GENETIC ANALYSIS OF DEVELOPMENT AND BEHAVIOR IN HYPOXIA AND CELLULAR CHARACTERIZATION OF ANOXIA INDUCED MEIOTIC PROPHASE ARREST IN Caenorhabditis elegans

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It was hypothesized that chronic hypoxia will affect various biological processes including developmental trajectory and behavior. To test this hypothesis, embryos were raised to adulthood in severe hypoxic environments (0.5% O₂ or 1% O₂, 22°C) and analyzed for survival rate, developmental progression, and altered behaviors. Wildtype hermaphrodites survive chronic hypoxia yet developmental trajectory is slowed. The hermaphrodites raised in chronic hypoxia had different phenotypes in comparison to the normoxic controls. First, hermaphrodites exposed to chronic hypoxia produced a significantly lower number of embryos and had a slight increase in male progeny. This suggests that chronic hypoxia exposure during development affects the germline. Second, animals raised in chronic hypoxia from embryos to young adults have a slight increase in lifespan when re-exposed to a normoxic environment, indicating that chronic hypoxia does not negatively decrease lifespan. Finally, hermaphrodites that were raised in hypoxia will lay the majority of their eggs on the area of the agar plate where the bacterial lawn is not present. This is in contrast to animals in normoxia, which lay the majority of their eggs on the bacterial lawn. One hypothesis for this hypoxia-induced egg-laying behavior is that the animal can sense microenvironments in hypoxia. To examine if various pathways are involved with chronic-hypoxia responses RNAi and assayed genetic mutants were used. Specifically, genetic mutations affecting oxygen sensing (egl-9), aerotaxis (npr-1), TFG-β signaling (dbl-1, daf-7) and predicted oxygen-
binding proteins (globin-like genes) were phenotypically analyzed. Results indicate that mutations in several of these genes (*npr-1, dbl-1*) resulted in a decrease in hypoxia survival rate. A mutation in *egl-9* also had a detrimental affect on the viability of an animal raised in chronic hypoxia. However, a similar phenotype was not observed in the *vhl-1* mutation indicating that the phenotype may not be due to a mere increase in HIF-1 levels, per se. A mutation in the globin-like gene (*glb-13(tm2825)*) suppressed the hypoxia-induced egg-laying phenotype. That is, the *glb-13(tm2825)* animal raised in chronic hypoxia laid eggs on the bacterial lawn at a significantly higher rate in comparison to wildtype controls, thus suggesting that globin-like molecules may be involved in the sensing of microenvironments. Together, this research lays the foundation for understanding the implications of chronic hypoxia in developing organisms.
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By

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INTRODUCTION

Oxygen Homeostasis

Oxygen Deprivation Terms

Oxygen homeostasis is regarded as one of the most fundamental physiological processes necessary for aerobic animals. Consequently, terminology has evolved to describe events that cause fluctuations in oxygen homeostasis. Oxygen deprivation is a state of reduced oxygen availability to tissues (hypoxia or anoxia) and cells that elicits physiological, biochemical or genetic responses. Oxygen deprivation is described by the terms hypoxia and anoxia. Hypoxia is defined as the following: 1. When $O_2$ deprivation limits electron transport, 2. A state of reduced $O_2$ availability or decreased oxygen partial pressure, 3. Reduction in $O_2$ levels that lead to abolishment or reduction in functions of cells, tissues and organs. Anoxia is often referred to as a state of “extreme hypoxia” wherein no $O_2$ is detected in the tissue or the environment that the organism is exposed (Höckel et al., 2001). In humans, cellular hypoxia and anoxia can be induced by a variety of factors such as reduced ability of blood to carry $O_2$ (anemia), inability of cells to use $O_2$ because of toxins (cyanide), low $O_2$ partial pressure in arterial blood due to high altitudes or pulmonary disease, reduced tissue perfusion (ischemia), alterations in blood flow due to alterations in vascular system (solid tumors) or trauma (suffocation).

Oxygen Deprivation and Metazoans

Many vertebrates are sensitive to oxygen deprivation and relatively few species can survive extended periods of hypoxia or anoxia. The few vertebrates that are oxygen deprivation-tolerant are characterized by their evolved mechanisms allowing them to
cope with low oxygen concentrations and reoxygenation without sustaining significant damage. Animals that are oxygen deprivation-tolerant cope with such via mechanisms that remain in a ready state and reduce response time; for example, maintain high levels of antioxidants to scavenge reactive oxygen species (ROS). Additionally, tolerant animals have anticipatory defense mechanisms that require an extended time period before producing a cellular response; for example, lactate dehydrogenase up-regulation to eliminate lactic acid accumulation as well as other end products of anaerobic metabolism.

