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Anoxia-Induced Suspended Animation in *Caenorhabditis elegans*

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1. Introduction

Some of the most complex biological processes were first elucidated in somewhat "simple" genetic model organisms. For example, where would we be in our molecular and cellular understanding of gene expression, cell division, embryo development and cell death if it were not for research using *E. coli*, Yeast, *Drosophila* and *C. elegans*? Due to the pioneering work by Sydney Brenner and others, the soil nematode *C. elegans* is now a well-known genetic and developmental model system (Brenner, 1974). Genetic approaches have contributed significantly to our advanced understanding of the mechanisms regulating gene function, organ development, microRNA function and signaling pathways regulating aging and stress (WormBook). The molecular advances and development of genetic tools such as RNA interference (RNAi) and protein expression analysis with the Green Fluorescent Protein (GFP) were initially worked out in *C. elegans* and thus firmly established this organism as a cornerstone of genetic models for unraveling the molecular mechanisms of many biological processes (Chalfie and Kain, 2006; Fraser et al., 2000; Jorgensen and Mango, 2002; Timmons and Fire, 1998). Research from many labs have clearly demonstrated that this small soil nematode has contributed significantly to our understanding of biology and that multiple types of molecular tools exist to elucidate mechanistic details. Further examples of the significant impact *C. elegans* has had in our understanding of biology are the fairly recent Noble Prize awards to six individuals (S. Brenner, M. Chalfie, A. Fire, R. Horvitz, C. Mello, J. Sulston) who made paradigm shifting discoveries using *C. elegans*. Through the effort of many within the *C. elegans* community, the molecular tools and genetic resources available in this model system have helped to address and elucidate the molecular mechanisms regulating multiple biological processes.

C. elegans, a soil nematode, was originally isolated in Bristol, England (N2 Bristol strain) (Brenner, 1974). In its natural environment oxygen levels fluctuate, therefore *C. elegans* likely evolved mechanisms to survive changes in oxygen levels (Lee, 1965). Indeed, it is known that *C. elegans* survive oxygen deprivation (hypoxia and anoxia) that have an impact on behavior, growth and development (Anderson, 1978; Padilla et al., 2002; Paul et al., 2000; Van Voorhies and Ward, 2000). *C. elegans* are able to sense various oxygen tensions and in fact prefer a 5-12% O_2 concentration (Gray et al., 2004). A lower level of oxygen in the

environment is stressful to the worm yet the animal has evolved mechanisms to survive the stress of hypoxia and anoxia. A review by Powell-Coffman nicely provides an overview of the signaling pathways and responses involved with oxygen deprivation in *C. elegans* (Powell-Coffman, 2010). In *C. elegans* and other metazoans a key to sensing low oxygen levels within the environment is the hypoxia-inducible factor HIF-1. The transcription factor HIF-1 is needed to induce the expression of a variety of genes so that the animal may survive low oxygen environments (Jiang et al., 2001). The mechanisms regulating HIF-1 activity are being worked out in a variety of systems including *C. elegans* and has been reviewed in many publications (Epstein et al., 2001; Semenza, 2007; Semenza, 2010) Interestingly, for *C. elegans*, the level of oxygen in the environment will dictate which genes are required for oxygen deprivation response and survival. For example, HIF-1 function is required for animals to survive and maintain normal developmental functions in .5% and 1% O_2 however, HIF-1 is not required for anoxia survival (Padilla et al., 2002). This review will focus on the response to anoxia and known mechanisms required for *C. elegans* to survive anoxia.

2. Methodology for studying anoxia response and survival in *C. elegans*

Given that the specific oxygen concentration affects the response *C. elegans* has to the environment it is important to discuss the methodologies used to produce an anoxic environment within the laboratory setting. Typically, several laboratory methods are used to expose *C. elegans* to anoxia. Figure 1 provides a general review of the methodologies commonly used and the pros and cons of each method. For example, a convenient and cost effective approach is to place *C. elegans*, which are grown on agar nematode growth media (NGM), into anaerobic biobags (Becton Dickson Company). These anaerobic biobags are typically used for growing anaerobic bacteria and use resazurin at an oxygen indicator. Another approach that allows one to study subcellular responses to brief periods of anoxia, in live animals, is to use an anoxia flow-through chamber in conjunction with a spinning disc confocal microscope. This method has been valuable in following chromosome structure in embryos and oocytes within adult hermaphrodites exposed to brief periods of anoxia. If one is planning to expose a large number of animals to anoxia, the use of a hypoxia chamber that can hold many *C. elegans* plates is the best approach. The use of a hypoxia chamber has been valuable for large-scale forward genetic or RNA interference screens. There are different types of hypoxia chambers; those that can be commercially purchased or those that can be tailor designed by the researcher. The chambers that are researcher designed can be made to allow a flow through of nitrogen to replace the air. The commercially available glove box chambers (Ex: Ruskinn Inc. anaerobic and hypoxia workstations) are useful if one needs to expose animals to oxygen deprivation for very long periods of time or if the animals need to be manipulated while exposed to anoxia. The primary disadvantage to the glove box chambers is that for some models a temperature below ambient can only be reached if the entire chamber is located within a low temperature room. Temperature during anoxia exposure is an important consideration given that temperature does influence anoxia survival rate (LaRue and Padilla, 2011). It is of interest to use more than one of these methodologies to verify observed anoxia responses and phenotypes are consistent.

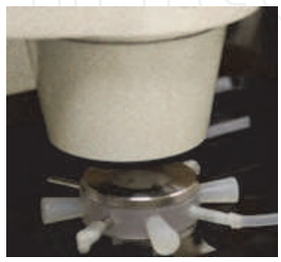
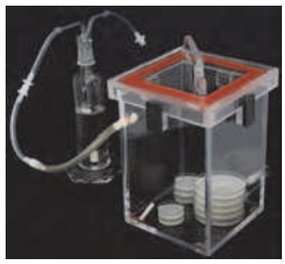
Method	Pros	Cons
Anaerobic biobag 	Inexpensive, commercially available Portable and can be placed in various temperature incubators Fairly consistent transition time to anoxia No need for gas tanks	Limited number of plates can be put into the environment A brief increase in temperature due to the chemical reaction used to remove oxygen within the biobag
Microscope chamber 	Visualize subcellular and cellular changes *In vivo* analysis of GFP fusion protein markers Hypoxia experiments also feasible	Exposure time is limited Potential issues such as sample dehydration Small number of animals analyzed
Flow-through chamber 	Researcher designed for specific experiments Hypoxia experiments also feasible Large number of animals can be simultaneously exposed Can be placed in a specific temperature controlled room	Transition time from normoxia to anoxia can vary
Glove box chamber 	Hypoxia experiments also feasible Manipulation of animals while in the anoxic environment Can immediately place animals into the environment with no transition time Large number of animals can be simultaneously exposed Commercially available from various sources	Costly Chamber temperature higher than ambient unless placed in room with reduced temperature

Table 1. Various methodologies used to expose *C. elegans* to anoxia.

3. *C. elegans* as a model to study anoxia-induced suspended animation

Some organisms, including metazoans, exposed to stresses or naturally occurring signals can arrest processes such as development, cell division or heartbeat (Clegg, 2001; Mendelsohn et al., 2008; Padilla and Roth, 2001; Podrabsky et al., 2007; Renfree and Shaw, 2000; Riddle, 1988). Suspended animation is the arrest of observable biological processes induced by either a cue or stress in the environment, or a signal from within the animal. In the case of *C. elegans*, animals exposed to anoxia will enter into a reversible state of suspended animation in which observable biological processes, such as cell division, development, eating, egg laying, fertilization and movement arrests until air is reintroduced into the environment. Suspended animation can be maintained for a few days, depending on the developmental stage of exposure; extended periods of anoxia exposure will lead to lethality. Figure 1 demonstrates the developmental arrest that is observed in *C. elegans* exposed to anoxia. In a hypometabolic state, such as suspended animation, homeostasis is maintained until the environment required to support energy requiring processes is resumed. However, hypometabolic states, including suspended animation, are not maintained indefinitely and at some point the animal may die if the environment is not shifted to a more conducive state to support biological processes. Embryonic diapause, another hypometabolic state that can be either obligate or environmentally induced, is a natural survival strategy to maintain populations and maximize offspring. Embryonic diapause can be thought of as a state of suspended animation in that development and cell divisions are arrested (Clegg, 2001). An example of a vertebrate that enters into an obligate diapause is the killifish embryo *Austrofundulus limnaeus*. *A. limnaeus* embryos in diapause II are remarkably tolerant to anoxia (Podrabsky et al., 2007). The mechanistic overlap between developmental arrests induced by anoxia in comparison to naturally occurring diapause remains to be determined.

