impart an enhanced anoxia tolerance compared to animals maintained at 20˚C (Fig. 2.5). Adults grown at reduced temperture and exposed to LTA at day 1 or day 2 had survival rates similar to age-matched controls cultured at 20˚C. The increase in survivorship seen at day 3 for 20˚C control animals was delayed in for animals grown at 15˚C until day 4 and corresponded to the onset of reproductive senescence.

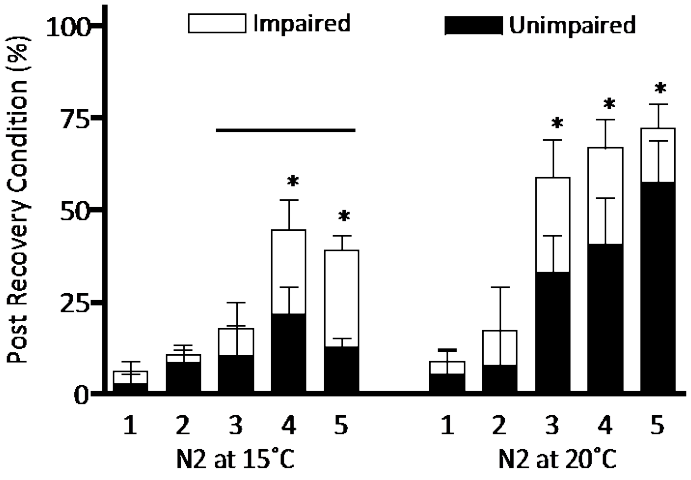

Fig. 2.5 Reduced culture temperature delays the age-associated increase in anoxia tolerance and suppresses LTA survival rates. The age-associated increase in long-term anoxia survivorship was delayed by one day for animals raised at 15˚C compared to controls raised at 20˚C. At both culture temperatures the dynamic increase in anoxia tolerance is correlated with cessation of self-fertilization and embryo production. X-axis values indicate day of adulthood at LTA exposure. For all experiments total number of animals tested is N>150 from three independent

experiments; error bars represent standard deviation. * indicate significant difference in survival compared to day 1 within each strain, $p<0.001$, Bar indicates significantly reduced survival compared to age-matched animals grown at 20˚C (One-Way ANOVA and Tukey's Multiple Comparison Test).

Consistent with previous reports, I observed that N2hermaphrodites grown at reduced temperature laid few eggs on day 4 and no eggs on day 5 (Larsen *et al.* 1995). Therefore, I compared LTA-survival rates of animals grown at 15˚C with survival rates of control animals that were 1 day-older. The survival rate at adult day 4 for animals grown at 15˚C was lower, although not significantly different, compared to day 3 controls reared at 20˚C. However, unlike animals grown at 20˚C the survival rate of animals grown at 15˚C did not continue to rise through day 5.

Aging Affects Anoxia-Survivor Phenotype in a Gene Specific Manner

Aging is characterized by onset of recognizable declines in somatic homeostasis such as reduced muscle function and reduction in proteome stability (Morely *et al.* 2002; Moronetti Mazzeo *et al.* 2012). In light of this I asked whether aging influences the post-recovery phenotype of LTA-survivors. To address this question I compared the motility phenotype of survivors exposed to LTA between adult day 1 and adult day 5. While young adults of both wild-type isolates examined, N2 and CB4856, had low overall survival rates at day 1 of adulthood, the percentage of survivors displaying unimpaired phenotype was greater than 56% for both strains and did not significantly differ for any age analyzed through adult day 9. Similarly, the percentage of unimpaired survivors in the *daf-16(mu86)* and *aak-2(gt33)* strains did not significantly vary across any age analyzed except for *daf-16(mu86)* at day 7 which had a

substantially decreased impairment level compared to other ages analyzed within the strain (Fig. 2.6). It should be noted that the standard deviation for the means of each age analyzed for all strains were large which reduced the likelihood of detecting a difference in impairment between the ages if one exist.

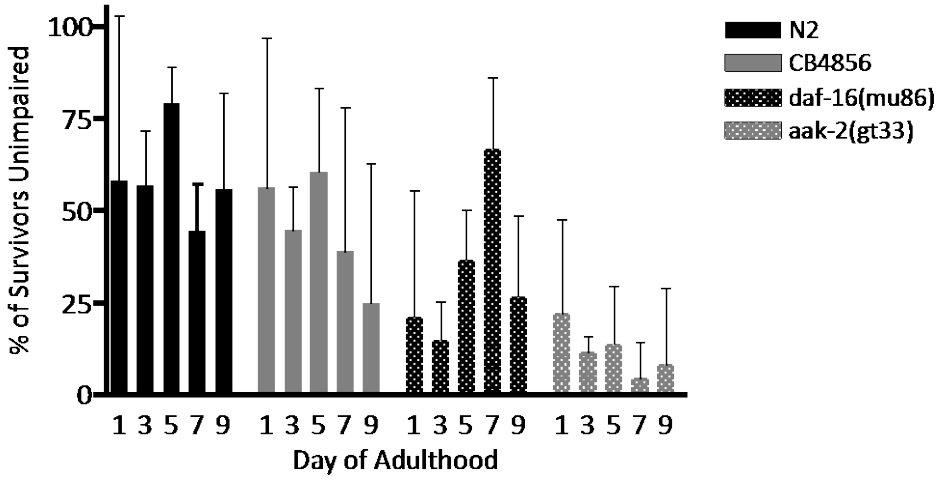

Fig. 2.6 Post long-term anoxia impairment phenotype is unaffected by aging in LTA-sensitive strains. Survivors of N2, CB4856, *daf-16(mu86)*, and *aak-2(gt33)* strains exposed to long-term anoxia at various adult ages were visually assayed for level of impairment. These data are a subset of dataset presented in Fig. 2.1 and Fig. 2.2.

For all LTA-tolerant strains analyzed the portion of survivors with an unimpaired phenotype was high at adult day 1 compared to N2 controls and remained consistently high through at least adult day 5 (Fig. 2.7). At day 9 *glp-1(e2141)* survivors showed a significant increase in impairment compared to day 1 within the same strain. Among the LTA-tolerant strains the effect of aging was seen earliest in the *fog-2(q71)* strain which had a 29% increase in impairment when exposed to anoxia at day 7 followed by a 40% increase when exposed to anoxia at day 9 when compared to animals exposed to anoxia at adult day 1. This increase in impairment beginning at day 7 was not seen in the LTA-sensitive strains.

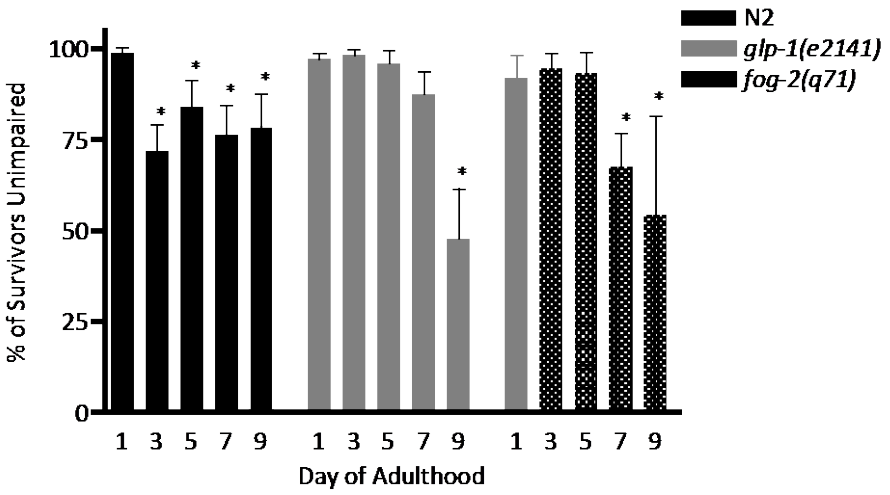

Fig. 2.7 Post long-term anoxia impairment phenotype is unaffected by aging in LTA-tolerant strains. Survivors of *glp-1(e2141)*, *daf-2(e1370)*, *fog-2(q71)* strains exposed to long-term anoxia at various adult ages were assayed for level of impairment. These data are a subset of dataset presented in Fig. 2.1 and Fig. 2.3. * indicates significant difference from day 1 within each strain (Tukey's Multiple Comparison Test, $p<0.05$,).

Males

C. elegans males are anoxia tolerant at day 1 of adulthood (Mendenhall *et al.* 2009) with survival and impairment rates similar to LTA-tolerant hermaphrodites. To investigate whether aging has a similar affect on males as seen for LTA-tolerant strains I exposed N2, CB4856, and *fog-2(q71)* males to long-term anoxia between the ages of day 1 and 7 of adulthood and assayed their post-recovery survival rates and level of impairment (Fig. 2.8). A benefit of utilizing males to investigate the effect of aging on anoxia tolerance is the absence of progeny production. At day 1 males of all strains analyzed had high survival rates and low levels of impairment similar to levels previously described. However, aging in males is associated with a significant drop in survivorship and increase in impairment for compared to aging hermaphrodites and females.

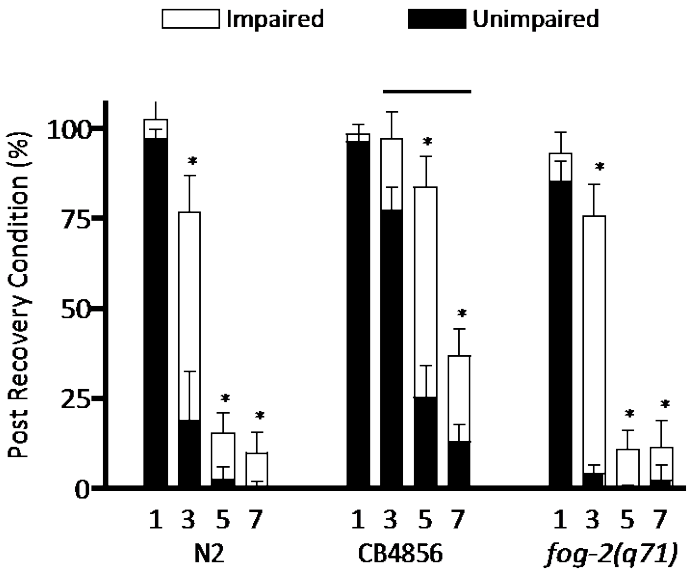

Fig. 2.8 Males show an age-related decrease in LTA tolerance. N2 (Bristol wild-type isolate), CB4856 (Hawaiian wild-type isolate) and *fog-2(q71)* males were raised to the specified age and exposed to 72h of anoxia followed by recovery in air and assayed for survival and impairment. Aging had an overall detrimental effect of LTA-tolerance in males of all strains analyzed. Aging CB4856 males tolerate anoxia better than N2 or *fog-2(q71)* males. For all experiments total number of animals tested is N>150 from three independent experiments; error bars represent standard deviation. * indicate significant difference in survival compared to day 1 within each strain, Bar indicates significant increase in survival compared to age-matched N2 and *fog-2(q71)* strains, p<0.001 (One-Way ANOVA and Tukey's Multiple Comparison Test).

Unlike *glp-1(e2141)* hermaphrodites and *fog-2(q71)* females that maintained a high survival rate through day 7 onset of LTA-sensitivity occurred early in adulthood for males (Fig. 2.8). At all ages examined male N2 and *fog-2(q71)* had similar survival rates and levels of impairment and both showed a decrease in survivorship and increased impairment at day 3. However, at day 3 survival rates and impairment levels remained high for CB4856 males. At day 5 survival rates for N2 and *fog-2(q71)* males dropped by 5-fold and 7-fold, respectively and remained low through day 7, although impairment levels remained constant. In contrast, despite a small but significant reduction in survival rate and increase in impairment CB4856 males retained a significantly higher survival rate than age-matched N2 and *fog-2(q71)* males at day 5 and 7.

These data indicate that age and genotype influence anoxia-tolerance in males. The discrepancy in the effect of aging between N2 and CB4856 males is further evidence of a geographic variation in stress tolerance among wild-type isolates of *C. elegans.*

Discussion

LTA Survival Rate at Day 1 is a Good Predictor of Aging Effects on LTA Viability

The effect of aging on stress-tolerance in humans is currently a topic of interest, especially in countries such as the USA which are experiencing a rise in average age of the population. In this study I report the first analysis of the effect of adult age on anoxia tolerance for wild-type and genetic mutant strains. At the population level, LTA-tolerance at day 1 of adulthood is a good predictor of the effect of age on LTA survivorship. Strains with high survival rates on day 1 of adulthood are able to maintain high viability rates with increasing age. In constrast, strains with low survival rates at day 1 showed an aging pattern characterized by punctuated increases in survival early in adulthood reaching a peak survival rate followed by either a gradual or rapid decline. Age associated increases in survivorship for wild-type isolates, *daf-16(mu86)* and *aak-2(gt33)* correspond to a reduction in self-fertilization. However, unlike This observation is in line with previously published results documenting increased anoxia tolerance in sterile mutant strains and strains with reduced ovulation. Interestingly, LTA-survival rates for post-reproductive hermaphrodites did not reach the levels seen for age-matched sterile mutant strains (Fig. 2.1 and 2.3) suggesting that reduced ovulation is not sufficient for high survival rates.