**Oxygen and Metabolism**

One common physiological response utilized by oxygen deprivation-tolerant animals is the suppression of metabolism. They conserve energy by repressing transcription and translation which are ATP costly cellular processes and therefore disadvantageous in a low ATP environment commonly generated during oxygen deprivation. By suppressing energy use during hypoxia exposure, fewer ATP molecules are used to sustain the same tissue compared to that needed in normoxic conditions. This hypometabolic state is accomplished through reducing several processes including membrane permeability and consequently the ATP demand for ion pumping in locations such as the mitochondria (Atkinson, 1973; Gnaiger et al., 1998). Similarly, metabolic flux through certain pathways such as glycolysis and gluconeogenesis are also altered. Reduction in glycolysis (the anaerobic process that breaks down glucose) requires less oxygen; however, it is an energy requiring process that disrupts blood sugar homeostasis (Hers, 1990). Likewise, reducing gluconeogenesis is beneficial, since it is
ATP demanding and inefficiently produces glucose during oxygen deprivation while glycolysis is suppressed.

Significance of Studying Oxygen Deprivation

Humans are sensitive to oxygen deprivation and it is understood that oxygen deprivation is a central cause for many human health related issues and death. Oxygen deprivation plays a role in the pathophysiology of conditions such as cardiac and pulmonary dysfunction, myocardial infarction, and the resistance of solid tumor cells to radiation and chemotherapy treatments. Although these pathologies affect a large number of individuals, the genetic and cellular mechanisms of these pathologies are poorly understood.

Oxygen Deprivation Tolerance-Physiological Stress Responses in Metazoans

Physiology Model Systems-Comparative Physiology

The August Krogh Principle states that for any question a scientist may ask, nature provides an ideal organism to study it (Krogh, 1929). Comparative physiology examines common physiological systems among diverse species, with each species offering unique characteristics to be investigated. For example, diving mammals provide a model system to study how air-breathing animals operate successfully in water for extended periods of time. Similarly, lab and field studies have used these animals to examine blood and tissue physiology before, during and after diving exercises. The results of these studies formed the foundation of our current understanding of diving
physiology and the discovery of three key physiological reflexes; apnea, bradycardia and hypoperfusion or ischemia of most peripheral tissues (Butler et al., 1982).

Many metazoans, including humans, exposed to prolonged oxygen deprivation experience death at the cellular and organismal level, yet several metazoans, such as diving mammals and migratory birds survive severe oxygen deprivation. (Bouverot et al., 1976; Hochachka et al., 2003b; Powell et al., 2004). These observations suggest that the ability to survive oxygen deprivation has been conserved across different phyla. For example, the harbor seal (*Phoca vitulina*), painted turtle (*Chrysemys picta belli*), brine shrimp (*Artemia franciscana*), zebrafish (*Danio reio*), fruit fly (*Drosophila melanogaster*), and nematode (*Caenorhabditis elegans*) are adapted to survive oxygen deprivation (Foe et al., 1985; Fuson et al., 2003; Hand, 1998; Hochachka et al., 2003a; Hochachka et al., 2003b; Padilla et al., 2002). Furthermore, the extent to which the animal survives oxygen deprivation is dependent on the species and stage of development. While zebrafish embryos survive 1-2 days of anoxia, the annual killifish (*Austrafundulus limnaeus*) survives 60 days of anoxia during embryonic development and the brine shrimp survives 4 years of continuous anoxic exposure (Hand, 1997; Padilla et al., 2001; Podrabsky et al., 2007).