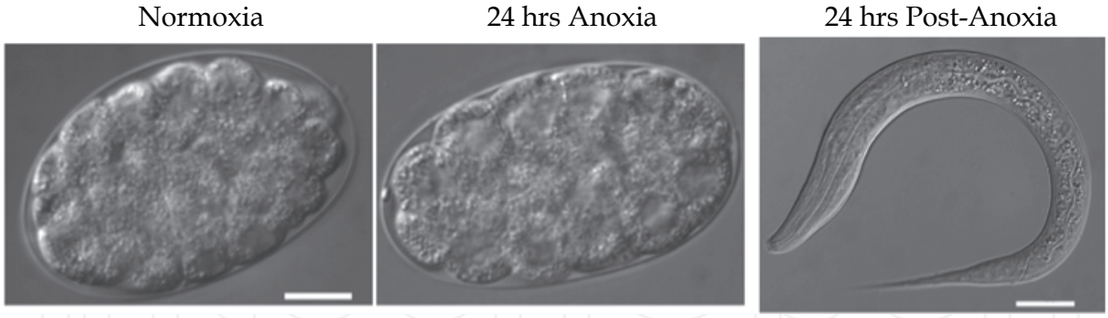


Fig. 1. *C. elegans* exposed to anoxia enter into a reversible state of suspended animation in which development and cell cycle progression will arrest. Shown are embryos collected from a gravid adult and exposed to normoxia or anoxia for 24 hours. The anoxia-exposed embryo will arrest development for several days and yet remain viable (shown is an embryo exposed to 24 hours of anoxia). The post-anoxia animal will resume development when air is added back to the environment. Scale bar = 10 μm for embryos and 20 μm for post-anoxia larva.

At every stage of development *C. elegans* survive 24 hours of anoxia exposure at a rate of 90% or greater (Foll et al., 1999; Padilla et al., 2002; Van Voorhies and Ward, 2000). The most anoxia tolerant developmental stages are dauer larvae and embryos (Anderson, 1978;

Padilla et al., 2002). The ability to survive longer bouts of anoxia depends upon developmental stage, growth temperature, diet, genotype and fertility; these factors will be elaborated on further in this chapter. In general, *C. elegans* are sensitive to anoxia (and hypoxia) if the temperature during exposure is increased (Ex: 28°C instead of 20°C) or if the duration of anoxia exposure is increased from one to three days (Mendenhall et al., 2006; Scott et al., 2002). After non-lethal exposures to anoxia the animals will resume biological processes such as cell division, development, eating, movement, and offspring production. How quickly animation resumes is dependent upon the anoxia exposure time. For example, an embryo that was exposed to one day of anoxia will resume cell cycle progression faster than an embryo that was exposed to three days of anoxia (Hajeri et al., 2005). Many aspects of anoxia response and survival are not understood. Listed below are questions that will be of interest to address in terms of molecular mechanisms regulating anoxia responses.

1. What genetic factors control entry, maintenance and exit from anoxia-induced suspended animation?
2. What cellular changes occur in animals exposed to anoxia and are such changes necessary and sufficient for anoxia-induced suspended animation?
3. In the embryo, how does a reduction in oxygen levels signal cell cycle arrest; via which cell cycle machinery?
4. How are developmental programs arrested and resumed in embryos and larvae exposed to anoxia?
5. How are complex tissues, such as muscles and neurons in adults, maintained during anoxia exposure?
6. What is the metabolic state of animals exposed to anoxia relative to duration and developmental state?
7. How do metabolic changes influence anoxia-induced suspended animation?
8. What molecular mechanisms balance offspring production and anoxia stress survival?
9. How can anoxia studies in *C. elegans* be used to better understand oxygen deprivation sensitivity in humans?

4. Anoxia-induced cell cycle arrest in the developing embryo

There are known environmental changes that influence cell cycle progression. For example, UV radiation will activate cell cycle checkpoint proteins and lead to a cell cycle arrest and repair of DNA damage so that the cell can progress through cell division (Hartwell and Weinert, 1989; Nurse et al., 1998). Also, exposing cells to drugs (Ex: Taxol, Nocodazole) was instrumental in the identification of cell cycle checkpoint genes. Identifying the fundamental regulation of cell cycle progression is central to the development of cancer treatments, thus understanding how oxygen levels affect cell division is of interest. Anoxia-exposed *C. elegans* embryos contain blastomeres that arrest at specific positions of the cell cycle: interphase, late prophase and metaphase. The lack of anaphase blastomeres indicates that the embryos are not progressing through the cell cycle further supporting that these embryos are indeed arrested. The phenomenon of anoxia-induced arrest is not unique to *C. elegans* since zebrafish (*Danio rerio*) and *Drosophila melanogaster* embryos also arrest cell cycle progression in response to anoxia or hypoxia (DiGregorio et al., 2001; Douglas et al., 2001; Foe and Alberts, 1985; Padilla and Roth, 2001). The use of cell biological techniques, such as indirect immunofluroescent assays or *in vivo* GFP fusion protein assays, showed that anoxia-

arrested blastomeres have specific characteristics or hallmarks (Hajeri et al., 2005). An overview of the anoxia-induced cellular changes observed in embryos is summarized in Table 2 and discussed in detail throughout this section.

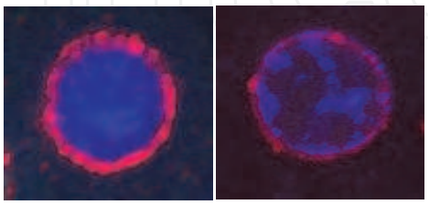
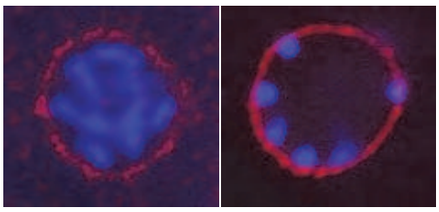
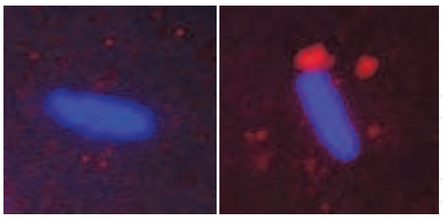
Cell cycle stage and environment	Characteristics of anoxia-arrested blastomeres and genes required
Interphase Nucleus: Normoxia Anoxia 	• Chromatin is condensed instead of dispersed throughout nucleus • Chromatin associates with inner nuclear membrane • Based on centrosome location, a specific stage of interphase arrest is not observed • Genes required for interphase arrest: none identified to date
Late Prophase Nucleus: Normoxia Anoxia 	• Chromosomes fully condensed • Chromosomes "dock" near the inner nuclear membrane • CDK-1 localizes to the chromosomes • Inactive form of CDK-1 (P^{Tyr15}) is detected • Genes required for prophase arrest: *npp-16* (NUP-50 homologue which is a component of the nuclear pore complex) and *hda-2* (histone deacetylase)
Metaphase Nucleus: Normoxia Anoxia 	• Nuclear pore complex aggregates, as detected by mAb 414 • Spindle microtubules are present but show some depolymerization • Spindle checkpoint protein SAN-1 localization altered • Reduced phosphorylation of various proteins including Histone H3 at Serine 10 and proteins recognized by MPM-2 antibody • Genes required: Spindle checkpoint genes *san-1* and *mdf-2*

Table 2. Characteristics of anoxia-induced cell cycle arrest. Shown are representative nuclei from normoxic and anoxic embryos. Nuclear membrane, recognized by mAb 414, is shown in red and DNA, recognized by DAPI, is shown in blue.

4.1 Cellular changes associated with interphase arrest

Interphase blastomeres of anoxia-exposed embryos contain chromatin that is highly condensed and the level of condensation appears to increase with longer periods of anoxia exposure (Foe and Alberts, 1985; Hajeri et al., 2005). Chromatin condensation is characteristic of inactive chromatin and thus it is likely that a global down regulation of gene expression occurs in anoxic embryos. A reduction in gene expression is likely a means to conserve energy and maintain metabolic homeostasis (Hochachka et al., 1996). We know little about the mechanisms that regulate arrest of interphase blastomeres and if the interphase blastomeres arrest at a specific position of interphase (G1, S or G2). A challenge with *C. elegans* embryos is that the onset of gap phases may be lineage dependent. Thus, cell lineage would likely need to be considered when trying to determine the position of interphase arrest.

4.2 Cellular changes and genes associated with metaphase arrest

Metaphase arrest or delay has been studied in other systems such as yeast or vertebrate cells in culture exposed to microtubule inhibitors. Through these studies the spindle checkpoint pathway and a greater understanding of cell cycle progression has been elucidated. The advantage of investigating anoxia-induced metaphase arrest in *C. elegans* embryos is that this can be studied in a developing organism and that oxygen deprivation is a stress that the organism must have adapted to in nature. The cellular changes observed in anoxia-arrested metaphase blastomeres are influenced by anoxia exposure time. For example, depolymerization of astral and spindle microtubules and a reduction of the spindle checkpoint protein SAN-1 at the kinetochore is more extensive in embryos exposed to three days of anoxia in comparison to one day (Hajeri et al., 2005). Likewise, there is a reduction in phosphorylation of certain proteins such as Histone H3 at Serine 10 and the mitotic proteins recognized by mAb MPM-2 (Padilla et al., 2002). These cellular changes could be due to a decrease in energy levels, in the form of ATP, with increased anoxia exposure time.

In many organisms including *C. elegans*, the mAb 414 recognizes FG repeats of specific nucleoporin proteins that are components of the nuclear pore complex (NPC) (Lee et al., 2000). In normoxic embryos, mAb 414 will recognize the NPC of interphase, prophase and prometaphase blastomeres; mAb 414 signal is diminished in metaphase and anaphase blastomeres and will reform in telophase blastomeres. Therefore, mAb 414 is an excellent marker for the NPC and cell cycle position. In anoxic embryos, the metaphase blastomere contains NPC aggregates recognized by mAb 414. The significance of these NPC aggregates is not known, but is a consistent characteristic of anoxia-arrested metaphase blastomeres. All of these anoxia-induced cellular changes are reversible when the embryos are re-exposed to normoxia. The arrested metaphase blastomere will transition to anaphase and chromosome segregation will take place.