Long-Term Anoxia Tolerance Differs Among Wild-type Isolates

In addition to the importance of genetic factors in LTA-tolerance, my analyses reveal that anoxia stress tolerance is not uniform across wild-type isolates and that the pattern of decline in survivorship seen with increasing age is not shared among all LTA-sensitive strains. The N2 strain is commonly regarded as the representative wild-type of *C. elegans* and analysis of N2 hermaphrodites is routinely included in data sets as controls in assays utilizing genetic mutant strains. Hermaphrodites of aging N2 (wild-type isolate from Bristol, England) were overall more tolerant of long-term anoxia than CB4856 (wild-type isolate from Hawaii, USA). In addition, survival rates decreased more gradually following a peak in survival for aging N2 populations than for other LTA-sensitive strains analyzed, including CB4856. The data reported in this study are among the first to demonstrate a difference in stress tolerance between wild-type isolates of *C. elegans* and provoke the question of why naturally occurring strains of this species display such disparate sensitivity to long-term anoxia. One explanation for these observations, encompassing both genetic and environmental factors, is that each strain is the product of selective forces encountered in the natural environment. The N2 strain was isolated by Sidney Brenner from Bristol, England, which lies at N 51˚ latitude and has a average annual temperature of 11.5 °C ranging between 2˚-22˚C throughout the year. In contrast, CB4856 was isolated from the Hawaiian archipelago, USA, which is lies between N 18˚-22˚ latitude and has a average annual temperature of 24.9˚C ranging between 20.5˚-29.2˚C throughout the year. The difference in latitude of the isolation sites causes divergent environmental conditions to be experienced by these two strains. It is possible that factors such as the wide range in annual temperature in England impart a selective advantage to genotypes that encode a phenotype

capable of tolerating fluctuating and potentially stressful environmental conditions that are not recurrently encountered in the more tropical environment of Hawaii.

C. elegans has traditionally been referred to as a soil-dwelling nematode. However, recent evidence suggests that *C. elegans* prefers a microenvironment of rotting fruit and other matter and may only incidentally be isolated from soil as it migrates between food patches and its preferred environment (Kiontke 2006). It is likely that the frequency of food patches differs between the two isolation sites through the year and perhaps within individual months being seasonally available in Bristol, England, and available year-round in Hawaii, USA. If so, the ephemeral nature of the England site may result in periods of reduced caloric intake and favor genotypes that confer ability to rapidly activate stress-tolerant pathways in response to environmental stresses. It would be of interest to determine if isolates from diverse environments exhibit physiologic differences in response to dietary restriction and environmental stresses, such as oxygen deprivation.

In addition to food availability the isolation locales of the two wild-type strains differ in average yearly temperature. The assays conducted in this study were carried out at 20˚C which is approximately 9˚C higher than the average annual temperature of the location from which the N2 strain was originally isolated, although it falls within the annual temperature fluctuation for the isolation locale. In contrast, 20˚C is at the lower end of the temperature range CB4856 would encounter in its natural environment and is approximately 5˚C cooler than the average annual for Hawaii. As shown above, N2 hermaphrodites grown at 15˚C are less tolerant of LTA than age-matched animals grown at 20˚C, suggesting that exposure to temperatures moderately above that normally encountered in the natural habitat promotes formation of an

anoxia tolerant physiology. This provokes the question of whether environmental conditions experienced by the N2 isolate has favored a phenotype that is LTA-tolerant when grown at 20˚C, while a different environmental selection has been imposed on the CB4856 isolate and if so, what are the genetic differences giving rise to these phenotypic differences? One test of this theory would be to grow CB4856 at 25˚C and compare survival rates to controls grown at 20˚C. If CB4856 has been selected to thrive in 20˚C we would predict that culture at an elevated temperature prior to LTA exposure would result in an increase in survivorship, perhaps to rates seen for N2 cultured at 20˚C. Such studies will likely provide insight into the variation in stress tolerance that exists among naturally occurring populations of *C. elegans.*

The difference in onset of LTA-sensitivity between the N2 and CB4856 wild-type isolates, along with the observation that N2 survival rates were significantly higher than age-matched CB4856, is the first report of variation in LTA tolerance among wild-type *C. elegans* isolates. If the two strains represent naturally occurring genetic variation among wild-type isolates from diverse locales then the differences in LTA-tolerance patterns may be useful to identify genetic requirements for oxygen deprivation tolerance. I acknowledge that the animals used for this study were obtained from stocks maintained in the laboratory for many generations and therefore the possibility that the differences between the two strains are due to genetic variation that arose since the progenitor animals were removed from the wild cannot be eliminated.

Activation of stress mechanisms requires specific gene expression profiles to maintain survival and health. It would be of interest to determine if N2 and CB4856, as well as other wild isolates, have genomic polymorphisms in genes known to play a role in anoxia tolerance, such

as *aak-2*, *daf-16*, *gpd2/*3, *glp-1, kri-1, daf*-12 along with several others. Until then, I propose that N2 and CB4856 can be used in a comparative approach to aid in elucidating the genetic basis of anoxia tolerance. Furthermore, it is apparent that age must be taken into consideration when designing and conducting experiments evaluating the role of particular gene products in anoxia tolerance.

Reproductive Senescence Does Not Suppress Age-Associated Decline in LTA-Tolerance

For all fertile strains analyzed, except *daf-2(e1370)*, an increase in LTA-tolerance was observed to correspond to the onset of reduction in self-fertilized reproduction. This is in line with the previously published report that sterility or reduced ovulation rate at day 1 of adulthood is associated with increased LTA-tolerance (Mendenhall *et al.* 2009). It is possible the increase in LTA-survival rates seen between day 3 and day 5 of adulthood is, at least in part, a reflection of the decreased fecundity due to sperm depletion and transition into a non-reproductive physiological state that promotes LTA-tolerance. Delaying this transition by mating hermaphrodites to males at adult day 2 was sufficient to suppress the adult day 3-increase in LTA-survival rates seen in untreated controls (Fig. 2.4). While the tissues involved and the specific cellular requirements needed to establish this anoxia-tolerant state are not yet known, it is apparent that reduction in reproduction contributes to its formation in early adulthood. It is of interest to note that peak LTA-survival rates for reproductively senescent hermaphrodites of fertile strains failed to reach the level of survival seen for age-matched sterile strains, indicating that the transition to a non-reproductive physiology is insufficient to establish the robustly anoxia-tolerant physiological state present in sterile strains. One

possibility is that the physiological state associated with oocyte maturation and ovulation is not fully abrogated by reproductive sense cense or that the environment required for successful reproduction suppresses anoxia tolerance and cannot be reset to the pre-reproductive condition once it is established.

While reduced reproduction confers an anoxia-tolerance benefit early in adulthood, the advantage is insufficient to prevent an aging-associated decline in anoxia tolerance. Similar to wild-type, the sterile *glp-1(e2141)* and *fog-2(q71)* strains displayed a marked decline in LTA-tolerance at day 7 and following, which is well past the age of reproductive senescence in fertile strains. This decline in tolerance is interpreted here as evidence of a suppressive effect of aging on LTA-tolerance. It would be of interest to determine whether the onset of physiological and anatomical changes associated with aging (such as protein aggregation, muscle function, metabolic byproduct accumulation, and others) is associated with the age-associated decline in LTA-tolerance. Also, an investigation of the physiological differences between reproductively senescent adults and age-matched sterile strains may lead to a better understanding of the cellular conditions required for robust anoxia tolerance. One valuable observation to be made from the data presented here is the importance of considering age when investigating the genetic requirements for LTA-tolerance, for example assays investigating the effect of gene loss, or gene over expression, may best be performed within the adult day 3 to day 7 window to eliminate the complicating effects of reproduction and aging.

Long-Term Anoxia Tolerance is Multifactorial

Anoxia-tolerance is under the control of many physiological processes, including

metabolic factors. LTA-survival requires an overall reduction in metabolic rate and ability to provide enough energy to sustain the animal through the oxygen deprivation period and allow maintenance of tissue integrity. In this study all fertile strains analyzed displayed an increase in LTA-survival early in adulthood followed by a decline with aging. However, the rate of decline varied among the wild-type and mutant strains analyzed, suggesting a variation in genetic contribution to the maintenance of an LTA-tolerant physiological state. For example, absence of a functional *aak-2* resulted in an early and rapid decline in survivorship indicating that ability to manage energy availability, specifically AMP:ATP ratios, is necessary to maintain an LTA-tolerant physiology in aging adult worms. In contrast, loss of *daf-16* (a transcription factor regulated through the insulin signaling pathway) resulted in an aging pattern similar to the wild-type N2 strain, (although with lower overall survival rates and increased impairment) suggesting that *daf-16*-induced gene expression influences LTA-tolerance via mechanisms distinct from the role of *aak-2*. This idea is supported by the findings of LaRue and Padilla (2011) in which suppression of the high LTA-survival rate and unimpaired phenotype of the *daf-2(e1370)* strain at adult day 1 required the combined loss of *daf-16* and reduction in *aak*-2. The need for the combined reduction in expression of these genes to reduce LTA-tolerance is further evidence that formation of an anoxia tolerant phenotype is an integrative process drawing on input from a variety of physiological processes.

A wide range of biological processes naturally, or in the mutant condition, enhance or reduce anoxia tolerance. Organisms that survive anoxic stress often have other stress resistant phenotypes as well. For example, the long-term anoxia tolerant strains *glp-1(e2141)* and *daf-2(e1370)* also share an increased longevity phenotype. However, longevity and anoxia-

tolerance phenotypes are not superimposable. The extended lifespan of *glp-1* requires the absence of a functional germline and presence of an intact somatic gonad. In contrast, *daf-2* mutants have full reproductive capacity and have nearly wild-type brood sizes. The long-term anoxia tolerant phenotype is *daf-16*-dependent in *daf-2* mutants, but *daf-16*-in-dependent in sterile mutants. Data presented in this study show the age-associated decline in LTA-tolerance seen for all strains is delayed in mutants that have an extended lifespan relative to wild-type and short-lived *fog-2(q71)* (Fig. 2.3). In addition, age affects *daf-2(e2141)* and *glp-1(e2141)* strains differently resulting in strain-distinct LTA-survival patterns. The *daf-2(e1370)* strain maintained a high LTA-tolerance through adult day 11 compared to the *glp-1(e2141)* strain that displayed a decline in LTA-tolerance as early as day 7.

The relationship between sterility-induced anoxia-tolerance and longevity is not yet clear and strains have been identified that carry one but not both characteristics. Unmated *fog-2*, for example, has a wild-type lifespan and presence of functional oocytes yet is long-term anoxia tolerant until mated. Similar to aging *glp-1(e2141)* animals the LTA-survival pattern for aging unmated *fog-2(q71)* females was high in early adulthood yet declined relatively early compared to *daf-2(e1370)*. Taken together these observations suggest an overlap in the mechanisms governing LTA-tolerance and longevity exists but that they are not identical. It is possible the delay in age-associated decline in long-term anoxia tolerance in longevity mutant strains is due to the same physiological state allowing extended lifespan phenotype. If so, the early onset of decline in survivorship seen in LTA-sensitive strains may be due, at least in part, to an inability to establish this physiological state.

Hermaphrodites, female and male *C. elegans* are differentially affected by anoxia exposure. Somewhat surprisingly, males of all three strains analyzed show increased anoxia sensitivity as they age. The pattern of anoxia tolerance across increasing age for males was recognizably unique from that seen for hermaphrodites. Although males and LTA-tolerant hermaphrodites were equally resistant to anoxia at day 1 of adulthood, males of all strains analyzed were more affected by aging than hermaphrodites. Predictably, N2 and *fog-2(q71)* males share the same LTA-survival pattern (Fig. 2.8), likely because males are unaffected by the *fog-2(q71)* mutation and are therefore express an essentially wild-type genome. In contrast, CB4856 males displayed less sensitivity to LTA exposure than males of either the N2 or *fog-2(q71)* stains.