**Metabolic Suppression**

Although one effect of oxygen deprivation is metabolic depression, the response to environmental anoxia is often different than the response to hypoxia. In anoxia some vertebrates and invertebrates enter into dormant states of inactivity where overall metabolic rates are reduced to 20% of the resting state (Storey et al., 1990). In contrast,
hypoxia occurs most frequently when an organism encounters suboptimal oxygen concentrations while glycolytic flux is working at normal capacity or higher; for example, an exercising animal that reaches exhaustion. Consequently, cellular responses to hypoxia include activation of enzymes such as phosphofructokinase (PFK) and pyruvate kinase. These enzymes influence the flux of metabolites through glycolysis. A change in cellular pH toward acidity (aerobic acidosis) also occurs (Goldhammer et al., 1979). Experiments with brine shrimp (Artemia franciscana) show creating artificial conditions of aerobic acidosis by elevating levels of CO₂ cause shutdown of carbohydrate metabolism (Carpenter et al., 1986). Since shutdown of metabolism is a characteristic of animals exposed to anoxia, pH could be a key signal in metabolic switching during oxygen deprivation.

**ROS and Reperfusion**

Atmospheric oxygen in its ground state is unlikely to react with organic molecules. This is because dioxygen contains two unpaired electrons with parallel spins and Pauli’s exclusion principle states a reductant would require two unpaired electrons with parallel spins opposite to oxygen, which is a rare occurrence (Takahashi et al., 1999). When dioxygen absorbs enough energy it can reverse the spin of one electron and become activated. This singlet oxygen molecule will readily react with organic molecules that have paired electrons with opposite spins. Reactive oxygen species (ROS) such as singlet oxygen, superoxide, hydrogen peroxide and hydroxide radicals react with organic substrates causing degradation of lipids, proteins and nucleic acids (Takahashi et al., 1999).
Cells and tissues encounter reperfusion injury after ischemic events. Ischemic events prevent blood supply from reaching tissues and consequently reduce the oxygen availability at cells and tissues. Reperfusion, restoration of circulation or blood flow, results in oxidative damage to cells and tissues at the site of injury through reactive oxygen species. The increase in ROS during reoxygenation is thought to be caused when damage to electron transport chain components results in inefficient transfer of electrons, generating superoxide (Murphy et al., 2008). The consequence of ischemia and reperfusion on cells and tissues is death.

The most prevalent adaptation to prevent reperfusion events such as electron transport damage is the up-regulation of antioxidant enzymes and small molecular weight metabolites such as ascorbate as oxygen scavengers. For example, turtles have constitutively high levels of antioxidants to help cope with reperfusion and prevent ROS formation through altered mitochondrial activity (Willmore et al., 1997). Results from studying the glutathione (GSH) cycle show anoxia depresses glutathione enzymatic activity. Turtles compensate by maintaining an endogenous high level of GSH. Many questions remain regarding the efficacy of antioxidants as a reperfusion treatment. Although some studies have found antioxidants and ROS scavengers such as superoxide dismutases (SODs) and catalases reduce infarct size in heart tissue and cell culture (Ambrosio et al., 1986; Bolli, 1998), there are also studies that show no reduction in injury (Downey et al., 1991).

Other protective physiological-adaptations to cope with oxygen deprivation and reperfusion have been documented. Physiological changes minimize damage caused during oxygen stress. For example, blood shunting from peripheral organs and muscles
to more oxygen sensitive tissues, such as, the brain and heart will supply oxygen to locations where oxygen perturbations are most likely to result in cellular death. Maintaining large calcium supplies in the shell and bones of anoxic turtles help to deal with lactic acid build up during anaerobic conditions (Willmore and Storey 1997). The calcium functions as a lactic acid sink allowing the cardiac and skeletal muscles to continue normal function by removing the detrimental acid build up. Also, different hemoglobin redox states are used during reperfusion by turtles. By attaching thiol groups to globin surfaces ROS species are sequestered and further damage is prevented (Riggs et al., 1964).

Oxygen Deprivation Tolerance-Genetic and Cellular Responses

Genetic Model Systems

C. elegans-Development

After fertilization, the life cycle of C. elegans (wildtype, N2 strain) begins with a 12-hour embryogenesis and upon hatching will develop through four larval molts (20°C). Each molt increases the size of the animal by a third and larvae resemble smaller versions of the adult. Once the embryo hatches, the L1 larvae contains an invariant cell lineage of 558 cells. Some of these cells will continue to divide to produce somatic tissues. The adult hermaphrodite contains multiple tissues composed of a nervous system with 302 cells, 95 striated muscles making the body wall, 16 somatic gonad muscles specialized for fertilization and egg laying, a 20-cell intestine and another 8 muscle types to make the pharynx. The hermaphrodite adult animal will contain 959 somatic cells and a variable number of germ cells in the gonad (Sulston et al., 1977).
The developmental trajectory of *C. elegans* is dependent on several abiotic and biotic factors. For example, environmental temperature is an influential abiotic factor that affects developmental timing. As temperature increases, the rate of development increases. A 10°C increase can decrease time from egg to adult by 48 hours. Several biotic factors such as crowding and food density can also influence developmental trajectory by inducing an alternative life stage, the dauer. This alternative larval stage occurs at the second molt in response to overcrowding, low food density and higher temperatures (Golden et al., 1984). Making the commitment to dauer in the first two larval stages can increase the time as larvae by 72 hours in mutant animals that are dauer constitutive.