An RNAi screen for genes on chromosome I that when knocked-down lead to an anoxia sensitivity phenotype showed that the spindle checkpoint is required for anoxia-induced metaphase arrest (Nystul et al., 2003). RNAi or genetic knockdown of the spindle checkpoint genes, *san-1* (mad-3/BubR1 homologue) and *mdf-1* (mad-1 homologue) leads to a decrease in the viability of embryos exposed to anoxia. The *san-1(RNAi)* as well as the *san-1(ok1580)* deletion mutant are sensitive to anoxia exposure. These embryos contain a dramatic

decrease in the number of arrested metaphase blastomeres and an increase in nuclei with abnormal nuclear phenotypes such as anaphase bridging. These studies were the first to demonstrate that the spindle checkpoint is active in metaphase blastomeres and that a reduction in oxygen signals spindle checkpoint function. The specific signal from a reduction of oxygen to the activation of the spindle checkpoint apparatus is not worked out. However, there is a reduction in microtubule polymerization in anoxic metaphase blastomeres, suggesting that a decrease in microtubule structure may be the signal to the spindle checkpoint proteins to initiate an arrest of metaphase blastomeres (Hajeri et al., 2005). This is inline with the findings by others that drugs that perturb the microtubule structure lead to an induction in spindle checkpoint function. Since various spindle checkpoint alleles are associated with predisposition to some cancers, the importance the of spindle checkpoint function and oxygen levels in regards to human health related issues is further underscored (Hardwick et al., 1999; Hardwick and Murray, 1995; Hartwell, 2004).

4.3 Cellular changes and genes associated with prophase arrest

In comparison to a metaphase arrest, an arrest of a prophase blastomere is less characterized. To further analyze prophase arrest the progression of prophase to metaphase must be understood. In *C. elegans*, the transition from prophase to prometaphase occurs when the chromosomes are fully condensed and nuclear envelope break down (NEBD) begins (Dernburg, 2001; Moore et al., 1999; Oegema et al., 2001). The progression of NEBD, which is a commitment to mitosis, can be followed using cellular analysis to detect nucleoporins, which are components of the nuclear pore complex. In an anoxia-induced prophase arrested cell the process of NEBD and the transition to prometaphase is arrested. To further understand prophase arrest two main approaches have been taken. First, cell biological analysis of nuclear structures was conducted to characterize the prophase arrest. Second, RNAi screens and analysis of genetic mutants were conducted to identify genes required for anoxia-induced prophase arrest. These approaches are of interest to identify molecular changes in the arrested prophase blastomere and to identify genes essential for anoxia survival.

A hallmark of an anoxia-arrested prophase blastomere is that the condensed chromosomes associate with the inner nuclear periphery; we refer to this phenotype as “chromosome docking” (Table 2) (Hajeri et al., 2005). Interestingly, anoxia-induced chromosome docking occurs in both the somatic cells of the developing embryo and in the oocyte of an adult hermaphrodite exposed to anoxia (Hajeri et al., 2010). In the embryo exposed to anoxia the chromosomes will condense prior to movement to the inner nuclear periphery. The chromosomes will remain docked at the inner nuclear membrane until returned to a normoxic environment. This is in contrast to the normoxic embryo in which the chromosomes move throughout the nucleus until NEBD occurs. In anoxia-exposed adult hermaphrodites the oocytes, which are in prophase I of meiosis, contain bivalent condensed chromosomes that localize to the inner nuclear periphery. In contrast, the oocytes of normoxic controls contain bivalent chromosomes that localize throughout the nucleus (Hajeri et al., 2010). *Drosophila* embryos exposed to anoxia also contain chromosomes that associate with the inner nuclear periphery indicating that chromosome docking in response to anoxia is not just a *C. elegans* phenomenon (Foe and Alberts, 1985). The relevance of chromosome docking in blastomeres that are exposed to anoxia is unknown but it is possible that chromosome docking is a means to maintain chromosome integrity or function

during anoxia exposure. While much is known about chromosomal territories in the interphase nucleus little is understood about chromosome location in prophase cells (Cremer et al., 2000; Geyer et al., 2011). It is not known if the mechanisms that regulate chromosome territories in interphase cells overlap with those regulating chromosome docking in arrested prophase blastomeres.

Given that anoxia induces chromosome docking in prophase blastomeres, indirect immunofluorescence has been used to characterize proteins associated with the nuclear membrane and chromosomes. Cell biological analysis shows that nuclear structures are altered in arrested prophase blastomeres relative to normoxic control embryos. Note that a relevant aspect of *C. elegans* chromosomes is that they are holocentric instead of monocentric in nature; this allows detailed cell biological analysis of chromosomes and chromosomal associated proteins. Using an antibody to recognize the kinetochore protein HCP-1 (CENP-F like) we determined that the kinetochore is altered in anoxia-arrested prophase blastomeres (Figure 2A). HCP-1 associates with chromosomes of normoxic prophase blastomeres but is not detected on the chromosomes of anoxia-arrested prophase blastomeres until embryos are returned to a normoxic environment (Figure 2A). In anoxia-arrested metaphase blastomeres HCP-1 is detectible indicating that the kinetochore changes observed in anoxia blastomeres is dependent on stage of mitosis (Hajeri, 2005).

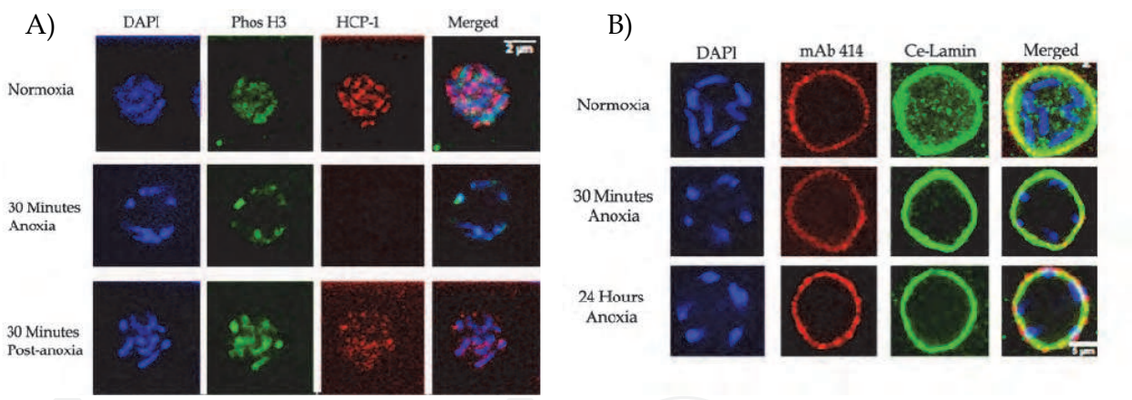


Fig. 2. Prophase blastomeres are altered in response to anoxia. A) Prophase blastomeres of embryos exposed to anoxia have diminished level of the kinetochore protein HCP-1. Embryos were collected from adult hermaphrodites and exposed to either normoxia or a brief period of anoxia and immediately fixed or allowed to recover in 30 minutes of air (post-anoxia) after anoxia treatment. After treatment the embryos were collected fixed and stained with DAPI to recognize DNA, mAb 414 to recognize the nuclear pore complex, anti phosphorylated Histone H3 at Serine 10 (Phos H3) to recognize mitotic nuclei and anti HCP-1 to recognize the kinetochore. Shown are representative prophase blastomeres analyzed using confocal microscopy. Scale bar = 2 µm. B) Lamin localization is diminished in the nucleoplasm of embryos exposed to anoxia. Embryos were exposed to normoxia or anoxia, for the specified time, and stained with DAPI to recognize DNA, mAb 414 to recognize nuclear pore complex and anti Ce-lamin to recognize lamin. Shown is a representative prophase blastomere from embryos exposed to noted environment and analyzed using confocal microscopy. Scale bar = 5µm.

Lamin, is an important inner nuclear protein that functions to maintain nuclear membrane structure and function. It is a target of post-translational modifications by CDK-1 during the complex process of cell cycle progression through mitosis (D'Angelo et al., 2006; De Souza et al., 2000; Gong et al., 2007; Heald and McKeon, 1990). In *C. elegans*, Ce-Lamin is localized to the inner nuclear membrane and nucleoplasm in normoxic prophase blastomeres. However, in the prophase blastomeres of anoxia-exposed embryos, Ce-Lamin is primarily localized to the inner nuclear membrane and there is a reduced level in the nucleoplasm (Figure 2B). The significance of reduced lamin in the nucleoplasm in anoxic blastomeres is not understood but does reflect alterations within the nucleus of anoxia-exposed embryos.

Antibodies that recognize nucleoporins associated with the nuclear pore complex can be used to monitor the nuclear envelop relative to cell cycle position (D'Angelo and Hetzer, 2008). We did not notice substantial change in the NPC of prophase-arrested blastomeres when assayed using mAb 414 (Table 2). However, using a commercially available antibody raised against human NUP50 we found evidence that an anoxia-arrested prophase blastomere differs in comparison to a normoxic prophase blastomere. The late prophase blastomeres of embryos exposed to normoxia have a reduced level of NPC that is detected by anti human NUP50, which is suggestive of NEBD occurring (Figure 3, arrow). Yet, in the anoxia-arrested prophase blastomere anti-NUP50 signal was present, suggesting that NEBD is not occurring and may be arrested (Figure 3, arrow head). Thus, a plausible mechanism to induce prophase arrest is to prevent NEBD and thus the transition to prometaphase. In both normoxic and anoxic embryos anti-NUP50 recognizes interphase nuclei in a similar manner.