Why aging affects N2 and CB48546 wild-type isolates differently is not immediately obvious. The answer may lie in the divergent environments in which the two strains evolved and their resultant life histories which ultimately impacts the role of males in each natural population. *C. elegans* is an omnivore consuming any food source it encounters and rapidly depletes environmental resources in an apparent effort to out compete others in the battle of progeny production (Lewis and Fleming, 1995). This theory is supported by the worm's rapid generation time, large brood size (~300 via selfing) and formation of dauer, a diapause larval stage that serves as the primary mode of population dispersal during periods of limited resources. Oocyte production is stimulated by mating thereby providing opportunity for an individual hermaphrodite's brood size to exceed 1000 (Kimble 1981). While males have rarely been isolated from the natural environment, in the laboratory they arise spontaneously at a frequency of 0.1%. Crossing has the benefit of increased genetic diversity and gives rise to

approximately equal numbers of hermaphrodite and male progeny. However, the percentage of males in populations often remains low due to self-fertilization giving rise almost exclusively to hermaphrodites (Hodgkin et al. 1979, Emmons & Sternberg 1997). Consequently, without a distinct selective advantage to male progeny, crossing may actually decrease the number of descendants passed to subsequent generations. This theory predicts that harsh environments with limited or patchy resources will favor the self-fertilization reproductive mode (hermaphrodite formation), while a uniform environment with predictable and abundant resources will be more favorable to the crossing mode of reproduction (production of males and maximizing genetic diversity).

The role of environmental factors such as temperature or food source and availability represent yet another genre of factors influencing anoxia tolerance. It is likely that environmental factors exert their influence by altering the rate of reactions associated with the biological processes discussed above. This idea is supported by the observation that wild-type embryos reared to adulthood at 15˚C have delayed development, including delayed onset of reproduction and reproductive senescence. For animals grown at either 15˚C or 20˚C cessation of reproduction corresponded with a significant increase in anoxia survival rate. However, the peak survival rates and levels of unimpaired survivors for animals grown at 15˚C did not reach the levels seen for controls by 5. Therefore, growth at a reduced environmental temperature did not promote formation of an LTA-tolerant physiological state and did not suppress tissue damage affect following anoxia exposure. One explanation for these observations is that the reduced temperature of 15˚C actually falls within the normal temperature range N2 animals would encounter in their natural habitat. The conventional lab culture temperature (20˚C) is at

the top of the temperature range likely to be encountered in the wild by N2 animals and may activate thermo stress-tolerating pathways allowing the animals to better withstand anoxia exposure. This theory is supported by the observation that N2 animals cultured at 25˚C have significantly higher LTA-survival rates than controls grown at 20˚C. Environmental factors such as temperature can be viewed as persistent modern reminders of the selective pressures to which organisms were obliged to adapt or die.

Applying these predictions to the N2 and CB4856 wild-type isolates offers an explanation for their discrepant LTA resistance. Briefly, compared to the tropical environment of Hawaii conditions in England can be considered harsh having a wide variation in temperature and seasonal depletion of food sources (such as rotting fruit). Having evolved under harsh and unpredictable conditions the N2 strain is predicted to be less likely to utilize the crossing mode of reproduction than the CB4856 strain. It is possible the N2 strain has experienced little selective pressure to evolve males capable of tolerating environmental stress. In contrast, the food rich environment and relatively unchanging Hawaiian climate is predicted to favor genotypes that produce males capable of persisting in the population even under condition of stress, such as oxygen deprivation (Graustein 2002). The phenotypic difference in anoxia tolerance displayed by these natural isolates provides opportunity to identify genomic characteristics and gene expression differences associated with anoxia tolerance. It will be interesting to test the hypothesis presented here by examining wild-type isolates from other global locales.

CHAPTER 3

GENETIC REQUIREMENTS FOR LONG-TERM ANOXIA TOLERANCE ARE AGE-DEPENDENT

Introduction

Adaptive Advantge of Stress Sensing Mechanism

Among the most devastating effects of physical trauma, oxygen deprivation is the cause of life-long disabilities and loss of human life. *C. elegans* has been instrumental in identifying biologic factors that influence anoxia tolerance, including environmental, metabolic, and reproductive factors. The ability to survive extended periods of anoxia is arguably un-adaptive if the animal is unable to resume normal activity such as foraging and reproduction after reoxygenation. It is reasonable to expect that adaptive mechanisms have evolved that protect or repair tissues when damage is incurred during stress. Specific genes have been recognized as required for post-anoxia health and they function in diverse biological processes. For example, *gpd-2* and *gpd*-3 are necessary for tissue maintenance during anoxic stress and function in the glycolytic pathway while *hyl-2*, a ceremide synthase required for short- term anoxia survival, functions in a seemingly unrelated manner to provide proper length fatty acyl chains which serve as the precursors of membrane sphingolipids and cell signaling molecules. Two biological processes have been identified as particularly influential in regulating stress response, metabolism and reproduction. Receptors have been identified whose activation state regulates flux through pathways associated with these processes and in an integrative manner influence anoxia resistance, for example, *daf-2* in the insulin/IGF signaling pathway and *glp-1* in the germ cell renewal pathway. These pathways work independently or in parallel to regulate the balance between a pro-stress resistant physiology and pro-reproductive physiology. One

protein common to both pathways is the transcription factor DAF-16, which is thought to function primarily by upregulating expression of genes required to active stress-tolerant pathways.

Sterility and LTA Tolerance

Although reproduction has been a focus of evolutionary biologist for many years, the molecular interplay between reproduction and stress tolerance is only beginning to be investigated. For example, sterility in *C. elegans* is associated with increased anoxia tolerance, although how reduced germline activity operates to prepare the soma for stress tolerance is not fully understood. The *glp-1* gene encodes a member of the LIN-12/Notch family of receptors that is required for cell fate specification in germline and somatic tissues and is essential in the germline for mitotic proliferation of germ cells and maintenance of germline stem cells (Austin & Kimble 1987; Priess 2005). Strains carrying the *glp-1(e2141ts)* allele have a somatic gonad but germline stem cells prematurely enter meiosis and fail to self-renew making the animal sterile (Austin & Kimble 1987; Hansen *et al.* 2004; Crittenden *et al.* 2004)). Sterility in the *glp-1(e2141ts)* strain confers both increased longevity and LTA tolerance, including high survival rates and an unimpaired phenotype following 4 days of anoxia exposure (Mendenhall *et al.* 2009; LaRue & Padilla 2011). Presence of a somatic gonad is necessary for loss of the germline to result in an extended lifespan indicating that sterility alone is insufficient to generate the phenotype (Hsin & Kenyon 1999). To date all sterile strains analyzed by our laboratory have displayed an LTA-tolerant phenotype, however, whether the somatic gonad is necessary for long-term anoxia tolerance in not yet known.

Regulation of DAF-16 Activity

The genetic requirements for long-term anoxia tolerance differ among strains in a manner dependent upon the physiological state of the animal. At day 1 of adulthood loss of *daf-16* suppresses the high survival phenotype of *daf-2(e1370)* highlighting the importance of stress pathway activation for anoxia tolerance in a reduced insulin-signaling physiology. Reduction in *aak*-2 gene expression via RNAi in *daf-2(e1370)* did not reduce survival rate however, it did result in an increase in post-recovery impairment. In contrast, loss of *daf-16* alone is insufficient to suppress high LTA survival rates in sterile *glp-1(e2141)*, yet if combined with a reduction in *aak-2* expression results in an increase in impairment following LTA exposure and partial suppression of survivorship following 4 days of anoxia exposure (LaRue & Padilla 2011). This indicates that although affected by loss of *daf-16*, the anoxia-tolerating mechanism utilized by *glp-1(e2141)* is less dependent on DAF-16-induced gene expression than the mechanism utilized by *daf-2(e1370).* Hsin and Kenyon (1999) reported that nuclear localization of DAF-16 is necessary for the longevity phenotype in both *glp-1(e2141)* and *daf-2(e1370)*, and as expected, strains carrying the *daf-16(mu86)* null mutation are neither long-term anoxia tolerant nor do they have an extended lifespan. However, having an extended lifespan is not a requirement for LTA survival. Sterile *fog-2(q71)* females, which produce oocytes but no sperm, survive LTA at high rates in an unimpaired condition yet are not long lived. Taken together these observations support that conclusion that overlapping but not identical genetic factors influences lifespan extension and anoxia tolerance.

Germline Signaling Pathway

DAF-16 appears to be activated in response to signals from at least two independent sensory pathways; the insulin/IGF pathway and germline signaling. These pathways appear to operate either independently or in parallel and converge at the intestine to regulate the animal's physiological state, yet how information from these independent pathways is integrated remains unclear. Berman and Kenyon (2006) identified a pathway by which the reproductive status of the gonad may be signaled to other body tissues, specifically to the intestine, and influence lifespan. The *kri-1* encodes the worm ortholog to human KRIT1 (Krev interaction trapped/cerebral cavernous malformation 1, CCM). KRIT1 is one of a few genes responsible for formations of CCM lesions that are characterized by abnormally enlarged and often leaky capillary cavities that predispose to seizures, focal neurological deficits, or fatal intracerebral hemorrhage (Goitre *et al.* 2010). Formation of the lesions is thought to follow a two-hit mechanism. The second hit may be exposure to local cellular stress factors, such as oxidative stress, resulting from inappropriate ROS scavenging. In *C. elegans* KRI-1 is required for DAF-16 nuclear localization and longevity in animals lacking a germline (Gerisch *et al.* 2001). Among the stress tolerance mechanisms upregulated by DAF-16 is ROS scavenging. Therefore, it is possible that KRI-1 serves a role in oxidative sterss tolerance. Reduction in *kri-1* expression in *daf-2(e1370)* mutants does not suppress lifespan extension, indicating KRI-1 plays a role in lifespan extension that is specific to the reproductive signaling pathway.

A second gene, *daf-9,* encodes a cytochrome P450 with homology to a steroidogenic/fatty acid hydroxylase is also required for DAF-16 nuclear localization in germline deficient animals (Antebi et al. 2000; Hsin & Kenyon, 1999). A third gene, *daf-12,* encodes a

nuclear steroid hormone receptor homologous to human Vitamin D receptor and among other functions promotes nuclear sequestering of DAF-16 (Gerisch et al. 2007). Fig. 3.1 shows a model for the interaction of proteins identified as required for DAF-16 nuclear localization in germline deficient animals (Berman & Kenyon (2006). In the presence of a functional germline DAF-16 is sequestered in the cytoplasm. However, in the absence of an active germline DAF-9 promotes the formation of an activating lipophilic ligand for the nuclear hormone receptor, DAF-12. With the aid of KRI-1, DAF-16 translocates into the nucleus where it cooperates with activated DAF-12 to upregulate a specific set of genes favoring the formation of a stress tolerant physiological state.

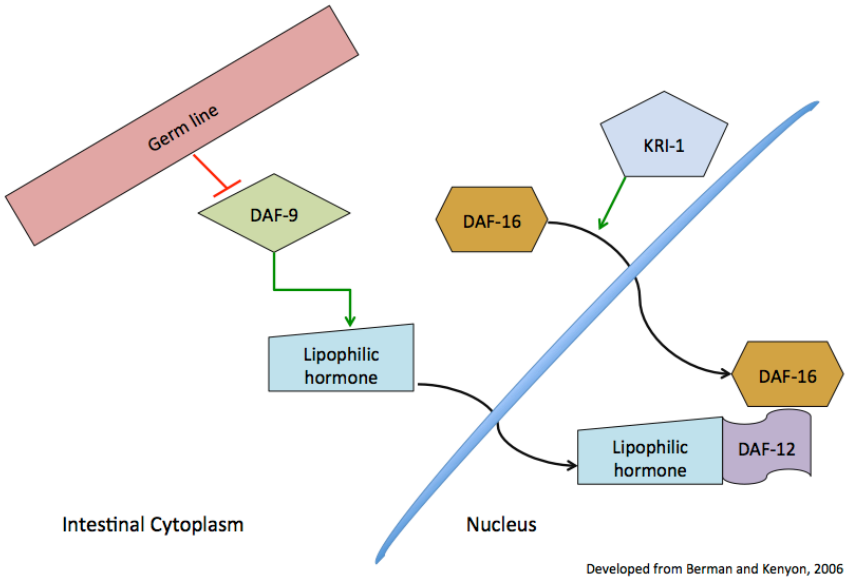

Fig. 3.1 Molecular interaction of players in the germline specific signaling pathway. Presence of an inactive germline inhibits the action of DAF-9 and prevents formation of a putative lipophilic signal. In germline deficient animals (due to ablation or genotype) production of the lipophilic signal allows activation of the nuclear hormone receptor DAF-12. Together DAF-12 and nuclear-localized DAF-16 regulate the expression of a subset of genes that promote lifespan extension. Developed from Berman and Kenyon (2006).

When *glp-1(e2141)* individuals are grown at a permissive temperature to the point of producing progeny then shifted to a nonpermissive temperature they display an extended lifespan compared to unshifted controls (Arantes-Oliveira et al. 2002). Therefore, absence of *glp-1(e2141)* activity (which results in absence of germline stem cells) during adulthood is sufficient to increase lifespan in *glp-1(e2141).* However, whether the absence of GLP-1 activity in adulthood is adequate for formation of an LTA-tolerant phenotype is not known. To address this question I utilized a temperature-shifting scheme to regulate the activity of *glp-1* in aging adults and assessed the effect on LTA-tolerance. I found that, as with lifespan extension, loss of GLP-1 function in adults is sufficient to induce an LTA-tolerant state.