Sperm production, mating and environmental cues influence fecundity in *C. elegans*. Wildtype (N2) gravid adult hermaphrodites contain two functioning gonads that together produce approximately 300-350 offspring over their adult lifespan. Egg production peaks on the second day of adulthood then tapers off. The number of offspring produced by an unmated hermaphrodite is determined by the quantity of sperm produced before oogenesis begins (Harvey et al., 2007). Sperm depleted hermaphrodites can still produce offspring if mated with a male. Environmental cues can also influence the fecundity of *C. elegans*. For an egg laying hermaphrodite, dietary restriction, low temperatures (<15°C) and high temperatures above 25°C result in decreased fecundity.

*C. elegans*-Signal Transduction

*C. elegans* development requires multiple signaling pathways of which many are
characterized. For example, the documented Notch signaling pathway in *C. elegans* mediates intercellular interactions to specify cell differentiation and tissue morphogenesis (Priess, 2005). There are multiple pathways characterized that influence larval development (Fielenbach et al., 2008). This is evident in genetic mutants that constitutively produce the long lived, stress resistant dauer larvae as opposed to molting into the third larval stage. Genetic analysis has elucidated four distinct molecular pathways, guanylyl cyclase signaling, TGFβ signaling, insulin signaling, and steroid hormone signaling, that can produce a dauer arrest (Antebi et al., 2000; Birnby et al., 2000; Kimura et al., 1997; Ren et al., 1996). When a developing animal is stressed one, or a multiple, of these signaling pathways is used in sensing and responding to the stress. These signaling pathways can be further analyzed to determine if they have a functional role in *C. elegans* oxygen deprivation responses.

**Oxygen Sensing-Transcriptional Responses, HIF-1**

Regulation of gene expression is a mechanism metazoans use to tolerate oxygen deprivation. Prolyl-hydroxylases (EGL-9 in *C. elegans*) are oxygen sensors that regulate the accumulation and degradation of a transcription factor called hypoxia-inducible-factor (HIF-1α) (Epstein et al., 2001a; Wang et al., 1993). The pathway that integrates O₂ sensing with cellular oxygen homeostasis is tightly regulated through the HIF pathway. Mammalian cells respond to hypoxia by increasing anaerobic energy production and increasing local angiogenesis, the increase in new blood vessels. The changes in gene expression required for these adaptations during oxygen deprivation are controlled by HIF (Wenger, 2002). For example, HIF regulates the erythropoietin
gene to signal expression of oxygen deprivation dependent genes responsible for sustained survival without oxygen (Epstein et al., 2001a; Maxwell, 2005).

C. elegans hif-1 Mutant Phenotype

The HIF signaling pathway is required for adaptation to hypoxia in C. elegans (Jiang et al., 2001). Although, wildtype embryos exposed to 1% O$_2$ hatch and grow to fertile adults in hypoxia, mutations in hif-1, for example, the hif-1(ia04) mutant, cause death in 66% of embryos exposed to hypoxia (1% O$_2$) (Jiang et al., 2001). The ia04 mutation deletes three exons and introduces a premature stop in the mutant mRNA resulting in complete lack of HIF-1 function (Jiang et al., 2001). Experiments in mice show that mammalian hif-1$\alpha$ is required for embryogenesis under normoxic environmental conditions as Hif-1$^{-/-}$ mice die in embryogenesis regardless of exposure to hypoxia (Iyer et al., 1998; Ryan et al., 1998). While hif-1 is not required for C. elegans cultured at standard laboratory conditions (in normoxia), hif-1 has other biochemical and functional similarities with mammalian HIF$\alpha$ homologues. For example, the C. elegans HIF-1 binds to the aryl-hydrocarbon receptor nuclear translocator, AHA-1, the mammalian ortholog of ARNT (HIF-1$\beta$), to form a HIF complex and HIF-1 protein levels are increased by hypoxia (Jiang et al., 2001). These results confirm that C. elegans hif-1 encodes a HIF-1 that is a functional homolog of mammalian HIF-1$\alpha$ and is required for adaptation to hypoxia.