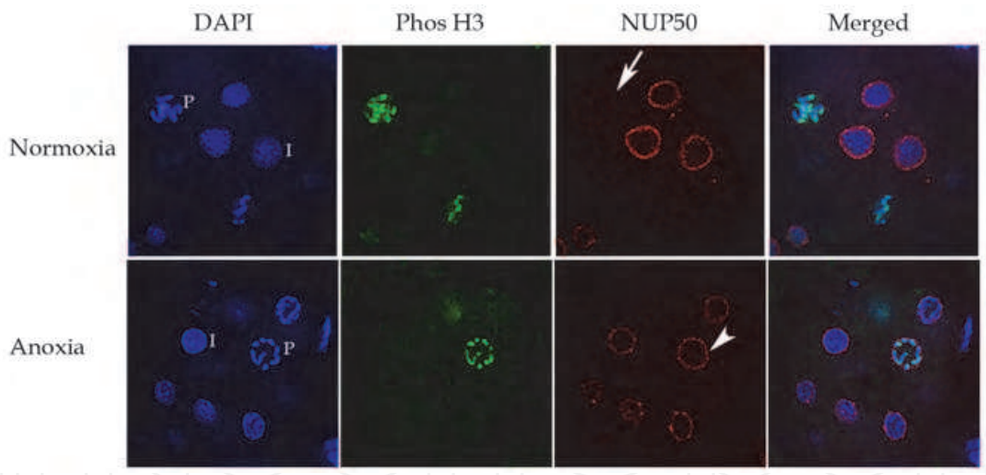


Fig. 3. Anti-NUP50 localizes to nuclear membrane and has a different pattern in anoxia exposed prophase blastomeres. The anti-human NUP50 antibody detects antigen localized to the nuclear membrane in interphase cells (I) which is then diminished by prophase (P, arrow) in normoxic embryos. In the prophase blastomeres (P) of anoxic embryos the antigen remained associated with the nuclear membrane (arrow head). Shown is a representative embryo analyzed by confocal microscopy.

Genetic analysis has been instrumental for identifying processes that regulate cell cycle arrest and progression. Previously, we determined that knockdown of the nucleoporin protein NPP-16/NUP50 by RNAi or genetic mutation results in a decrease in embryos that survive anoxia exposure (Hajeri et al., 2010). Additionally, in the anoxia exposed *npp-*

16(RNAi) embryo, there is an increase in abnormal nuclei and a reduction in arrested prophase blastomeres (Figure 4). The number of arrested metaphase blastomeres is not significantly different than wild-type embryos exposed to anoxia indicating that *npp-16* function is required specifically for prophase arrest (Hajeri et al., 2010).

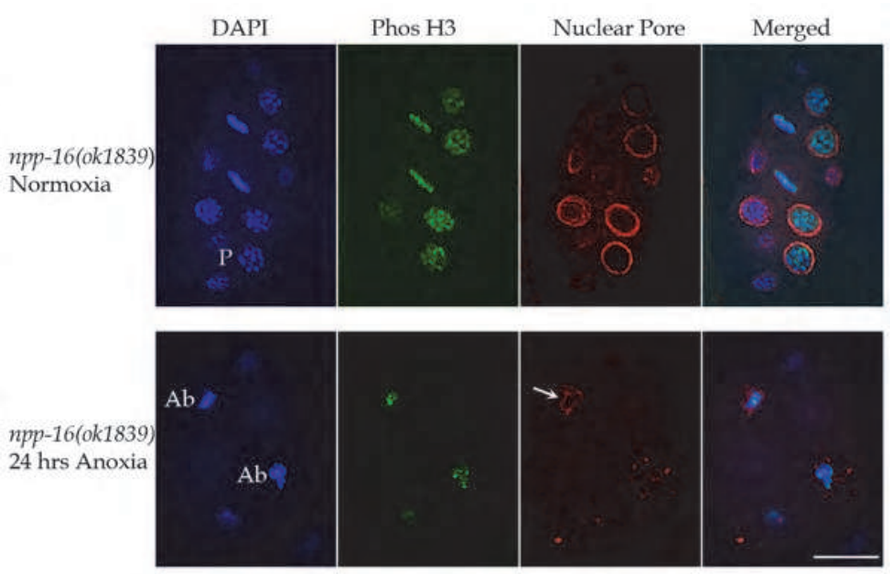


Fig. 4. The gene encoding the nucleoporin, *npp-16*/NUP50, is required for anoxia-induced prophase arrest. *npp-16(ok1839)* embryos were exposed to normoxia or anoxia and then stained with DAPI to detect DNA, Phos H3 to detect the mitotic marker phosophorylated Histone H3 at Serine 10, and mAb 414 to detect NPC. The *npp-16(ok1839)* embryos exposed to normoxia contain normal prophase (P) and yet the *npp-16(ok1839)* embryos exposed to anoxia contain a decrease in prophase blastomeres and an increase in abnormal nuclei (Ab) and NPC structure (arrow). Scale bar = 20 μm.

What is the role of NPP-16 in anoxia-induced prophase arrest? A key to addressing this question was noting that the predicted NPP-16 human homologue NUP50 was shown to interact with $p27^{kip1}$, a CDK inhibitor, suggesting a role of NUP50 with cell cycle regulation (Smitherman et al., 2000). In mammalian cells, CDK-1/cyclinB regulates the G2/M transition and NEBD by targeting a multitude of substrates (Lindqvist et al., 2009; Lindqvist et al., 2007). In *C. elegans* embryos, the NPC protein gp210, which is phosphorylated by CDK-1/cyclin B, is important for the depolymerization of lamin and required for NEBD (Galy et al., 2006). Data suggest that NPP-16 and CDK-1 have a role in anoxia-induced prophase arrest and that anoxia-induced arrest of NEBD is compromised in *npp-16* mutants. The use of antibodies that recognize CDK-1 showed that in wild-type embryos exposed to anoxia the protein is localized near the chromosomes in prophase blastomeres; this localization is reduced in the *npp-16* mutant exposed to anoxia. Second, an antibody that recognizes the inactive form of CDK-1 (anti CDK-1 P^{Tyr15}) was localized to prophase blastomeres of anoxic embryos but was absent from the prophase blastomeres of normoxic controls or the *npp-16* embryos exposed to anoxia. This indicates that not only is CDK-1 regulated differently in anoxic embryos but that this regulation differs in the *npp-16* mutant which does not arrest properly in response to anoxia. Although the specific signaling

between NPP-16 and CDK-1 is not yet understood this work does provide evidence that anoxia influences cell cycle machinery.

Chromatin modifications have major affects on chromatin structure and function (Geyer et al., 2011). Modifications of histones are highly conserved in eukaryotes and influence many cellular processes such as gene expression and chromosome condensation. For example, the phosphorylation of histone H3 at Serine 10 is known to correlate with mitotic and condensed chromosomes. Previously, we showed that the phosphorylation of histone H3 at Serine 10 is reduced in mitotic blastomeres of embryos exposed to long-term anoxia. Alteration in the phosphorylated state of proteins may reflect that energy-requiring processes are reduced in anoxia and that cellular signals change in anoxia-exposed embryos. Acetylation of histones is another example of how post-translational modifications regulate cellular functions. Histone acetylation and deacetylation by Histone Acetyl Transferases (HATs) and Histone Deacetylase (HDAC), respectively, modulate chromatin and influence gene expression via the addition or removal of acetyl groups on histones (Ferrai et al., 2011). There are several *C. elegans* genes that are involved with histone modifications and many of these genes are essential. We found that the gene *hda-2,* when knocked down using RNAi, does not lead to embryo lethality or obvious defects in normoxic embryos. However, when these embryos are exposed to anoxia there is an increase in abnormal nuclei (Figure 5). The specific role *hda-2* has in anoxia response and survival in the embryo needs to be further analyzed.

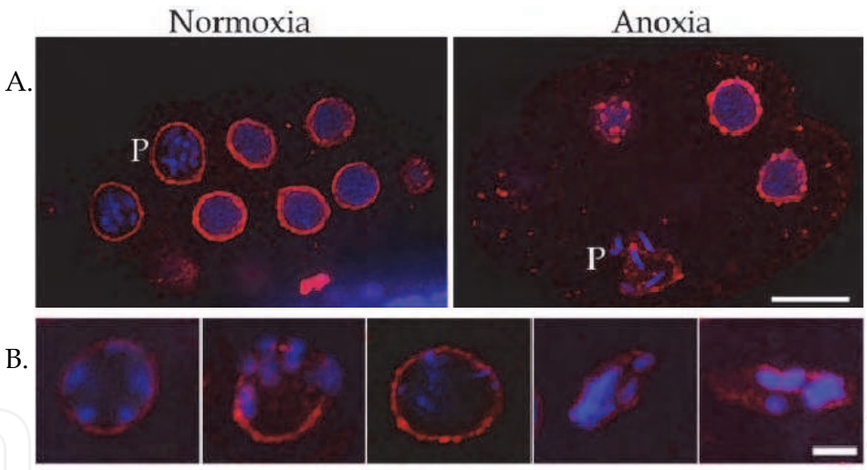


Fig. 5. *hda-2(RNAi)* embryos are sensitive to anoxia. Embryos were obtained from gravid adults and fixed and stained with DAPI to detect DNA and mAb 414 to detect the nuclear pore complex. A) Unlike normoxic controls, the *hda-2(RNAi)* embryos exposed to anoxia have abnormal blastomeres and prophase (P) blastomeres in which the chromosomes do not associate with the NE. Scale bar = 19 μm. B) Enlarged image of nuclei observed within the *hda-2(RNAi)* embryos exposed to anoxia. A significant number of the blastomeres contain a variety of abnormal nuclei. Scale bar = 5 μm.