To further investigate how loss of germline signaling promotes an LTA-tolerant state I asked if loss of genes known to be required for lifespan extension were also required for LTA tolerance. One benefit of utilizing sterile strains is that experiments can be conducted utilizing aging adults without the compounding effects of reproduction or reproductive senescence. As reported in Chapter 2 adult age is a determining factor in LTA-tolerance even for sterile strains. I hypothesized that younger animals may have different molecular requirements for formation of an anoxia-tolerant physiology than do animals of increased age. To investigate if adult age is a compounding factor in anoxia tolerance I determined the effect of loss of *kri-1* or *daf-12* (both are required for lifespan extension in sterile *glp-1(e2141)*) on LTA-tolerance of *glp-1(e2141)* between day 1 and day 5 of adulthood. I found that loss of either *kri-1* or *daf-12* has a suppressive effect on the LTA-tolerant phenotype of *glp-1(e2141)* in an age-dependent manner.

Results

Germline Signaling Suppresses LTA Tolerance

From studies of germline stem cells (GSCs) in *C. elegans*, *Drosophila* and mice conserved themes of cell renewal, proliferation regulation and germline maintenance are becoming known. GSCs are usualy sequestered in a microenvironment termed a niche where they are supported by somatic cells. In *C. elegans*, the distal tip cell serves as the niche for GSCs where they closely assoicat with the somatic gonad (Kimble & White 1981). Complex regulatory interactions with both the niche and the environment modulate germline stem cell function. Through a clever bit of temperature-shifting one study demonstrated that germline stem cells exert an inhibitory influence on lifespan (Arantes-Oliveira *et al.* 2002). To ask if germline stem cells have a similar influence on anoxia tolerance I developed a similar temperature-shift regimen to control the activity of the temperature sensitive *glp-1(e2141)* allele and manipulated the presence of germline stem cells in adults (see Materials and Methods). The wild-type and *glp-1(e2141)* animals grown at the permissive temperature were actively producing progeny when shifted to nonpermissive 15˚C. Of the embryos laid in the first 18h after the shift to 25˚C approximately one-third hatched and the remaining embryos failed to hatch (were dead) over the next 36h (data not shown). All embryos laid between 18h and 24h after the shift to 25˚C failed to hatch over the next 36h. Since GLP-1 is required for embryonic development, I took this to mean that by 18h after the shift to 25˚C the GLP-1(*ts*) protein was mostly inactive. Therefore, animals were exposed to anoxia no less than 6h after the loss of functional GLP-1, although that period may have been longer. Loss of GLP-1 native conformation was not confirmed by independent means. The shift up to the nonpermissive

temperature results in misfolding of the temperature sensitive GLP-1 protein and significantly increased survival rate for *glp-1(e2141)* compared to unshifted controls however, survival rates remained lower than for *glp-1(e2141)* animals reared continuously at 25˚C (Fig. 3.2).

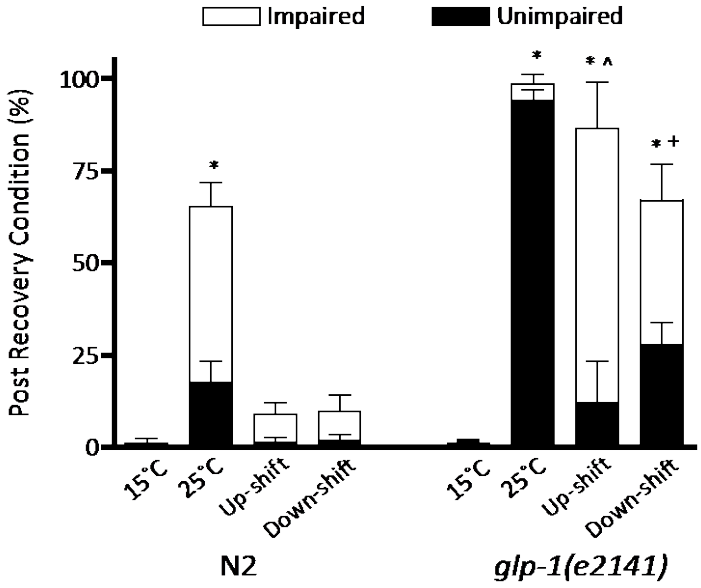

Fig. 3.2 Absence of GLP-1 in adulthood is sufficient for long-term anoxia tolerance. Wild-type and *glp-1(e2141)* hermaphrodites were cultured at 15˚C or 25˚C to day 1 of adulthood then either Up-shifted to 25˚C or down-shifted to 15˚C for 24h immediately prior to 72h of anoxia exposure. All animals were exposed to long-term anoxia at day 2 of adulthood. For *glp-1(e2141)* the Up-shift in temperature resulted in a significant increase in survival rate relative to unshifted 15˚C controls. The Down-shift in temperature resulted in significantly decreased survival rate for both strains analyzed, and for *glp-1(e2141)* a higher percentage of survivors with an impaired phenotype compared to unshifted 25˚C controls. For all experiments the total number of animals assayed is *N*>150 from three or more independent experiments; error bars represent standard deviation. * indicates significant difference from 15˚C control within each strain; One-Way ANOVA and Tukey's Multiple Comparison Test; (p<0.001), ^ indicates significant difference from 25˚C control (p<0.01), $^{+}$ indicates significant difference from 25˚C control (p<0.001).

Survival rates for N2 hermaphrodites shifted in the same manner did not significantly increase, indicating that reduction in GLP-1 function, and not the temperature shift itself, promoted increased anoxia tolerance. The shift down resulted in significantly decreased survival rates for

N2 and *glp-1(e2141)* animals compared to unshifted controls. The decrease in survivorship for down-shifted N2 was substantially greater than for down-shifted *glp-1(e2141)*, however, anoxia tolerance in *glp-1(e2141)* animals were clearly negatively affected by regaining GLP-1 activity, given that the portion of unimpaired survivors declined by almost 7-fold. It is possible that LTA survival rates of up-shifted *glp-1(e2141)* animals may have continued to rise and perhaps reached levels equivalent to age-matched animals grown at 25˚C from embryos. It should be noted that when shifted away from 25˚C the N2 hermaphrodites were actively producing progeny while the *glp-1(e2141)* individuals were not due to loss of germline stem cells. During incubation at the permissive temperature N2 animals continued producing progeny while no embryos were seen on the plates for *glp-1(e2141)* animals, although some individuals had cell-like structures in the gonad which may have been pre-oocytes (data not shown). I took these observations to support the idea that loss of a functional GLP-1 in adulthood was sufficient to enhance LTA-tolerance.

KRI-1 and DAF-12 are Required for LTA Tolerance in glp-1(e2141)

Presence of a functional germline inhibits LTA viability through a pathway involving the LIN-1/Notch signaling. To determine if the reproductive signaling pathway identified by Berman and Kenyon (2006) is required for long-term anoxia tolerance in germline deficient animals, and protein KRI-1 is required for DAF-16 nuclear localization in animals lacking a germline (Berman & Kenyon 2006). Adult *kri-1(ok1251)* hermaphrodites do not survive long-term anoxia during any age of adulthood (Fig. 3.3). Loss of *kri-1* in aging *glp-1(e2141)* (day 3 and 5) suppressed

survival and significantly reduced the ability of survivors to maintain an unimpaired phenotype following anoxia exposure.

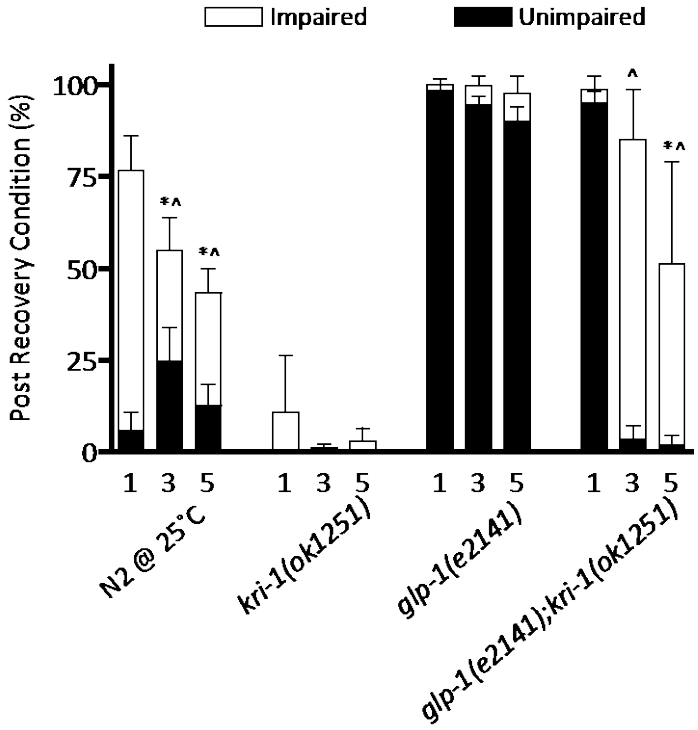

Fig. 3.3 Loss of kri-1 suppresses the anoxia tolerant phenotype in glp-1(e2141) in an age dependent manner. Wild-type, *kri-1(ok1251)*, *glp-1(e2141)* and *glp-1(e2141);kri-1(ok1251)* strains were raised at 25˚C and exposed to 72h of anoxia (20˚C) at the indicated ages. Survivors were scored for post-recovery level of impairment. X-axis indicates adult age at anoxia exposure. Loss of *kri-1(ok1251)* suppresses the anoxia resistance of *glp-1(e2141)*. For all experiments the total number of animals assayed is *N*>150 from three or more independent experiments; error bars represent standard deviation. * indicates significant difference in survival from day 1 within each strain, $p<0.001$, ^ indicates significan tdifference in percent of survivors with an unimpaired phenotype compared to day 1 within each strain $p<0.001$ (One-Way ANOVA and Tukey's Multiple Comparison Test).

The *glp-1(e2141);kri-1(ok1251)* 1 day old animals survived long-term anoxia and were able to move, however they needed to be prodded with a worm pick to move (data not shown). In contrast, survival rates and activity among survivors remained high for age matched *glp-1(e2141)* controls. Given that *kri-1(ok1251)* animals are sensitive to anoxia and the kri-1

mutation suppresses anoxia tolerance in *glp-1(e2141)* animals we wanted to further investigate the role kri-1 has in anoxia tolerance. We tested if *kri-1* males were sensitive to anoxia and determined that at day 1 of adulthood they were not (Fig. 3.4). It will be of interest to determine if *kri-1* is necessary to survive anoxia exposure in aging males.

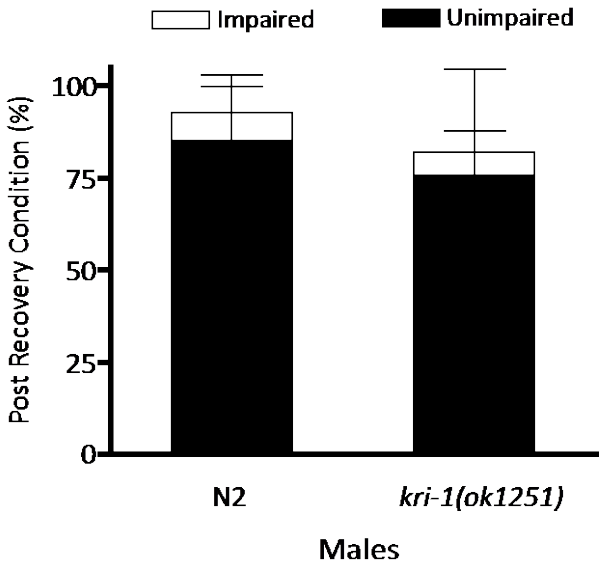

Fig. 3.4 The long-term anoxia tolerant phenotype of male C. elegans is kri-1-independent. Wild-type and *kri-1(ok2151)* males were grown at 20˚ to day 1 of adulthood and exposed to 72h of anoxia. Survivors were scored for post-recovery level of impairment. Loss of *kri-1(ok1251)* did not suppress the anoxia tolerance of wild-type males at day 1 of adulthood. Total animals examined is greater than *N*>280 animals from three independent trials; error bars represent standard deviation. No significant difference, One-tailed t-test.

Sterile *glp-1(e2141)* strain is anoxia resistant even when exposed to 4 days of anoxia indicating the physiological state present in *glp-1(e2141)* is robustly anoxia tolerant (Larue & Padilla 2011). LaRue and Padilla reported DAF-16 and AAK-2 to be necessary for maintaining anoxia tolerance in the *glp-1(e2141)* strain when exposed to 4 days of anoxia. The steroid hormone receptor DAF-12 is homologous to human vitamin D receptor and together with DAF-16 is required for gonad-dependent adult longevity (Gerish *et al.* 2001; Gerish *et al.* 2007).