When oxygen levels are physiologically normal HIF-1$\alpha$ is targeted for degradation through proline residue hydroxylation. Hydroxylation occurs through EGL-9/PH (EGL-9 in C. elegans), which is a member of a superfamily of 2-oxoglutarate-
dependent dioxygenases. Once hydroxylated, the von Hippel-Lindau (VHL) protein (VHL-1 in *C. elegans*) targets HIF-1α for proteasomal degradation through ubiquitination (Bishop et al., 2004). Therefore *egl-9* and *vhl-1* mutants express HIF-1α at constitutively high levels. The regulation of HIF-1α through the EGL-9/VHL pathway is conserved between vertebrates and invertebrates (Epstein et al., 2001a). When *C. elegans* are exposed to a hypoxic environment EGL-9 no longer hydroxylates HIF-1α for targeted degradation and HIF-1α forms a stable heterodimeric DNA-binding complex with HIF-1β. Slight induction of HIF-1 levels begins around 5% O₂ in 16 hours but maximal induction occurs at O₂ levels less than 1% in 4 hours (Epstein et al., 2001a).

The HIF transcriptional complex binds to specific DNA sequences termed Hypoxia Response Elements (HREs). In mammalian systems HRE’s contain the sequence 5’-RCGTG (Wang and Semenza 1993). In *C. elegans*, a survey of the sequences 200-2000 base pairs 5’ to the predicted translational start site show the motif 5’-TACGTG is present in 46% of *hif-1*-dependent genes from hypoxic microarray analysis (Shen et al., 2005). Therefore, this HIF targeting mechanism is evolutionarily conserved between mammals and worms. Targets of the HIF transcription factor include multiple prolyl 4-hydroxylase enzymes that modify collagen in the endoplasmic reticulum and the EGL-9/PHD that modifies HIF-1α (Shen et al., 2005). Positive regulation of *egl-9* by HIF maintains EGL-9 activity when oxygen is limiting. Microarray analysis comparing wildtype and *hif-1* mutant animals showed several classes of genes regulated by HIF-1. These include genes required for collagen synthesis and genes for post-transcriptional changes to proteasomes (Shen et al., 2005). These data suggest
that changes in cellular structure and protein synthesis are likely important for adaptation to low oxygen concentrations.

Misregulation of hif-1 and egl-9 are associated with several phenotypes in C. elegans. egl-9 (lf) mutants although first isolated by having an egg laying defect, are more resistant to hydrogen cyanide, hydrogen sulfide and heat. Loss of hif-1 function in these mutant studies suppresses these phenotypes (Budde et al., 2010; Epstein et al., 2001a; Treinin et al., 2003; Zhang et al., 2009). Reduction and over expression of hif-1 also have several affects on adult lifespan in C. elegans. For example, over-expression of hif-1 has been shown to produce a dose-dependent increase in adult lifespan (Zhang, Shao et al. 2009). Expression above moderate levels becomes detrimental to longevity. Additionally, in normoxia it was shown that hif-1 loss of function results in a longer lifespan (Mehta et al., 2009). Therefore, wildtype animals live shorter lives than animals with an overexpression of HIF-1 and loss of function of HIF-1. The effect of HIF-1 overexpression or reduction is not qualitatively the same, as the effects of HIF-1 misregulation require different genes to influence lifespan (Zhang, Shao et al. 2009). This provides evidence that misregulation of HIF-1 under normoxic conditions alter stress responses and aging.

In addition to affecting wildtype normoxic lifespan, HIF-1 is also required for the increased longevity observed in mitochondrial respiration mutants. CLK-1 is a mitochondrial protein directly involved in production of ubiquinone (Ewbank et al., 1997). Mutations in the clk-1 gene results in increased longevity by slowing respiration rate and metabolic potential (Braeckman et al., 1999). In a genome-wide screen for genes that affect HIF-1 activity in C. elegans, it was discovered that mutations in clk-1(qm30) and
other respiration mutants such as *isp-1(qm150)* (another mitochondrial mutation in complex III) activate HIF-1 in normoxic conditions (Lee et al., 2010). Furthermore, this research shows that mutations in genes for mitochondrial proteins such as *clk-1* and *isp-1* increase reactive oxygen species (ROS). Using a HIF-1 transcriptional reporter, it is shown that the elevated ROS levels in respiratory mutants is enough to activate HIF-1 activity in normoxia and increase longevity (Lee et al., 2010). These data suggest a link between respiratory stress and longevity.