There is evidence that modulation of the electron transport chain (ETC) activity has a role in cellular arrest. Exposure of embryos to ETC inhibitors (Ex: sodium azide) lead to cell cycle arrest and docking of prophase chromosomes. However, the embryos do not remain arrested and die within an hour of exposure (Hajeri et al., 2010). Thus, ETC inhibitors do not

phenocopy anoxia exposure, suggesting that anoxia-induced suspended animation is partially regulated by changes in the ETC. Unanswered questions regarding anoxia-induced cell cycle arrest include: what is the specific signal between reduced oxygen levels and docking of prophase chromosomes? Is chromosome docking essential for anoxia survival? How are cell cycle checkpoint proteins regulated in the anoxia-exposed embryo? Further genetic analysis of *C. elegans* embryos exposed to anoxia can lead to answers to these questions.

4.4 Metabolic and environmental changes that influence anoxia survival in the embryo

In the embryo exposed to anoxia, not only does the cell cycle machinery need to respond to the reduction in oxygen levels but metabolic pathways must do so as well. Embryos exposed to anoxia have a reduction in the ratio of ATP/AMP; this reduction will affect metabolic pathways (Padilla et al., 2002). Given the central importance of carbohydrates to metabolism, carbohydrate homeostasis is likely to be important for anoxia survival. Indeed it was found that sugar levels are altered in anoxia exposed embryos. For example, glycogen levels decreased to approximately 20% of intitial levels after a 24 hour exposure to anoxia (Frazier and Roth, 2009). A sufficient level of carbohydrates, perhaps in the form of glycogen, is likely important for maintaining metabolism during anoxia exposure. The gene *gsy-1* encodes glycogen synthase and when this gene is knocked down by RNAi the animal has a reduction in glycogen stores are sensitive to anoxia. (Frazier and Roth, 2009). Mutations in other genes that have reduced glycogen levels were also sensitive to anoxia further supporting the idea that glycogen homeostasis is important for anoxia survival. The Frazier and Roth study also demonstrated that the environment to which the parent is exposed can influence the anoxia survival rate of its embryos. For example, when L4 larvae develop to gravid adhulthood in a high salt environment (300 mM sodium chloride) their embryos are sensitive to anoxia (Frazier and Roth, 2009). It is likely that alterations of other central metabolic macromolecules are important for anoxia survival and the use of *C. elegans* genetics to alter metabolic pathways will shed light on metabolic pathways required for anoxia survival.

Embryos are able to survive anoxia and hypoxia (0.5% O_2), yet when exposed to to severe hypoxia (100 to 1000 ppm of O_2) embryos will die (Nystul and Roth, 2004; Padilla et al., 2002). Whereas anoxia is inducing suspended animation a .5% O_2 hypoxic environment is sufficient to support the signal for developmental activities. It is possible that the embryos exposed to 100 to 1000 ppm of O_2 are exposed to oxygen levels that are too high to induce suspended animation but not high enough to support normal growth and development. In the initial experiments of embryos exposed to 100 to 1000 ppm of O_2 the embryos were on media and not within the adult (Nystul and Roth, 2004). However, when gravid adults were exposed to 100 to 1000 ppm of O_2 the embryos within the uterus survived by arresting (Miller and Roth, 2009). These results indicate that the O_2 microenvironment differs between the uterus of an adult and the surface of NGM media and that embryos differentially respond to O_2 levels.

5. Anoxia tolerance in adult animals

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.

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 physiological mechanisms that increase the efficiency of oxygen removal from the environment (Hochachka, 2000; Hochachka et al., 1996). The execution of these strategies involves modulation of a wide range of cellular pathways. For example, animals switch from 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).

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 (one 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 and Ward, 2000). Interestingly, Foll et al., (1999) reported a higher mortality for adult worms exposed to 24 hours of anoxia (20˚C) and a subsequent sharp rise in mortality for slightly longer exposure when assayed in liquid culture. The discrepancy between the reported survival rates of the two studies is likely due to differences in methodology. One possibility is that the process of crawling across agar medium requires less energy than swimming in liquid medium. If so, the additional energy expenditure while swimming in liquid media may compromise anoxia tolerance. While adult hermaphrodites are anoxia-tolerant the 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). The anoxia-survival limitation is taken advantage of to identify genetic mutations that lead to anoxia sensitivity (mutants that cannot survive 24 hours of anoxia) and anoxia tolerance (mutants that can survive long-term anoxia, > 3 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 and Ward, 2000; Vanfleteren and De Vreese, 1996). 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 a long-term anoxia tolerant mutant strain and carries a reduction-of-function mutation in the insulin-like receptor (see section 6 below), 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 and 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). It has been observed that animals remain active in hypoxia but enter suspended animation in anoxia. 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). For example, among the adaptations adults 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 and Dusenbery, 1977; Suda et al., 2005; Van Voorhies and Ward, 2000). However, once the environment becomes anoxic, metabolic rate drops to as low as 5% of that in normoxic conditions and recover in a slow linear fashion only after removal from anoxia (Van Voorhies and Ward, 2000).

5.1 The anatomical and physiological impact of anoxia exposure

While in a state of suspended animation, the immobile *C. elegans* often adopt linearly extended bodies or a bent or curved-sickle shape (Figure 6, arrow). Upon re-oxygenation survivors will resume movement and the overwhelming majority exposed to 24 hours of anoxia will move normally several hours post recovery (Figure 6B). 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 long-term anoxia 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.

Recovery of anatomical and organ function at different rates may compromise the viability of the animal. For example, if ovulation precedes the ability to lay eggs, the accumulated eggs within the uterus may lead to embryos hatching within the uterus (bagging out phenotype) and further compromise organs such as neurons, muscles or the intestine. It is possible that long-term anoxia survivors are better able to resume anatomical and physiological processes.

The extent to which animals regain motility after anoxia exposure can vary within an experimental cohort and is influenced by duration of anoxia and genotype (see section 6). As the duration of anoxia exposure is lengthened the number of individuals that regain normal motility decreases (as visualized via a dissection microscope). In regards to wild-type adult animals, recovery within minutes can be observed after a 24-hour anoxia exposure. However, the few wild-type animals that survive long-term anoxia may not begin moving for several hours after re-oxygenation. This variability has been a useful tool in assessing the post-anoxia condition of survivors. Several approaches have been described for categorizing worm locomotion phenotypes (Gerstbrein et al., 2005; Herndon et al., 2002; LaRue and Padilla, 2011). In each system animals are categorized based on their movement and response to touch. For example, an animal is scored as dead if it is not moving and does not respond to gentle touch with a platinum wire. If the animal moves in response to touch it is scored as alive and then further classified based on level of motility; this classification provides an assessment of how well the animal moves after anoxia exposure. That is, animals capable of completing the typical sine wave motion similar to that of untreated adults are classified as having "unimpaired" movement while animals that move abnormally or move only a portion of the body are classified as having "impaired" movement. Utilizing this method one can assess how well an animal tolerates anoxia by monitoring its ability to recover and execute the fundamental process of movement. Often the impaired worms do not move and will consume the bacteria nearby leaving a fan-shaped area emptied of food (Figure 6D). The underlying cause (Ex: a compromise of muscle and/or neuronal function) of anoxia-induced impairment is yet to be determined.

It is also possible that post-reoxygenation impairment is due, at least in part, to the organism's inability to execute the processes required for the maintenance of cellular integrity thus leading to a loss of tissue structure (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 (Figure 7). However, long-term anoxia tolerant strains do not show the same tissue disorganization and appear to be better able to maintain tissue structure. This is presumably accomplished by either sustaining a homeostatic physiology during the anoxic period or by activating tissue maintenance and repair pathways post-reoxygenation.

In addition to the loss of coordinated movement and incurring tissue damage, anoxia exposure affects multiple aspects of fertility. When eggs are exposed to 24 hours of anoxia and then allowed to mature to adulthood the onset of first reproduction is significantly delayed compared to normoxic controls. The anoxia treated animals also have a reduced reproduction rate and reduced fecundity compared to untreated controls (Van Voorhies and Ward, 2000). It is possible these changes are the result of a programmed response to the stress or may simply be the result of damaged meiotic cells.