Anoxia tolerance in *daf-12(rh61rh411)* animals has not yet been determined. However, in preliminary trials I found that loss of *daf-12* did not significantly reduce anoxia tolerance in 1-day old *glp-1(e2141)* animals exposed to 3 days of anoxia. In an effort to maximize sensitivity to the effects of loss of *daf-12* I exposed *glp-1(e2141);daf-12(rh61rh411)* animals to 4 days of anoxia between day 1 and day 5 of adulthood. I found that loss of a *daf-12* suppressed the anoxia resistance of *glp-1(e2141)* at all ages analyzed (Fig. 3.5)

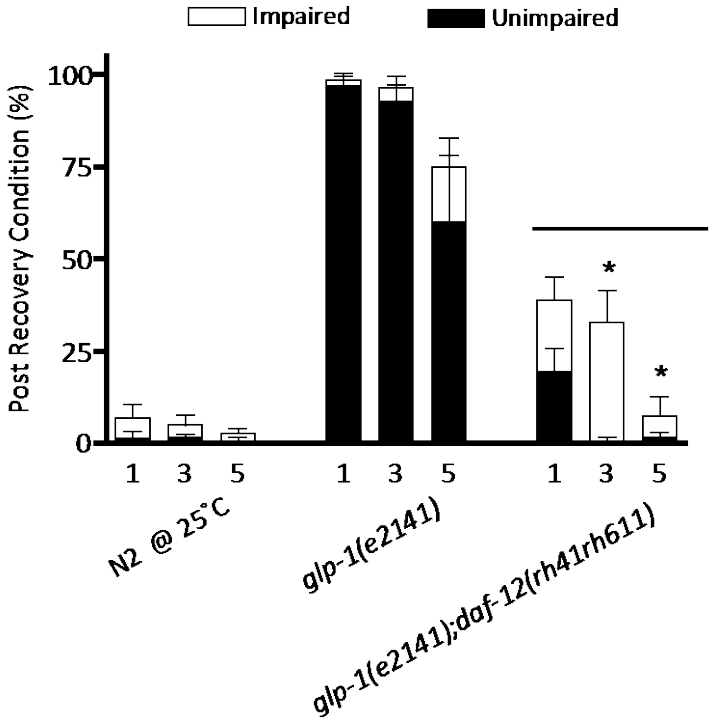

Fig. 3.5 Loss of daf-12 suppresses the enhanced anoxia tolerant phenotype of glp-1(e2141) in an age dependent manner. Wild-type, *glp-1(e2141)* and *glp-1(e2141);daf-12(rh61rh411)* strains were grown at 25˚C and exposed to 96h of anoxia at the indicated ages. Survivors were scored for post-recovery level of impairment. Loss of *daf-12* significantly reduced anoxia tolerance of *glp-1(e2141)* adults at all ages analyzed. X-axis values indicate adult age at anoxia exposure. Total animals tested is N>150 from three independent experiments; error bars represent standard deviation; experiment ANOVA ($F_{8,59}$ = 20.42; p<0.0001). Bar denotes significant difference in survivorship compared to age-matched *glp-1(e2141)* controls (One-Way ANOVA and Tukey's Multiple Comparison Test, p<0.001); * denotes significant difference survivorship compared to day 1 within the strain (Tukey's, p<0.001).

At day 1 of adulthood survival rates for *glp-1(e2141);daf-12(rh61rh411)* were suppressed to less than half that seen for *glp-1(e2141)* controls. Aging in *glp-1(e2141);daf-12(rh61rh411)* caused increased impairment in anoxia survivors and by day 5 survival rates and impairment levels were not different from wild-type controls. It will be of interest to determine the effect of loss of daf-12 on anoxia tolerance for aging *glp-1(e2141)* when challenged with 3 days of anoxia. Note the *glp-1(e2141);kri-1(e2151)* and *glp-1(e2141);daf-12(rh61rh411)* animals remain sterile, thus their suppression of *glp-1(e2141)* anoxia resistance is not due to induction of a functional germline. Both mutant strains display abnormal phenotypes including slow growth, lethargy or an egg laying defect (data now shown). However, it is not clear if these phenotypes are linked to the observed anoxia sensitivity phenotype.

Discussion

Loss of GLP-1 Activity in Adulthood is Sufficient for LTA Tolerance

Ecologists have long understood that animals face the persistent dilemma of maximizing the number of offspring passed to the next generation while conserving energy resources needed to sustain life (Cody 1966). Ability to integrate signals from a variety of sensory pathways and respond by regulating reproductive effort is predicted to be evolutionarily favored. Berman and Kenyon (2006) proposed that in the absence of signals relaying the action of a functional germline, either naturally due to sperm depletion or in germline deficient mutant strains or in germline oblated animals, *C. elegans* activates stress-tolerating mechanisms in a manner dependent upon DAF-16 nuclear localization. The *glp-1(e2141)* strain carries a temperature sensitive allele. This strain has been useful for studying the mechanism

for sterility induced LTA tolerance. When cultured at 25˚C hermaphrodites of these strain fail to form a functional germline, neither oocytes nor sperm. By shifting hermaphrodites between 15˚C and 25˚C culture temperatures I manipulated the presence of germline stem cells. In the absence of functional germline signaling the sterile hermaphrodites were LTA tolerant. However, when sterile *glp-1(e2141)* hermaphrodites were shifted back to 15˚C LTA viability was suppressed. LTA tolerance of wild-type N2 hermaphrotides grown and shifted in the same manner was unaffected by the temperature shift indicating that the change in environmental temperature itself did not suppress LTA viability. Instead, the change in *glp-1(e2141)* LTA viability wasl likely due to presence of properly folded GLP-1 protein and resumption of germline signaling. In a complimentary experiment, *glp-1(e2141)* grown at 15˚ and shifted up to 25˚C at day 1 of adulthood for 24h were more LTA toleranct than ushifted 15˚C culture controls. This indicates that the 24h incubation at 25˚C was adequate to reduce the amount of functional GLP-1 protein due to the temperature sensitive nature of the protein. Animals that were actively reproducing gain a significant degree of LTA tolerance in the 24h preconditioning period, presumably due to loss of GLP-1 activity. These data indicate that loss of *glp-1(e2141)* at adult stages is sufficient to render the animals LTA tolerant, and the LTA tolerant physiological state is not due to an action resulting from loss of *glp-1(e2141)* during larval development. Furthermore, these data indicate that the effect of the change in germline signaling operated quickly to establish LTA viability. This makes sense. A rapid somatic response to signaling processes that have evolved to make the animal stress toleant would be predicted. Finally, these experiments suggest that the LTA tolerant phenotype of sterile animals is likely due to

reduced signaling from the germline stem cells and may not directly involve signals from oocytes indicating fertilization and oocyte maturation.

Repduction suppresses LTA viability, but the mechanism of the signaling is not yet clear. In this study I have demonstrated that two proteins thought to work exclusively in the germline sensing pathway, KRI-1 and DAF-12, are required for surviving anoxia and maintaining an unimpaired phenotype in aging sterile *glp-1(e2141)* animals. It has not yet known been determined if these proteins are necessary for the anoxia resistant phenotype in the insulin-signaling mutant *daf-2(e1370)* or the sperm deficient sterile *fog-2(q71)*. I hypothesized that the loss of *kri-1* or *daf-12* will have a suppressive effect on *fog-2(q71)* and only modest effects on the anoxia tolerance of *daf-2(e1370)*. I found that loss of either *kri-1* or *daf-12* did suppress the LTA tolerant phenotype of *glp-1(e2141)*. This suggests LTA tolerance is enhanced in germline deficient animals through a signaling pathway that involves nuclear hormone activation and KRI-1 activity, perhaps the KRI-1 assisted nuclear localization of DAF-16.

Three Days of Anoxia May Be Inadequate Stress to Detect the Need for Some Genetic Factors

The question of whether genotype or environment has the greater impact on an organism's stress tolerance rages on. It is likely that environmental factors exert their influence by altering the rate of reactions associated with the biological processes discussed above. Once can view these environmental factors as persistent modern reminders of the pressures to which organisms were obliged to adapt or die. Having evolved under the influence of a range of environmental pressures it is not surprising that multiple mechanisms persisted to cope with diverse environmentally induced stressed. Through the work conducted in our laboratory,

evidence has been presented that anoxia stress tolerance in *C. elegans* is determined by a complex interplay between these two contributing categories. Consequently, investigations that seek to identify the role of specific factors, such as a particular gene or pathway, in stress tolerance require well-designed experiments. For example, detecting the role of *daf-16*, *aak-2*, *kri-1*, and *daf-12* in a *glp-1(e2141)* anoxia tolerance required single and double knockouts, both 3 days and 4 days of anoxia exposure at various adult ages. These observations emphasize the impact of an animal's age and the duration of stress exposure on the molecular requirements for anoxia tolerance. For example, younger adults may be inadequately challenged by 72h of anoxia to allow detection of required gene products, or perhaps younger adults are intrinsically better able to handle stress, again making it difficult to detect the need for specific gene expression. Animals with reduced caloric intake (which may mimic reduction in insulin signaling) such as the dauer stage of larval development animals carrying a reduced function *eat-2* allele (animals have a reduced pharynx pumping rate and therefore, reduced food intake), have been found by our lab to have an elevated anoxia survival rate. Complimentary to this observation is that long-term anoxia tolerant strains have elevated levels of fuel source carbohydrates relative to long-term anoxia sensitive strains. It is possible that, in a genotype- and age-dependent manner, previously unstimulated *C. elegans* adults are intrinsically able to meet the cellular and tissue demands imposed by a stress (anoxia for example). How long an animal can endure the stress likely depends upon several factors, including which metabolic and stress pathways were active when the stress was encountered, the level of energy the animal has stored (ATP, carbohydrates, fats) and the efficiency with which the animal can

access the stored recourses. At some point during the stress exposure a threshold may be reached after which stress tolerant mechanisms must be activated.

Anoxia Tolerance is Multifactorial

Finding this theoretical threshold point is necessary for designing experiments to accurately determine the role of a gene in stress tolerance. I suggest that for anoxia tolerance studies, each strain to be utilized be pre-characterized for maximal anoxia tolerance across the adultspan prior to experiments examining the effect of gene knock-out or knock-down. This approach will maximize the efficiency of performing experiments and prevent improperly assessing a phenotype to be gene-independent.

It is likely that a LTA tolerant strain is able to survive anoxia stress at a high rate due to one or more of the biochemical processes that lead to an anoxia tolerant phenotype. In Fig. 3.6 I present a model depicting the integration of many factors in establishing an anoxia tolerant phenotype. The nature of signals sent from each branch may be influenced by environmental or genetic factors.

A consequence of multiple pathways contributing to a physiologic state is formation of an indiscrete spectrum of phenotypes. In the context of anoxia tolerance this may present as a continuum of LTA tolerances among analyzed strains. In addition, each branch contributing to the anoxia tolerant phenotype may also influence other characteristics as well, such as longevity or pathogen resistance. Resulting in an overlap of genetic requirements between tolerance mechanisms. Ultimately, it is likely that *C. elegans* survives oxygen deprivation via the integration of multiple signals.

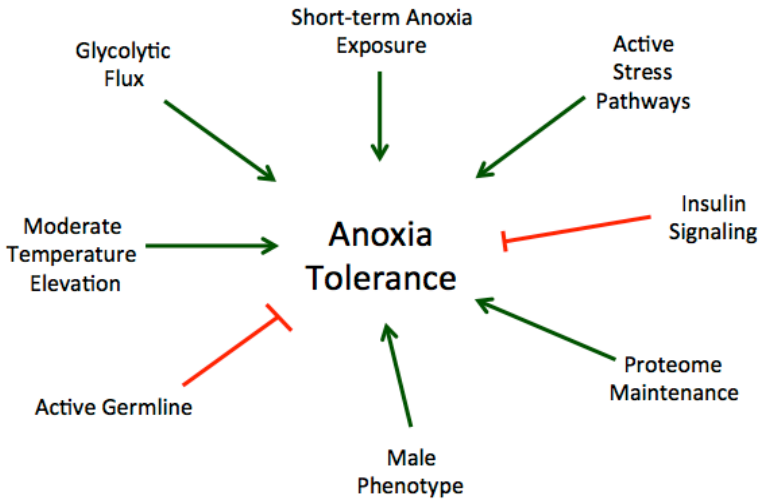

Fig. 3.6 The anoxia tolerant phenotype is multifactorial in nature. Biological factors that promote enhanced anoxia survival are shown as green activating arrows, while factors or conditions that decrease the rate of survival during anoxia exposure are shown as red inhibiting blunt-ended lines. Anoxia tolerance is likely determined by the combinatorial integration of these and other as yet unidentified factors.