**Significance of HIF-1 Studies**

One aspect of cardiovascular disease is impaired oxygen transport to the body. Cardiovascular disease has a negative impact on the lives of humans; however, therapies to improve hypoxia resistance or improve oxygen transport during and after myocardial infarction have not been discovered. HIF is required for increases in formation of blood cells and vascularization during oxygen deprivation. Consequently, studies in *C. elegans* that contribute to our understanding of HIF regulation and function are important. All of the above HIF studies have led to a better understanding of oxygen sensing in mammalian systems; either in mice models or in cell culture. Once a mechanism, for example, oxygen sensing and gene regulation via EGL-9/PH and VHL-1, is identified in *C. elegans* it is then characterized in mammalian models. This rapidly increases the discovery of genes required to increase resistance to oxygen deprivation. New discoveries can lead to potential treatment of all types of vascular diseases.
Oxygen Sensing and Behavioral Studies in *C. elegans*

Wildtype animals behaviorally respond to O$_2$ levels in aerotaxis assays by avoiding low (<2%) and high (>14%) concentrations of O$_2$. Genetic studies have shown how *C. elegans* senses oxygen tension within the environment and will avoid oxygen levels >14% (Gray et al., 2004). Consequently, these researchers have termed 14-21% O$_2$ as hyperoxic for the worm. Specific neurons such as URX, AQR and PQR of the worm express soluble guanylate cyclases, *gcy-35* and *gcy-36*, that sense oxygen. For example, mutations in *gcy-35* prevent animals from sensing and avoiding oxygen concentrations higher than 14% (Gray et al., 2004). Oxygen concentration signal neuronal responses causing animals to avoid >14% O$_2$ and migrate to a preferred oxygen concentration of ~7% O$_2$.

Aerotaxis response to oxygen concentration is also regulated by the presence of food. Wildtype animals grown on thick bacterial lawns show moderate bordering behavior defined as the majority of animals located on the thickest part of the bacterial lawn. Bordering behavior is suppressed at 7% O$_2$ (Chang et al., 2006; Gray et al., 2004). Wildtype animals avoid hyperoxia (>14% O$_2$) in the absence of food but not in the presence of bacterial food. In aerotaxis assays *egl-9(lf)* mutants avoid hyperoxia in the presence or absence of food. Hyperoxia avoidance in *egl-9* mutants is dependent on the *hif-1* transcription factor (Chang et al., 2008). These results show that hypoxia can regulate aerotaxis regulations by food.

Animals with mutation in *egl-9* were first isolated based on defective egg-laying behavior (Trent et al., 1983). For example, *egl9(sa307)* animals hold late stage embryos
in utero and frequently die from these embryos hatching within the uterus. Like $egl$-9(sa307) animals, hyperoxia avoidance is also regulated by the neuronal TGF-β homolog DAF-7, a secreted mediator of crowding and stress responses. Mutations in $daf$-7 suppress hyperoxia avoidance on food much like $egl$-9. Analysis of mutants in $daf$-3(If), the downstream transcription factor of $daf$-7, revealed these two pathways regulate hyperoxia avoidance by different mechanisms (Chang et al., 2006). Interestingly, $egl$-9(If) mutants show reduced $daf$-7::GFP fluorescence in neurons and gonad cells.