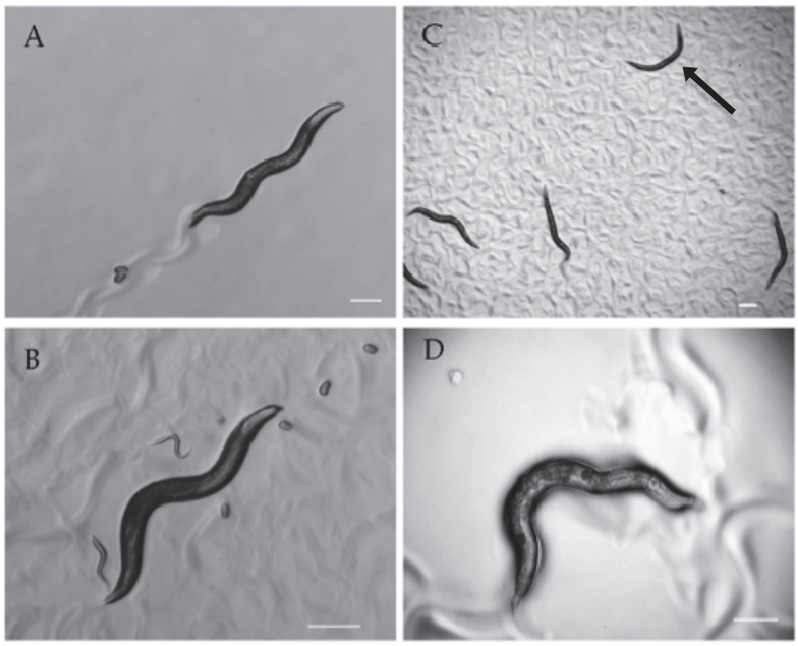


Fig. 6. Wild-type *C. elegans* adults survive and fully recover motility after 24 hours of anoxia whereas animals exposed to 72 hours of anoxia have a reduced survival rate and impaired motility. Wild-type animals were raised to one-day old adults then exposed to anoxia for 24 hours or 72 hours. A) One-day 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).

5.2 Environmental factors affect anoxia tolerance

The environment to which an organism is exposed can have profound affects on phenotype. Environmental changes that induce a stress response include changes in temperature, water availability, diet and nutrient content, oxygen levels and exogenous molecules including toxins or pharmaceutical agents. If an organism is exposed to these factors during development and/or adulthood, the responses to and ability to survive the stress can be affected. In some cases a preconditioning environment, a low level of stress, can improve stress tolerance.

In the laboratory *C. elegans* are typically grown at 15-20°C and provided the *E. coli* OP50 strain as a food source. 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 and Padilla, 2011). Wild-type *C. elegans* grown at 20°C and fed the *E. coli* OP50 diet are very sensitive to long-term anoxia in that the majority of the

Fig. 7. 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.

animals die and among those that survive most have an impaired phenotype. However, if the animal is grown at 25°C and fed the *E. coli* HT115 strain throughout larval development there is a significant increase in long-term anoxia survival and an unimpaired phenotype. The animals grown at 25°C and fed the *E. coli* OP50 strain 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. There is evidence to support the idea that metabolic stores are altered in *C. elegans* raised at 25°C and fed the *E. coli* HT115 diet during development.

First, it is known that 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). Second, staining with carminic acid, which is used to detect carbohydrate stores within the intestines, indicates that carbohydrate levels are increased in the intestine of animals grown at 25°C and fed the *E. coli* HT115 diet in comparison to those raised at 20°C (LaRue and Padilla, 2011). Further analysis is needed to determine mechanistically how preconditioned metabolic and physiological changes within the nematode contribute to the enhanced long-term anoxia phenotype.

While the temperature 25°C is not typically considered a stressful environment for *C. elegans*, some physiological processes are likely different between animals grown at 25°C or greater in comparison to those grown at 15-20°C. An increased temperature such as >28°C is likely stressful to the animal. It is known that animals exposed to one day of anoxia or severe hypoxia at 28°C instead of 20°C leads to markedly decreased survival rate (Mendenhall et al., 2006; Scott et al., 2002). Animals that are exposed to more than one stress at a time typically have a decrease in survival. However, such a condition has been useful in identifying genetic changes that increase viability in this environment. Alleles that affect the insulin signaling pathway increase viability when exposed to anoxia and 28°C (Scott et al., 2002). The insulin signaling pathway is known to be important for many stress responses and it will be of interest to tease apart the molecular factors that are specific to anoxia response and survival.

6. Genetic factors associated with anoxia survival in adult animals

A major strength of the *C. elegans* model system is the ability to dissect biological processes using a genetic approach. The use of forward genetic screens, RNAi genomic screens, suppression or enhancer screens, and analysis of transgenic fusion proteins is fundamental for discovering mechanisms regulating biological processes. These approaches have been used by many within the *C. elegans* community to unravel the mechanisms regulating complex processes such as programmed cell death, aspects of embryo and larvae development, chemosensing, dauer development, meiosis and many other processes. *C. elegans* is also a wonderful model to study stress responses, environmental and genetic factors that influence lifespan and the overlap between mechanisms regulating stress responses and lifespan. In terms of the biology of the adult hermaphrodite, it contains meiotic cells that can differentiate into oocytes and sperm. The hermaphrodite, which produces approximately 300 offspring, typically lays the majority of the embryos within the first two days of adulthood. with offspring production tapering off after the third day of adulthood. Males exist within the population thus genetic crosses can be conducted. In typical laboratory conditions the adult hermaphrodite has an average lifespan of approximately 15-20 days. The capability to manipulate genotypes, a rapid lifecycle and capacity to produce a large number of offspring make *C. elegans* an excellent system to analyze stress responses in relationship to specific biological function, such as germline and metabolic capacity. Here we expand upon the genetic approaches used to analyze anoxia responses in adult *C. elegans*. Table 3 summarizes genotypes discussed further in this chapter and their respective phenotypes relative to anoxia tolerance, germline function and lifespan.

Genotype	Summary of Reproductive Capability and Lifespan
Long-term anoxia tolerant strains: high survivor rate and unimpaired phenotype after three days of anoxia and recovery	
daf-2(e1370)	Enhanced anoxia tolerance (5 days of anoxia); Brood size is slightly less than wild-type; intermittent progeny production late in life; lifespan is extended relative to wild-type
fem-3(q20)	Complete sterility; mutant produces sperm but no oocytes; wild-type lifespan
fog-2(q71)	Mutant is female and rarely lays unfertilized oocytes; male has functional sperm; wild-type lifespan
glp-1(e2141)	Complete sterility; somatic gonad present; primordial germ stem cell production abnormal; mutants have extended lifespan
ksr-1(ku86)	Reduction in meiotic progression and reduced progeny production
wild-type males	Have functional sperm; mean lifespan of 16 days is density dependent
wild-type fed FUDR	Exposure of hermaphrodites to FUDR at L4 stage induces sterility
Anoxia survival rate similar to wild-type: High survivor rate and unimpaired phenotype after 24 hours of anoxia but long-term anoxia sensitive	
wild-type hermaphrodite	Produce both oocytes and sperm; mean lifespan approximately 20 days
daf-2(e1370);daf-16(mu86)	Null *daf-16* allele suppresses *daf-2(e1370)* longevity and long-term anoxia tolerance
daf-2(e1370);gpd-2/3(RNAi)	*gpd-2/3(RNAi)* suppresses *daf-2(e1370)* high temperature and anoxia tolerance
daf-16(mu86)	Wild-type brood size; mean lifespan approximately 15 days
hif-1(RNAi)	Anoxia survival rate comparable to wild-type, sensitive to hypoxia
Anoxia sensitive: low survival rate and/or impaired phenotype after 24 hours of anoxia	
aak-2(rr48)	Survives anoxia but highly impaired after exposure; wild-type lifespan
gpd-2/3(RNAi)	Survives 24 hour of anoxia well; arrest motility immediately upon encountering anoxia

Table 3. Summary of anoxia tolerance, reproductive capacity and lifespan for specific genotypes discussed in this chapter.

6.1 Reduction in insulin-like signaling favors anoxia tolerance

Arguably the most well known pathway in the study of anoxia tolerance in *C. elegans* is the insulin/IGF-1-mediated signaling (IIS) pathway. Research led to identification and characterization of the molecular nature of the genes functioning in the insulin-like signaling pathway revealing that the pathway regulates metabolism, lifespan, stress responses and the development of the dauer state, which is a stress-resistant larva in diapause (Gottlieb and Ruvkun, 1994; Kenyon et al., 1993; Kimura et al., 1997; Riddle et al., 1981; Tissenbaum and Ruvkun, 1998). Much is known about the genes that function in *da*uer *f*ormation pathway (*daf*). 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, 2001; Larsen et al., 1995; Lin et al., 1997; 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). The activation of functional DAF-2 results in phosphorylation of cytosolic DAF-16, an action that prevents its translocation into the nucleus keeping it sequestered in the cytoplasm. However, when signaling through the IIS pathway is reduced, for example during periods of food deprivation or in the presence of null or severely reduced function forms of the DAF-2 receptor, this inhibition does not occur and DAF-16 is translocated into the nucleus of the cell 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 *daf-2(e1370)* mutant allele confers a greatly extended lifespan (from 18 to 42 days) when 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). Both exceptional phenotypes are DAF-16 dependent and animals carrying a null mutation allele of *daf*-16 show no extension of lifespan and are long-term anoxia sensitive (survival = ~2%). It is noteworthy 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.

6.2 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 one 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 knocked down by 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. The extent to which adaptive response programs are activated should correspond to the level or intensity of the encountered stresses. 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. Stress triggers of 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 and Padilla, 2011). These observations implicate AMPK as a player in anoxia tolerance and necessary for preventing loss of coordination during anoxia treatment.