CHAPTER 4

RECURRENT SHORT-TERM ANOXIA EXPOSURE ENHANCES LONG-TERM ANOXIA TOLERANCE

Introduction

Oxygen deprivation is common to diseases that affect humans of all ages and development of preventative treatments that reduce the impact of acute oxygen deprivation may lead to reduced mortality and lessen the long-term post trauma effects. Several environmental factors have been shown to have a preconditioning effect on *C. elegans* long-term anoxia tolerance, resulting in increased survival rates and reduced post-recovery impairment. Previously identified factors include aspects of culture condition including food source and culture temperature (LaRue & Padilla 2011). *C. elegans* is adapted to survive anoxia exposure as evidenced by high survival rates and low level of impairment following exposure to 24h of anoxia at 20˚C. Since the natural habitat of *C. elegans* is decaying fruit, compost and the surrounding soil it frequently encounters oxygen deprivation of varying durations. One goal of this study was to determine the biological effect of repeated exposure to sub-lethal bouts of anoxia.

The Hormetic Effect of Mild Stress

Understanding the role of mild stress exposure in preconditioning for acute stress tolerance is a current topic of interest. The concept that physiologic stress may be a beneficial component of one's environment remains a relatively unintuitive concept however, the preconditioning action of mild stress has been well documented (Mattson 2008; Calabrese et al. 2011,). The physiological impact of stress varies with several factors including the type and

intensity of the stress, as well as the age at which the stress is encountered (Arumugam et al. 2006;). There is accumulating evidence that the beneficial effects of stresses such as dietary restriction on lifespan extension operate through activation of pathways that pre-establish a physiological state that alleviates the effects of stress and decreases metabolic rate (Kenyon 2005; Oliveira et al. 2009; Van Voorhies & Ward 1999). For example, caloric restriction has been shown to activate AMPK (AMP-activated protein kinase) that stimulates production of ATP through catabolism of glucose and fatty acids while inhibiting energy consuming pathways (Steinberg & Kemp 2009; Hardie 2011). In addition to energy metabolism, AMPK operates in a broad range of cellular process such as increased autophagocytosis of damaged cellular organelles, upregulation of thioredoxin, and inhibition of edoplasmic reticulum stress and inflammation (Mihaylova & Shaw 2011; Li et al. 2009, Dong et al. 2010, Salminen et al. 2011). That these cellular processes are affected by aging is evidenced by metabolic diseases being most common in elderly people.

Environmental and Genetic Factors Precondition for LTA Tolerance

The insulin/IGF pathway of *C. elegans* influences lifespan through stress tolerance mechanisms that integrate metabolic and environmental cues, such as food availability and ATP levles (Apfeld *et al.* 2004; Ayyadevara *et al.* 2008). Dietary restriction or mutations that reduce DAF-2 activation promote translocation of DAF-16 into the nucleus where it links with other molecules to promote the upregulation of specific subsets of genes, some of which act in stress protective pathways. Our lab group has previously demonstrated that genetic and environmental factors can precondition wild-type *C. elegans* hermaphrodites for anoxia

survival. Mutations resulting in reduction in signaling through the insulin/IGF-like pathway (such as *daf-2(e1370)*) or reduction in ovulation due to mutations that cause sterility either by loss of a functional germline (such as *glp-1(e2141)* and *glp-4(bn2ts)*) or inability to produce fertilized oocytes (such as *fog-2(q71)* and *spe-9(hc52ts)*) results in long-term anoxia tolerance (Mendenhall *et al.* 2009). LaRue and Padilla (2011) reported that 1-day old wild-type adults cultured at an elevated temperature (25˚C) survive long-term anoxia better than age-matched controls cultured at 20˚C. This preconditioning effect was enhanced when animals were fed the HT115 strain of *Escherica coli* compared to a diet of the OP50 strain of *Escherica coli*. These observations support the idea that environmental factors can work synergistically to precondition for anoxia tolerance.

At day 1 of adulthood hermaphrodites survive 24h of anoxia at 100% and recover normal movement, as visually determined. However, if anoxia exposure is extended to 72h survivorship drops to less than 10% (Mendenhall *et al.* 2009). To better understand how an anoxia-tolerant physiological state can be formed in adult *C. elegans* I asked if short-term oxygen deprivation stress would precondition animals to survive long-term anoxia exposure. If bouts of anoxia act to promote formation of an anoxia-tolerant physiology then animals exposed to one or more anoxic bouts should have higher survival rates and less post-recovery impairment than untreated animals. To test this prediction I exposed aging adults to 1 or more 24h bouts of anoxia followed by a 72h anoxia challenge (long-term anoxia, LTA) and compared survivorship to age-matched adults that did not experience anoxic stress. I report here that exposure to 24h bouts of anoxia increased LTA tolerance both a strain-dependent and dose-dependent manner.

Results

Recurrent Short-Term Anoxia Exposure Enhances Long-Term Anoxia Tolerance

It is becoming increasingly clear that mild stress exposure can act hormeticly, confering a beneficial effect on stress tolerance (Arumugam *et al.* 2006). To assess the ability of mild-anoxic stress to enhance long-term anoxia tolerance I developed an exposure scheme by which animals were exposed to 1, 2 or 3 periods of 24h anoxia followed and 24h recovery in normoxia (referred to here as a bout) prior to long-term anoxia exposure. Details of the bout timeline are presented in Fig. 4.1. To confirm that exposure to 24h of anoxia had no inherent detrimental health effect on adult animals, the lifespan of animals exposed to anoxia bouts was compared to untreated control. Animals exposed to one anoxic bout showed a shift in lifespan equivalent to the duration of the anoxia exposure, indicating that the sub lethal exposure did not result in a detectable loss of health nor did it act to increase lifespan. These observations also support the idea that *C. elegans* does not appreciably age during periods of anoxia-induced suspended animation.

As shown above in this study, an organism's age can influence the biologic effect of encountered stress. To control for the potential effect of age at the onset of anoxia bouts I exposed animals to an initial anoxia bout as either young adults (immediately following the L4 molt) or at day 1 of adulthood. Exposure to even 1 anoxia bout was sufficient to significantly increase survival rates of animals challenged with LTA at day 1, day 2 or day 4 of adulthood (Fig. 4.2). At all ages assayed, except day 3, animals that experienced anoxic bouts had increased survivorship compared to controls. Survivorship at day 4 showed an additive effect of bouts. From these observations I concluded that recurrent exposure to anoxia stress operates in a

hormetic manner to precondition animals for increased LTA viability in a dose-dependent manner.

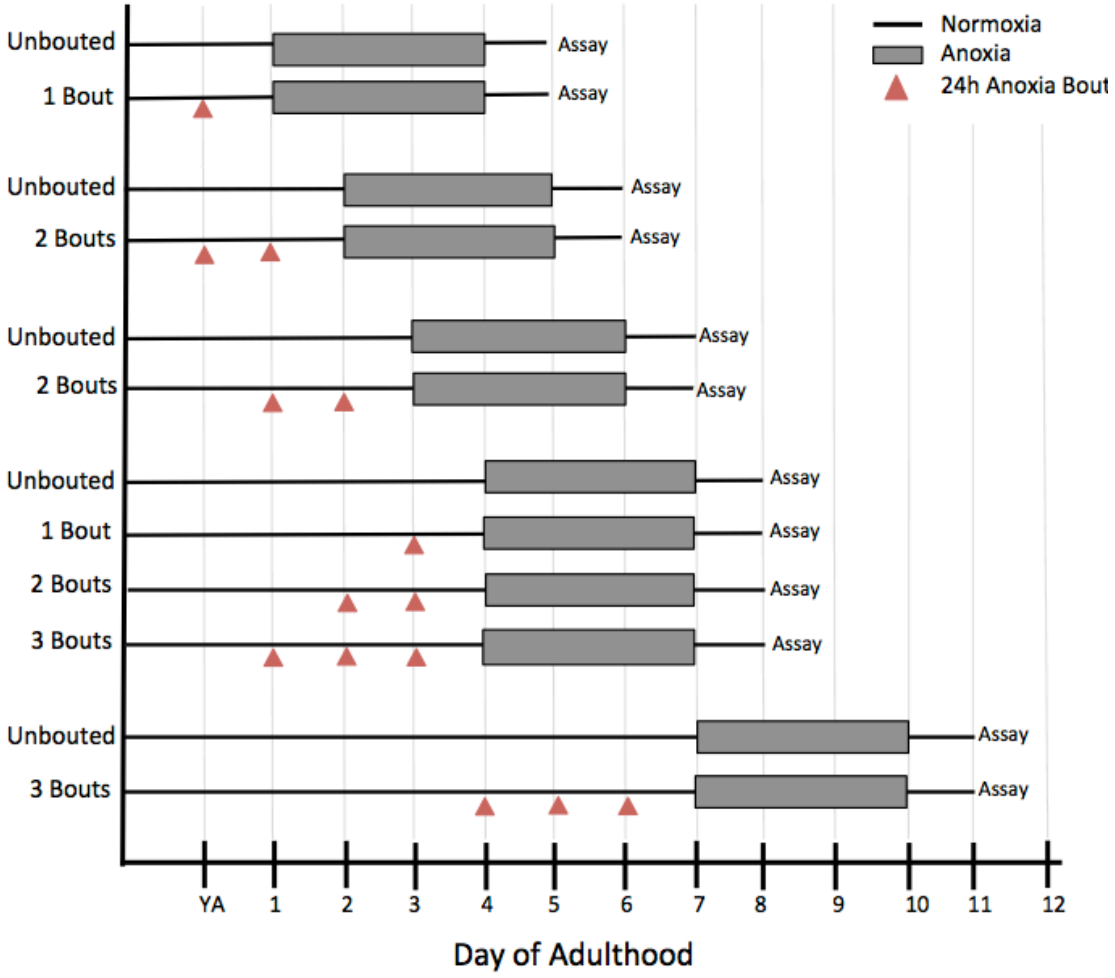

Fig. 4.1 Recurrent Anoxia Timeline. Black lines represent aging in normoxia. Animals were exposed to 0 (untreated controls), 1, 2 or 3 bouts of anoxia at the ages indicated by triangles. Each bout consisted of 24h of anoxia followed by 24h recovery in normoxia. At the indicated ages untreated and bout-exposed animals were challenged with 72h of anoxia (gray bars) followed by recovery in normoxia. Survivors were assayed for level of impairment. All phases of the experiments were conducted at 20˚C.

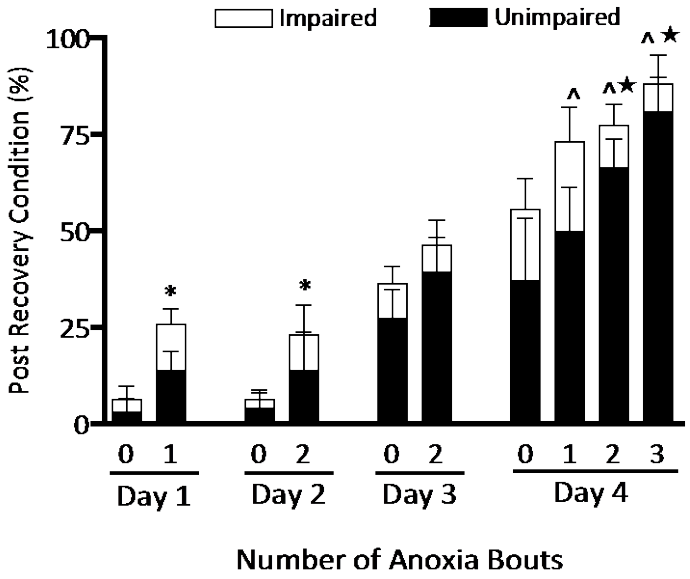

Fig. 4.2 Recurrent anoxia bouts precondition for long-term anoxia survival. Wild-type hermaphrodites exposed to 0 (controls), 1, 2 or 3 bouts of 24h of anoxia as described in the text, were challenged with 72h of anoxia at the adult day as indicated on the X-axis. Exposure to 1 or more bouts resulted in increased long-term anoxia survival rates compared to age-matched untreated controls for all ages assayed expect day 3 of adulthood. Numeric values along X-axis indicate number of anoxia bouts animals experienced prior to 72h anoxia challenge. Total animals examined is N>150 in three independent experiments; error bars represent standard deviation; experiment ANOVA ($F_{9,76}$ = 89.27; p<0.0001); * denotes significant increase compared to untreated controls within an age group (Tukey's, p<0.05), ^ denotes significant decrease compared to untreated controls within an age (Tukey's, p<0.001).

Short-term Anoxia Exposure Enhances Long-Term Anoxia Tolerance in a Gene-Dependent Manner

Anoxia tolerance is influenced by multiple factors including genetic factors. Recurrent bouts of anoxia abbrogated the difference in survivorship between aged wild-type animals and age-matched LTA-tolerant strains. Survival rates for 4-day old wild- type adults exposed to 3 anoxic bouts reached a level not different from rates seen for *daf-2(e1370)* and unmated *fog-2(q71)* of a similar age (day 5 of adulthood). Wild-type adults exposed to 3 anoxic bouts reached a level not different from rates seen for *daf-2(e1370)* and unmated *fog-2(q71)* of a similar age (day 5 of adulthood). Exposure to multiple anoxic bouts had the beneficial effect of

reducing the number of survivors with an impaired phenotype in a dose-dependent manner. Animals exposed to 2 and 3 anoxic bouts were significantly less impaired than controls or animals exposed to 1 bout.

This further supports the idea that exposure to multiple short bouts conferred a tolerance benefit in a dose dependent manner. To better understand the role of genotype in the beneficial effect of recurrent anoxia exposure I asked if LTA-sensitive strains could be preconditioned to survive LTA at rates at least as high as control wild-type hermaphrodites. Adults of *daf-16(mu86)* and *aak-2(gt33)* were exposed to short-term bouts of anoxia following the same exposure scheme and timeline used to precondition wild-type adults for LTA exposure at day 2 of adulthood (Fig. 4.3).

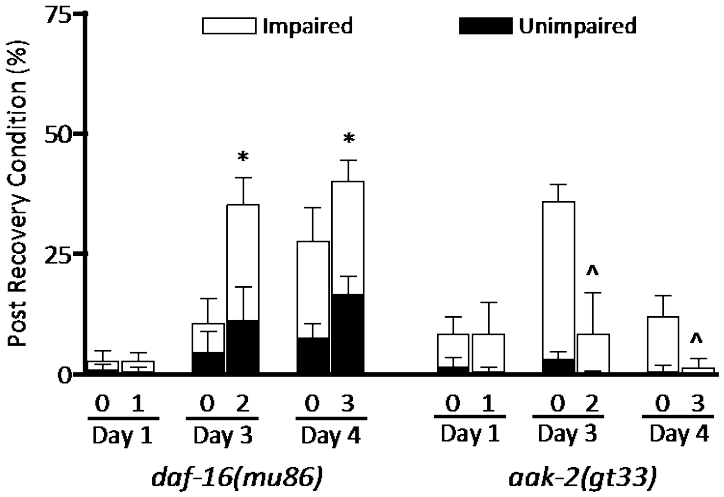

Fig. 4.3 The beneficial effect of recurrent anoxia bouts is daf-16-indepdendent and aak-2-depdendent. Adults if *daf-16(mu86)* and *(aak-2(gt33)* mutant strains were exposed to 0 (untreated controls), 1, 2 or 3 bouts of 24h of anoxia prior to challenge with 72h of anoxia at. Survivors were assayed level of impairment. Numeric values along X-axis indicate number of anoxia bouts animals experienced prior to 72h anoxia challenge. Total animals examined is N>125 from three independent trials; error bars represent standard deviation; experiment ANOVA ($F_{11,84}$ = 43.32; p<0.0001); * denotes significant increase compared to untreated controls within an age group (Tukey's, p<0.001), ^ denotes significant decrease compared to untreated controls within an age (Tukey's, p<0.001).

At day 3 and day 4, *daf-16(mu86)* animals exposed to anoxia bouts had higher survival rates compared to age-matched controls. However, the survival rate of *daf-16(mu86)* adults that experienced 2 bouts of anoxia and entered long-term anoxia at day 3 of adulthood was not significantly different from rates of 4-day old adults that experienced 3 bouts and was equivalent to that seen for 5-day old control adults that did not experience bouts (Fig. 2.2). Therefore, while exposure to anoxia bouts increased survivorship within an age group of *daf-16(mu86)* (at day 3 or day 4 compared to untreated controls), recurrent anoxia exposures did not increase survival rate above the maximum seen for untreated controls. This indicates that a functional *daf-16* is necessary to establish an LTA tolerant physiology via recurrent anoxia exposure.

Mechanisms utilized by animals to survive stressful environments usually include substantial alterations to metabolic activities (see chapter 1 for a review of these mechanisms). A common factor in these mechanism is suppression of processes that require ATP. It was not surprising to find that repeated exposure to anoxia had a detrimental effect on the *aak-2(gt33)* strain, which has reduced function of AMPK, the energy balance sensor kinase. Unlike other strains exposed to anoxia bouts, the *aak-2(gt33)* strain had a decrease in proportion of survivors with an unimpaired phenotype compared to untreated controls following 1 bout. Exposure to 2 or 3 bouts of anoxia resulted in a significant decrease in *aak-2(gt33)* survival rate. The *aak-2(gt33)* animals challenged with long-term anoxia at adult day 4 following 3 anoxia bouts had a survival rate of only 1.3%. I concluded from these observation that a functional *aak-2(gt33)* is required for the preconditioning effect of recurrent anoxia exposure.

Anoxia Exposure Reduces Fertility in a Dose-Dependent Manner

While conducting these experiments I observed that animals exposed to repeated anoxia bouts were laying fewer embryos than untreated controls. To confirm this observation I conducted brood assays for wild-type and *daf-16(mu86)* adults exposed to 3 short-term anoxia bouts. Bouts were initiated as either young adults or 1-day old adults. In line with other published results the mean brood size for untreated N2 hermaphrodites was 319 embryos laid between young adult and day 7 of adulthood.

Animals exposed to short-term bouts of anoxia had significantly smaller brood sizes compared to untreated controls (Table 4.1).

Table 4.1 Brood size analysis for hermaphrodites exposed to three recurrent short-term bouts of anoxia. Values are average number of eggs laid across all trials during the age interval indicated. Numbers shown in bold indicate embryos produced during a short-term anoxia bout or the following 24h period.

	Age at first anoxia bout (N)	Day of adulthood							Mean Brood Size	Reduction (%)
		YA	1	2	3	4	5	6		
Wild-type (N2)	Control (97)	79	**152**	57	10	2	1	1	319	--
	Young adult (21)	83	**114**	17	2	0	0	0	214*	-105 (33%)
	Adult day 1 81)	66	73	31	2	0	0	0	172*	-147 (46%)
daf-16(mu86)	control (18)	59	**145**	68	2	0	-	-	274	--
	Adult day 1 (16)	81	76	17	1	0	-	-	175^	-99 (36%)

N = number of animals assayed over five independent trials for N2 and two independent trials for *daf-16(mu86)*; YA denotes young adult stage immediately post L4 to adult molt; superscripts denote signficiant difference compared to untreated controls; Unpaired t-test, * denotes $p<0.0001$, ^ denotes $p<0.005$.

The age at which anoxia bouts were initiated made a difference in the overall effect on brood size. Mean brood size was reduced by 33% when males (controls) and the number of offspring produced during the subsequent 48h period were determined. Hermaphrodites of both groups laid an equal number of embryos during the first 24h following anoxia exposure (males were present) (Table 4.2). During the 48h-72h post-anoxia period survivors cultured with males produced significantly more offspring than controls.

Table 4.2 Number of offspring produced by hermaphrodite and males following long-term anoxia exposure. Values indicate number of offspring produced in the time period indicated.

Treatment	Post Anoxia Period		% male progeny
	24h	48h-72h	
Hermaphrodites (*N*)			
No males present (6)	6.8	1.5	0
Males present (7)	4.3	42.7*	45.8
	Time with females		
	24h	48h-72h	
Males (*N*)			
Untreated males (10)	113	162	51.2
Anoxia exposed males (10)	0	0	-

N = number of animals assayed over three independent trials; ANOVA ($F_{3,26}$ = 17.71; p<0.001); * denotes significant difference from control (Tukey's p<0.05)

Post-anoxia mating was confirmed for survivors cultured with males by presence of approximately 46% males in the brood produced compared to no males produced by control animals. I then determined whether 1-day old adult wild-type males exposed to long-term anoxia were capable of mating and fertilization of oocytes. Following exposure to long-term anoxia at adult day 1 each male was placed with 1-day old unmated *fog-2(q71)* females. The number of offspring produced during the following 48h-72h period was assayed. During this period no mating attempts by males were observed and no offspring were produced. In

contrast, wild-type males not exposed to long-term anoxia produced normal size broods when placed with unmated *fog-2(q71)* females. It is not clear whether failure of anoxia-exposed males to fertilize offspring is due to reduced sperm viability or if the anoxia exposure resulted in behavioral changes that prevented males from mating.

Short-term anoxia bouts enhance long-term anoxia tolerance in post-reproductive adults. The mechanism by which anoxia bouts enhance long-term anoxia is not clear. It ispossible that repeated anoxia exposure leads directly to an as yet undetermined change in somatic physiology that imparts long-term anoxia tolerance. However, it is also possible that bout exposures are beneficial to adults by reducing fecundity, a characteristic previously shown to increase anoxia tolerance. If this hypothesis is accurate then hermaphrodites that have reached self-reproductive senescence are predicted to not show an increase in long-term anoxia tolerance following exposure to anoxia bouts. To test this prediction I exposed 4-day old wild-type hermaphrodites to 0 (control) or 3 short-term bouts as described above. Establishing proper controls for these experiments required addressing the course of aging in animals exposed to multiple short bouts of anoxia. If one considers aging to halt during suspended animation then aged animals exposed to anoxia bouts were at adult day 7 when challenged with long-term anoxia. However, if one considers the aging process to continue during suspended animation then the aged adults were challenged at day ten of adulthood. To increase stringency in the analysis I chose to compare survival rates and impairment levels of bout exposed animals to the more robust 7-day old adults. Exposure to anoxia bouts had a beneficial effect on post-reproductive hermaphrodites, resulting in a 60% increase in survival and a significantly greater proportion of survivors with an unimpaired phenotype compared to

untreated controls (Fig. 4.4). Furthermore, bout exposed wild-type adults had a significantly higher survival rate and reduced level of impairment compared to 7-day old LTA-tolerant *fog-2(q71)*, but not for 7-day old *daf-2(e1370)* or *glp-1(e2141)*. These observations are in contrast to results expected if short-term anoxia bouts enhance anoxia tolerance simply by reducing fecundity. These data support the idea that the physiologic state of the animal at the time of anoxic stress exposure is a determining factor in tolerance level.

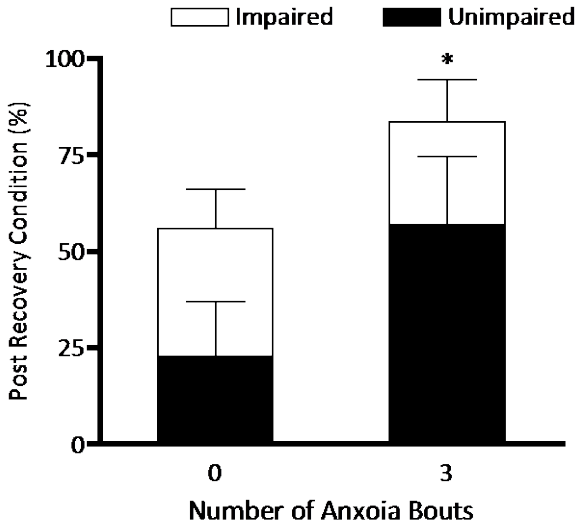

Fig. 4.4 Exposure to short-term anoxia bouts preconditions aged adults for long-term anoxia tolerance. At day 4 of adulthood wild-type hermaphrodites were exposed to zero (controls) or three 24h bouts of anoxia prior to challenge with long-term anoxia at day 7 of adulthood. Exposure to anoxia bouts enhanced survival rate and portion of survivors with unimpaired phenotype relative to untreated controls. Total animals examined is $N>100$ from three independent trials; error bars represent standard deviation; * denotes significant difference compared to controls, (Unpaired t-test, $p<0.001$).

Discussion

The environment to which an organism is exposed can have profound effects on phenotype. Animals exposed to fluctuating environmental factors such as temperature, diet,

and toxin concentration or oxygen availability often display activated stress responses. Here I report that *C. elegans* exposed to short-term anoxia bouts significantly increased survival rates when challenged with long-term anoxia exposure. Conditioning bouts operated in a dose-dependent fashion to establish a physiological state allowing wild-type hermaphrodites to tolerate long-term anoxia at least as well as age-matched *daf-2(e1370)* hermaphrodites, that have a reduction in flux through the insulin/IGF signaling pathway, or age-matched *fog-2(q71)* hermaphrodites, that are sterile. However, the survival rate of bout exposed N2 hermaphrodites failed to reach the level seen for *glp-1(e2141)* of similar age (day 5 of adulthood).

Recurrent Anoxia Exposure Reduces Brood Size

Repeated exposure to short-term anoxia bouts affected animals in a gene-dependent manner. The beneficial effect of anoxia exposure was evident in the increased survival rates of early adult *daf-16(mu86)* hermaphrodites compared to untreated age-matched controls, suggesting a preconditioning effect that is independent of DAF-16-activated stress pathways. However, even when exposed to multiple preconditioning bouts maximum survivorship of *daf-16(mu86)* hermaphrodites remained well below that of age-matched preconditioned wild-type (N2) or LTA-tolerant strains. I interpret these observations to indicate that the effect imparted by recurrent anoxia bouts operates on at least two levels, via genotype-independent and genotype-dependent factors. An important genotype-independent factor is likely to be the inhibitory effect of anoxia on reproduction. For both N2 and *daf-16(mu86)* hermaphrodites exposed to recurrent anoxia bouts had significantly reduced brood sizes in a dose-dependent

manner. Reduction in reproductive effort is associated with anoxia tolerance and the increase in survivorship seen in early adulthood for bout exposed *daf-16(mu86)* may represent the contribution of reduced reproduction to the formation of a long-term anoxia phenotype.