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 and Storey, 1995). *C. elegans'* simple body design localizes many of the functions accomplished by a variety of organs in higher eurkaryotes almost exclusively to the intestine, including carbohydrate storage (McGhee, 2007). In the long-term anoxia 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 long-term anoxia tolerant strains including *daf-2(e1370)*. Carminic acid is a fluorescent derivative of glucose that binds to glycogen and trehalose. As expected, animals exposed to long-term anoxia 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. When *daf-2(e1370);aak-2(RNAi)* animals were exposed to 3 days of anoxia they showed impaired motility compared to *daf-2(e1370)*

controls. Furthermore, *aak-2* knockdown suppressed the *daf-2(e1370)* long-term anoxia tolerant phenotype when exposed 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 preconditioning at 25°C may operate 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 IlS mutants such as *daf-2* in a *daf-16*-independent manner (Apfeld et al., 2004). AMP/ATP ratios do not differ between wild-type and *daf-2*, suggesting that the longevity phenotype of *daf-2* mutants is not simply due to an altered ratio of the two nucleotide molecules. Furthermore, individuals with the *daf-16(mu86);aak-2(ok524)* genotype have a reduced lifespan compared to wild-type or individuals carrying each mutation separately. While the long-term anoxia tolerant phenotype of *daf-2(e1370)* is completely suppressed by loss of *daf-16*, loss of *aak-2* does not reduce the overall survival rate but instead significantly affects post-anoxia health. Taken together these observations suggest the genes function to influence lifespan and anoxia-tolerance phenotypes via separate pathways. It will be of interest to determine how other metabolic mutants, such as *daf-16(mu86);aak-2(ok524)* fares in long-term anoxia.

It is possible that alternative forms of carbohydrates naturally present in *C. elegans* may play a role in the extreme phenotypes of longevity and long-term anoxia tolerance. For example, trehalose is a glucose disaccharide that is thought to participate in a wide variety of stresses including heat, desiccation, hypoxia, oxidative stress and others. It has been proposed that trehalose exerts its stress-protective effects through protein stabilization (Hottiger et al., 1994; Singer and Lindquist, 1998). Lifespans of young-adult animals were optimally extended (by 32%) when the animal was exposed to 5mM trehalose but not by other disaccharides (Honda et al., 2010). A decrease in age-associated decline was seen within a few days of initial exposure to the sugar and the lifespan extension effect was greater in older animals than younger. Furthermore, trehalose-treated animals had an extended reproductive span that was not due to reduced daily progeny production but by prolonged self-fertility. Animals fed trehalose also showed other evidences of slowed aging and senescence, including delay of age-associated decline in pharyngeal pumping and reduced rate of accumulation of age-pigment. Interestingly, *Drosophila* adults overexpressing *tps-1*, trehalose-phosphate synthase, and with a confirmed increase in trehalose production had a reduced recovery time following anoxia exposure (Chen and Haddad, 2004). Furthermore, they present evidence and an argument supporting the role of trehalose as a protein stabilizer that operates during stress such as anoxia. The role of trehalose in the anoxia tolerance of *C. elegans* has not yet been clearly established. Considering the importance of metabolic factors in anoxia tolerance, it would be of interest to determine if trehalose plays a role in establishing the anoxia-tolerant phenotype.

6.3 Loss of ceramide signaling confers hypersensitivity to anoxia

The alleles identified that influence anoxia tolerance are mutations that lead to an increase in anoxia survival and were previously shown to influence stress responses, germline function or lifespan. Recently, a mutation in the *hyl-2* gene was isolated that leads to

anoxia sensitivity in the adult hermaphrodite (Menuz et al., 2009). In carrying out a genetic screen, the researchers specifically sought mutations that suppressed 24 hour anoxia-survival at 20°C; this led to the identification of the *hyl-2(gnv1)* allele. The *hyl-2* gene encodes one of three ceramide synthases and has homology to Lag1p (yeast longevity assurance gene). Two alleles of a related ceramide synthase, *hyl-1(gk203)* and *hyl-1(ok976)*, carry loss of function mutations. In contrast to *hyl-2(gnv1)* the two loss of function alleles actually conferred an increased tolerance to 48 hours and 72 hours of anoxia. The HYL-1 and HYL-2 synthases operate to efficiently produce ceramides and sphingomyelins of different lengths. Presence of a functional *hyl-1* gene is not sufficient to rescue the anoxia sensitive phenotype of *hyl-2* deficient worms. This suggests that *hyl-2* operates to synthesize a specific ceramide required for anoxia tolerance. This is supported by the observation that the *daf-2(e1370);hyl-2(gnv1)* double mutant has a reduced anoxia survival compared to the *daf-2(e1370)* further suggesting that *hyl-1* and *hyl-2* work in parallel to affect anoxia tolerance. Ceramides have been implicated as effectors of several biological processes and it is possible that chemical interactions between ceramides of a specific chemical composition and other molecules may result in regulation of pathways specific to anoxia tolerance. It will be useful to clarify the role of ceramide-signaling in oxygen-deprivation tolerance as an approach to understanding the function of small lipophilic molecules in the regulation of biological processes.

6.4 The germline influences anoxia tolerance

As 1-day old adults, wild-type hermaphrodites actively reproduce via self-fertilization. Through the process of gonadal sheath contraction and dilation of the spermatheca mature oocytes move into the spermatheca to complete fertilization and are ovulated into the uterus theoretically making room for the next proximal maturing oocyte to take its place. These steps are 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 and 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 mutations of *glp-1* gene cause germ cells, located in the distal gonad that would normally undergo mitosis, to prematurely enter meiosis thus preventing formation of self-renewing germ cells and a functional germ line. Therefore, 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 long-term anoxia tolerant with a survival rate of approximately 98% (Mendenhall et al., 2009). LaRue and Padilla (2011) were able to partially suppress the *glp-1(e2141)* long-term anoxia tolerant phenotype when the *aak*-2 was knocked down via RNAi in the double mutant *glp-1(e2141);daf-16(mu86)*. It is worth noting that in addition to having an anoxia-tolerant phenotype, sterile *glp-1(e2141)* mutants also have an increased lifespan relative to wild-type animals (Arantes-Oliveira et al., 2002).

Sterile genetic strains may exist as temperature-sensitive genetic mutants such as the germ-line deficient mutant strain *glp-1*(*e2141*), or as gonochoristic mutant strains such as *fog-2*(*q71*) in which females are incapable of producing self-sperm and thus the strain is maintained by mating with males. Additionally, treatments such as feeding animals the cell-cycle inhibitor drug FUDR or laser ablation of the germline precursor cells in L1 larvae will result in sterile animals. There is not only a relationship between sterility and anoxia survival but sterility also has an influence on increased lifespan. Interestingly, the longevity phenotype of sterile mutants is not due to merely the absence of producing offspring. Laser ablation of the germline precursor cells results in animals without a germline yet still possessing an apparently fully developed somatic gonad; such animals show the lifespan extension phenotype. However, ablation of both the germline and somatic gonad precursor cells results in sterile adults with a wild-type lifespan. Since both ablation treatments render the worm sterile the difference in lifespan cannot be attributed just to reproductive cost. Instead these studies present substantial evidence that the somatic gonad and germline both influence lifespan in contrasting manner (Hsin and Kenyon, 1999; Kenyon, 2010). While absence of a germline in *glp-1(e2141)* results in an long-term anoxia tolerant phenotype the role, if any, played by the somatic gonad in anoxia tolerance has not been determined.

The anoxia-tolerant phenotype of the unmated *fog-2(q71)* is suppressed by mating with a fertile male. This observation supports the theory that the maternal soma is under the regulatory control of the germline. While the mechanism by which the germ line regulates maternal log-term anoxia sensitivity is not yet known, exceptions to the observation that sterility induces long-term tolerance are known. First, mutant strains have been identified that are sterile yet long-term anoxia sensitive. 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 long-term anoxia sensitive (survival rate= 23.4% and 2.6%, respectively). This sensitivity is presumably due to an altered physiology triggered in the somatic tissues in response to signals originating in the germline. Second, in a contrasting exception, the mutant strain *daf-2(e1370*) is not only long-term tolerant but also fertile (Larsen et al., 1995). At 15°C *daf-2*(*e1370*) has a slightly smaller brood size than wild-type (81% of wild-type). Furthermore, the *daf-2*(*e1370*) animals lays eggs over an extended period of adulthood (from adult day 1- 6) compared to wild-type (from adult day 1- 4) (Larsen et al., 1995; Tissenbaum and Ruvkun, 1998). It is unlikely that a reduction in average daily progeny production alone is sufficient to account for the strong long-term anoxia tolerant phenotype seen in a 1 day old adult *daf-2*(*e1370*). Instead, the reduction in function of DAF-2 is probably operating by acting on substrates and in pathways not yet identified. Finally, It is important to note that unlike *daf-2(e1370)* the anoxia-tolerant phenotype of these sterile reproductive mutants is *daf-16*-independent. Evidence thus far suggests that the long-term anoxia tolerant phenotype can be established via multiple pathways that may genetically overlap but which are not identical.

6.5 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 male lifespan is dependent upon whether the individual male is solitary or within a group of other males (Gems and Riddle, 2000). Gems and Riddle interestingly found that males that are solitary have a longer lifespan than males that are cultured as a group of other males indicating that male-male interactions reduce lifespan.

Survival of long-term anoxia also differs between wild-type adult hermaphrodites and males. One-day old wild-type and *daf-16(mu86)* mutant hermaphrodites survive 72 hours of anoxia at approximately 10% and 7% respectively, and are considered long-term anoxia sensitive. In contrast, wild-type and *daf-16(mu86)* mutant males survive long-term anoxia with a viability >98% (Mendenhall et al., 2009). The animals maintain normal motility and demonstrate an unimpaired phenotype after long-term anoxia exposure. Furthermore, the males that were raised in the presence of hermaphrodites and likely had an opportunity to mate still maintained an increased capacity to survive long-term anoxia relative to age matched hermaprodites indicating that mating and interaction with other males did not compromise the long-term anoxia survival phenotype. 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)* animal survived long-term anoxia similar to that of wild-type males indicating that something inherent about the male phenotype confers anoxia tolerance.

Combined, these studies provided further evidence that anoxia tolerance is strongly influenced by physiology and genotype. The ability of an individual to survive anoxic stress is determined by the interplay of multiple pathways in a complex fashion as evidenced by the wide range in biologic function attributed to the many encoded proteins and enzymes recognized to influence anoxia tolerance.

7. The multifactorial architecture of anoxia tolerance

As additional work is conducted to identify the mechanisms by which anoxia tolerant animals survive, recover, and protect tissues it is unlikely that a single important regulatory gene will be identified. Instead, we propose that anoxia survival is by way of a complex interaction of multiple physiological processes that animals are able to survive oxygen-deprivation stress and specifically, anoxia. In this chapter we have discussed a wide range of biological processes that naturally, or in the mutant condition, enhance or reduce anoxia tolerance. We have also related the observation that 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 germ line 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* independent in sterile mutants. The differences in physiology 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. 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. The unmated *fog-2* mutant has a wild-type lifespan and functional oocytes, yet this mutant is long-term anoxia tolerant unless induced to have offspring by mating. Not all lifespan extended mutant strains have an increase in anoxia tolerance relative to wild-type animals, suggesting that at least an overlap in the mechanisms governing the two phenotypes exists but that they are not identical. Currently, the mechanism by which the germline is regulating anoxia tolerance remains unclear.

Anoxia-tolerance is also under the control of metabolic factors. Reduced signaling through the insulin-IGF pathway confers long-term anoxia tolerance. Animals with reduced caloric intake (which may mimic reduction in insulin signaling) such as the dauer stage of larval development and a mutation in *eat-2* (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. These elevated carbohydrate stores and the associated long-term anoxia tolerance can be suppressed by mutations in *aak-2*, the kinase subunit of the AMPK energy sensor, in some but not all long-term anoxia tolerant strains. 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*.

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 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. We 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 persist to cope with diverse environmentally induced stresses.

Long-term anoxia 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. Figure 8 depicts a model of the multifactorial character of the anoxia tolerant phenotype. It is likely that a long-term anoxia tolerant strain is able to survive anoxia stress at a high rate due to one or more of the biochemical branches that lead to an anoxia tolerant phenotype. Within each branch specific adaptive responses occur, governed by a particular set of genes that may overlap but are not identical to the set of genes working in the other branches. Therefore, not all long-term anoxia tolerant strains of *C. elegans* survive via a common mechanism.

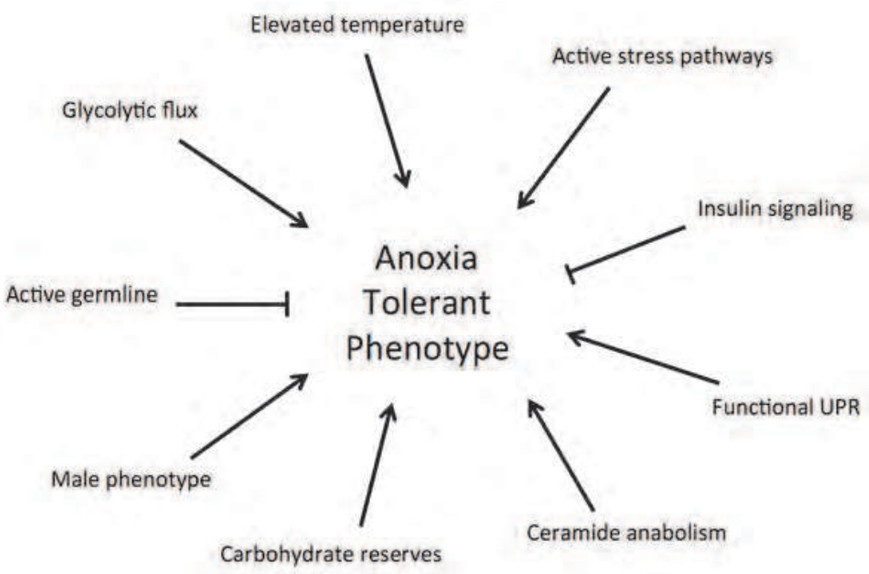


Fig. 8. The anoxia tolerant phenotype is multifactorial in nature. Biological factors that promote enhanced anoxia survival are shown as activating arrows, while factors or conditions that decrease the rate of survival during anoxia exposure are shown as inhibiting blunt-ended lines. We propose that the level of anoxia tolerance for any particular strain is a function of the interaction of the various factors shown in the diagram.

8. Conclusions - suspended animation and human health related issues

Oxygen deprivation is central to many life-threatening human health issues (Semenza, 2010). The extremely high economic and social cost associated with traumas such as blood loss, drowning, suffocation and toxins that affect pulmonary or cardiac function, in addition to diseases that compromise pulmonary and cardiac function underscores the significant importance in understanding responses to oxygen deprivation. In addition to these obvious human health related issues oxygen levels also influence the progression of tumors in individuals afflicted with cancer. It is known that microenvironments within solid tumors exist and that cells in regions of low oxygen are often more resistant to chemotherapeutic or radiation treatment. The solid tumor cells that are further away from the vascular system not only have less chemotherapeutic drugs being delivered but also have a decrease in oxygen levels. This reduction in oxygen can influence the progression of cell division leading to a population of cancer cells that are not rapidly dividing yet remain viable and quiescent. When these cells are re-exposed to oxygen it is possible that they resume rapid cell cycle progression and seed further tumor progression. Therefore, the understanding of how cells, tissues and whole organisms respond to and survive oxygen deprivation is of not only scientific interest but vital in the context of human health related issues.

There are many important and significant approaches that researchers are taking to understand the implications and effects oxygen deprivation has on organisms. Use of *C. elegans* as a genetic model system allows one to characterize many aspects of hypoxia and anoxia responses including the influence on development, cell cycle progression and organ structure and function. Furthermore, the capacity to use cellular and genetic tools to dissect

molecular pathways that are involved with oxygen deprivation survival further underscores the value of *C. elegans* as a model system. Finally, the ability to generate a state of anoxia tolerance through genetic manipulation or chemical means will aid in understanding how organisms with complex tissues respond to and survive oxygen deprivation.

The induction of suspended animation in *C. elegans* and zebrafish led to the pursuit of molecules that induce suspended animation in more complex systems (Roth and Nystul, 2005). The general idea is to treat individuals experiencing a traumatic event, such as blood loss leading to severe oxygen deprivation to vital organ systems, by inducing a state of suspended animation so that cellular processes (such as cell death) arrest or slow. Induction of suspended animation may "buy time" until other treatments can be administered. One molecule under intense investigation for inducing a state of suspended animation or a hypometabolic state is hydrogen sulfide (Blackstone et al., 2005; Roth and Nystul, 2005). Remarkably, hydrogen sulfide can reversibly induce a hypometabolic state in which core body temperature can be reduced in mammals (Blackstone et al., 2005; Blackstone and Roth, 2007). Hydrogen sulfide, or molecules with similar capabilities, provides a possible therapeutic approach to treating individuals with life-threatening events that compromise oxygen delivery to vital organs (Aslami et al., 2010; Szabo, 2007; Wagner et al., 2009). Like many new ideas that address biologically and medically complex problems, the ability to use basic sciences from model systems to identify treatments and therapeutics for the benefit of human health related issues is going to be costly, perhaps controversial and quite complex at the biological level (Olson, 2011). However, given the profound effect oxygen deprivation, including anoxia, has on living systems it is of great interest to continue the onward march toward understanding the molecular nature of anoxia tolerance in biological systems.

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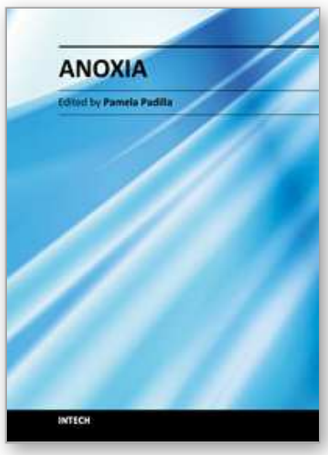

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This book reviews how severe oxygen deprivation affects biological systems - from the molecular to the ecological level. The contributing authors come from diverse regions of the world, which proves the interest in the academic analysis of oxygen deprivation. The diversity in the experimental approach scientists take, in order to understand the influence oxygen deprivation has on living systems, is apparent throughout this book. One of the presented ideas deals with the exploration and examination of the physiological, cellular and genetic characteristics of killifish embryos and nematodes exposed to anoxia. Furthermore, the book includes material on the mechanisms regulating hypoxia and anoxia tolerance and their implications of on human health issues. Finally, new methodologies to examine oxygen deprivation and the impact of human-related activities on oxygen level, within important ecological systems such as Lake Victoria, are presented. There is no doubt that the oxygen molecule is central to every stratum of biological systems.

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