Recurrent Anoxia Exposure Preconditions in a Gene Dependent Manner

This preconditioning benefit is DAF-16-indepdent. However, the inability of recurrent bouts to raise peak survival rates of the *daf-16(mu86)* null strain to levels seen for bout exposed wild-type or LTA-tolerant strains is evidence of DAF-16-dependent factors in the preconditioning process. It appears that while DAF-16-dependent gene activation is not required for the beneficial effect of bouts received in early adulthood, it is necessary to elicit a maximal preconditioning effect. In contrast to the *daf-16(mu86)* strain, the *aak-2(gt33)* strain exposed to 2 or more anoxia bouts had a significant decrease in LTA-survival rate indicating that AMPK activity is necessary for the early adult preconditioning effect and emphasizing the importance of energy sensing and AMP:ATP ratio management for the preconditioning effect associated with reduced reproduction.

Preconditioning by Recurrent Anoxia Exposure is Age Dependent

The effect of recurrent anoxia bouts differs between young and aged hermaphrodites. Aged wild-type (N2) hermaphrodites exposed to 3 recurrent bouts of anoxia after reaching self-fertilization senescence displayed a significant increase in both survival rate and portion of survivors with an unimpaired phenotype compared to untreated age-matched controls and unmated *fog-2(q71)* females, which are considered LTA-tolerant. Furthermore, the survival rate

of bout exposed aged N2 hermaphrodites reached levels not significantly different from rates seen for age-matched *daf-2(e1370)* and *glp-1(e2141).* The cause of this robust increase in tolerance is uncoupled from the effects of reduced reproduction. Instead, the increase reflects physiological alterations capable of transforming wild-type hermaphrodites into a state that is phenotypically indiscernible from that generated by reduced insulin-signaling or germline depletion. It is unclear why recurrent anoxia exposure encountered early in adulthood does not raise survival rates to the same level as when encountered by aged adults. One explanation for this observation is that the physiologic state required for reproduction and the state necessary to execute stress resistant pathways are mutually exclusive. If so, the anoxia induced decrease in embryo production seen in early adulthood may be indicative of a transition in physiological state that is neither favorable toward reproduction nor stress resistance.

Recurrent Bouts of Anoxia are Hormetic

Overall, repeated exposure to short bouts of anoxia appears to be operating in a hormetic fashion. Anoxia exposure is biphasic, with short duration intermittent exposures stimulating formation of a preconditioned physiological state, and long duration exposure being lethal. In its natural environment, *C. elegans* is likely to encounter short episodes of varied stresses, owing to the ephemeral nature of its environment. Consequently, the ability to respond to stressful environmental conditions by delaying oocyte maturation until more favorable condition are encountered would be an evolutionary stable strategy acting to maximize fitness. Such a system would require sensory and signaling mechanisms sensitive to environmental cues. In this study I have presented data supporting the role of *daf-16* and *aak-2*

in such pathways. It remains unclear whether different environmental perturbations result in activation of common stress-tolerant pathways.

Selection has likely operated differently upon males than hermaphrodites. Interestingly, in preliminary trials I was unable to increase the survival rate of wild-type males by exposing them to recurrent anoxia bouts (data not shown). It is not unreasonable to consider that males may not have evolved to respond to preconditioning stress in the same manner detected in hermaphrodites. Instead, selection may have worked at the level of producing males capable of producing large amounts of sperm along with a behavior that drives males to attempt fertilization with all hermaphrodites encountered (Chasnov 2002; Hosono *et al.* 1982). It is been established that the percentage of males in a population increases soon after the onslaught of a variety of stresses and that male *C. elegans* tenaciously pursue hermaphrodites (Emmons & Stemberg 1997). In such a system, hermaphrodites that mature in low stress conditions can maximize the number of offspring passed to future generations through self-fertilization which minimizes the benefit of crossing and favors production of hermaphrodite offspring instead of males. However, when a hermaphrodite encounters a stressful environment (either a recurring event or an acute exposure) selection would favor suspending reproduction until more favorable environmental conditions are restored. In populations recovering from a perturbing stress the percentage of males in the first offspring produced approaches 50%, favoring genetic recombination that is usually associated with maximizing stress resistance of future generations. The hypothesis presented here is one explanation of the data presented in this study, and I cannot rule out alternative explanations.

Given that adult hermaphrodites of the Hawaiian wild-type isolate strain CB4856 are less tolerant of LTA exposure than N2 hermaphrodites it would be of interest to determine whether exposure to anoxia bouts results in a preconditioning effect in. If CB4856 hermaphrodites respond differently to recurrent bouts of anoxia than the N2 strain then further comparison of the genotypic differences between the strains (for example, SNP analysis) may provide insight into the genetic basis of anoxia tolerance. Furthermore, the assay developed for this study represents a new method of evaluating the role of target genes in formation of an oxygen deprivation tolerant physiology.

CHAPTER 5

MATERIALS AND METHODS

Caenorhabditis elegans Strains and Culture Conditions

The following genetic strains were obtained from the *Caenorhabditis elegans* Genetics Center: N2 Bristol (wild-type), CB1370 [*daf-2(e1370)*], CB4037 [*glp-1(e2141)*], CB4108 [*fog-2(q71)*], CB4856 (wild-type isolate), CF2065 [*kri-1(ok1251); glp-1(e2141*)], CF2278 [*daf-16(mu86);glp-1(e2141);daf-12(rh61rh411)*], CF2288 [*daf-16(mu86);glp-1(e2141);daf-9(rh50)*], TG38 [*aak-2(gt33)*], CF1038 [*daf-16(mu86)*]. All strains were cultured on nematode growth media (NGM) plates seeded with *Escherichia coli* (*OP50*) and raised at 15˚C, 20°C or 25°C as indicated for each experiment.

Oxygen Deprivation Experiments

For all experiments age appropriate adults were exposed to anoxia at 20°C using anoxia Bio Bags (Becton Dickinson) as previously described (Padilla *et al.* 2002). Transition time to anoxia in these bags is <2h as determined by a resazurine anoxia indicator (<0.001kPa of O_2 detection limit). Briefly, four 60mm petri dishes containing seeded NGM and 30-50 worms were placed without lids in Bio Bags (Becton Dickinson) as previously described (Mendenhall *et al.* 2006). Prior to sealing the bag an uncapped 15ml conical centrifuge tube was positioned between the anoxia generator and the petri dishes serving to minimize the transfer of heat from the generator to the petridishes. Likewise, the anoxia catalyst device was placed in a centrifuge tube cap and positioned to the side of the petri dishes. The anoxia generators were activated after the Bio Bag was heat-sealed. For the purpose of these experiments I refer to

long-term anoxia (LTA) as 3 or 4 days of anoxia at 20°C. After anoxia exposure animals were allowed to recover for 24h in air at 20˚C prior to being scored for viability and motility as previously described (Mendenhall *et al.* 2009). Briefly, animals were scored using a standard dissection stereomicroscope as dead (no response to prodding by a worm pick) or as survivors. Survivors were further classified using a stereomicroscope as unimpaired (no detectable motility defects) or impaired (detectable morphological, behavioral, or motility defects).

Age Determination

Synchronized larval populations were obtained by collecting embryos from hypochlorite-treated adults or by collecting embryos laid by 5 to 10 hermaphrodites during a 2h to 3h period. Strains were cultured at 20˚C (unless otherwise stated) and animals collected 22-26h after the L4 to adult molt were designated as 1-day old. Strains carrying temperature-sensitive mutations were maintained at 15˚C and newly laid embryos were held at 15˚C for 24h then shifted to 25˚C for 48h and designated as 1-day old adults. Males were collected at the L4 larval stage or as young adults from a synchronized population of hermaphrodites and males. For all strains animals were considered to age by 1 day for every 24h and were not considered to increase in age during anoxia-induced suspended animation.

Mating Suppression Analysis

To determine the effect of extended reproduction on long-term anoxia survival rates N2 and *fog-2(q71)* hermaphrodites were grown to day 2 of adulthood then transferred individually to breeder plates along with 5 males of the same strain. At day 3 of adulthood hermaphrodites

were transferred to fresh breeder plates and exposed to long-term anoxia and assayed for survival rate and motility as described above. Mating was confirmed for each hermaphrodite by presence of ~50% males in the offspring produced during the 24h mating period.

Temperature Shift Assays

To determine whether presence of germline stem cells influences anoxia tolerance I used a temperature-shift regimen (adapted from Arantes-Oliveira *et al.* 2002) to control the activity of a *glp-1(ts)* allele prior to 3 days of anoxia exposure and recovery. Briefly, *glp-1(e2141)* embryos were collected by allowing adults cultured at the permissive temperature (15˚C) to lay eggs on a plate for 2-3h. The adults were removed and embryos were held at 15˚C for 24h to allow normal embryonic development. Larvae were then either maintained at 15˚C or shifted to 25˚C for 48h at which time the shifted animals were considered to be 1-day old adults. Animals maintained at 15˚C matured more slowly than their shifted-cohorts and were considered to be 1-day old adults 24h after the L4 to young adult molt (approximately 5 days after being laid). At day 1 of adulthood, animals that had been shifted to 25˚C were divided into two groups. Half of the animals were maintained at the nonpermissive temperature for an additional 24h (25˚C control) and the remaining half were returned to the permissive temperature (15˚C) for 24h (down-shifted). Similarly, when animals maintained at 15˚C reached day 1 of adulthood they were divided into two groups; half were maintained at 15˚C for an additional 24h (15˚C control) and half were shifted to 25˚C for 24h (Up-shifted). Immediately prior to the temperature shift animals that had been reared at 15˚C were moved to fresh plates so that embryos laid during the elevated temperature period could be monitored. At 18h after

the shift to 25˚C the adults were again moved to fresh plates and the old plates carrying embryos and larvae were held at 25˚C for 36h, at which time the embryos deposited on the plates were assayed for hatching. In the same manner, at the conclusion of the 24h temperature exposure period adults were again moved to fresh plates and embryos on the old plates assayed for hatching. Immediately following the shifting treatment animals were exposed to 3 days of anoxia (20˚C) and 24h of normoxia recovery. After recovering animals were assayed for survival and level of impairment.

Recurrent Anoxia Exposure Assays

Age synchronized adults of indicated strains were exposed to 1 or more 24h bouts of anoxia prior to long-term anoxia exposure and assayed for survival and motility as detailed in Fig. 4.1. Anoxia exposure was carried out in Bio Bags as described above. Previous work has shown that effective preconditioning requires recurrent insults be separated by a recovery period (Centeno *et al.* 1999). Our lab has previously determined that most strains of *C. elegans* survive 24h of anoxia at high levels with no permanent visible effect on behavior or body movement. For these experiments an anoxia bout was defined as 24h of anoxia (20˚C) followed by a 24h recovery in air (20˚C). The age of animals at the initial bout varied according to experimental condition. For experiments involving multiple exposures each subsequent bout began immediately following the recovery period of the previous bout. Animals were exposed to LTA immediately following the recovery period of the final bout and assayed for viability and impairment as described above. Worms entered suspended animation during each anoxia bout and for the purpose of statistical analysis were considered to not age during the bout.

Brood Assays

Twenty control L4 hermaphrodites were placed individually on 35 mm petri dishes (breeder plates) and moved daily to fresh breeder plates until no embryos were laid within a 24h period. F1 progeny of each individual were counted daily. An additional twenty experimental L4 hermaphrodites were cultured in the same manner to either young adult or day 1 of adulthood. At the indicated age individuals were moved to fresh breeder plates and exposed to 24h of anoxia followed by a 24h recovery in air (one bout). For subsequent bouts adults were moved to fresh breeder plates and the bouting process repeated. After the final bout adults were moved to fresh plates daily until no embryos were produced in a 24h period. For each individual total F1 progeny produced before, during and after bouting were counted.

Post-Anoxia Reproductive Viability Analysis

Long-term anoxia survivor N2 and *fog-2(q71)* hermaphrodites and males were transferred individually to seeded NGM breeder plates following a 24h recovery in air. Five untreated 1-day old adult males of the same strain were placed onto plates with each hermaphrodite and 3 untreated 1-day old adult *fog-2(q71)* unmated hermaphrodites were placed onto plates with each male. Plates were monitored for visual evidence of mating. Adults were moved to fresh plates daily and number of offspring produced during the following 72h was determined. To confirm newly laid embryos by survivor hermaphrodites were the result of post-anoxia mating the percentage of males in the offspring produced was confirmed to be approximately 50%.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 4 for Macintosh, 1994-2005. One-way ANOVA followed by Tukey's Multiple Comparison Test determined effect of aging on LTA tolerance, significance level set at $p<0.05$. Comparison between two survival rates was determined using one-tailed t-test, significance level set at $p<0.05$.

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