Globin-like Molecules

*C. elegans* has a globin-gene family composed of 33 putative members with large variations in protein structure beyond the globin motif (Hoogewijs et al., 2007). Quantitative Real-Time PCR experiments demonstrated all 33 putative globin-like genes are expressed and ten are anoxia responsive (Hoogewijs et al., 2007). Data from transcriptional globin::GFP reporters show that the majority of *C. elegans* globin-like genes are transcriptionally expressed in the nervous system (Hoogewijs et al., 2008). While these results conclusively show globin-like gene transcripts are differentially expressed in anoxia, the authors did not analyze protein levels. Two globin::GFP translational fusions have been reported. Both $glb$-1 and $glb$-26 GFP translational reporters show distinct expression patterns in muscles and neuronal tissue (Geuens et al., 2010). Only $glb$-1 and $glb$-26 have been described under hypoxic conditions. Quantitative Real-Time PCR following a 12 hours exposure to hypoxia (0.1% $O_2$) showed $glb$-1, but not $glb$-26, is upregulated in hypoxia.
Natural variations in several genes affect *C. elegans* foraging behavior in response to oxygen levels. Wildtype (N2 Bristol strain) animals move slowly on food while the Hawaiian isolate CB4856 (HW) moves rapidly on food. The HW strain slows movement speed on food at 19.2% or 17.4% O$_2$ (Persson et al., 2009a). Another aspect of foraging is turning behavior. N2 and HW animals have similar rates of turning at 21% O$_2$. Yet shifting these two strains between 21% to 20% O$_2$ causes N2 animals to increase turning rate and HW to suppress turning (McGrath et al., 2009). Two genes were found to regulate foraging behaviors during the shift of oxygen from 21% to <20% O$_2$. One of these genes encodes a hexacoordinated neural globin protein, GLB-5, that acts with the atypical soluble guanylate cyclases such as GCY-35 (McGrath et al., 2009; Persson et al., 2009a). The other gene, *npr-1*, encodes a predicted G protein-coupled receptor, which functions with GLB-5 to change locomotory activity. Together, *glb-5* (HW high activity) and *npr-1* (HW low activity) allow animals to increase movement when O$_2$ rises and slow movement as O$_2$ falls below 21%. Changes in rate of movement allow selection of oxygen concentration and food location.

*Cell Cycle and Developmental Arrest*

*C. elegans* Responses to Anoxia

*C. elegans* survives anoxia (< 0.001 kPa O$_2$) by entering into a state of suspended animation during which all microscopically observable processes such as development, feeding, motility and cell cycle progression arrest (Padilla et al., 2002). Surprisingly, upon re-exposure to normoxic conditions (20.8% O$_2$) they proceed with development in a manner indistinguishable from untreated nematodes (Figure 1.1). This
ability of *C. elegans* to enter into suspended animation exists during all stages of development including embryos, L1 larvae, L2 larvae, dauer larvae, L4 larvae and adults. Animals in suspended animation survive 24 hours of anoxia at the rate of 90%. However, with the exception of embryos, starved L1 larvae, dauer larvae and adult males, the capacity for *C. elegans* to survive 72 hours of anoxia is reduced. This shows developmental stage influences the ability to survive long-term anoxia (>3 days).

Cell Cycle Arrest

*C. elegans* embryos survive anoxia exposure and are a model to study anoxia-induced cell cycle arrest. In normoxic conditions the *C. elegans* embryo undergoes asynchronous cell division and contains blastomeres at different stages of the cell cycle. Consequently, characterizing blastomeres arrested at various stages of the cell cycle is a tool to monitor what stages of the cell cycle are influenced by anoxia. The analysis of *C. elegans* embryos exposed to brief, intermediate, and long term anoxia helped to identify and order the initial and later changes at the sub-nuclear level taking place in relation to length of anoxia exposure time (Hajeri et al., 2005).

Anoxia-induced cell cycle arrest is an early response, occurring within 30 minutes of exposure. Wildtype embryos exposed to anoxia contain blastomeres arrested at interphase, prophase and metaphase of the cell cycle (Hajeri et al. 2005). Anoxia causes observable changes at different stages in the cell cycle such as chromatin condensation at interphase and microtubule depolymerization at prophase. The location of chromosomes near the nuclear membrane is also a characteristic of arrested anoxic prophase blastomeres in all embryos exposed to anoxia for either brief or longer periods.
of anoxia (Figure 1.2). However, the mechanism and functional role involving the sensing of anoxia and chromosome re-localization from the nucleoplasm to nuclear periphery is still unknown.

Initial experiments analyzed sub-cellular structures including: nuclear localization of chromatin, phosphorylation of histone H3, spindle microtubule structure, and the localization pattern of a spindle checkpoint protein, SAN-1. Typically mitotic nuclei in many organisms including C. elegans show an increase in the phosphorylation of histone H3 at serine 10 recognized by the antibody MPM-2. Exposure to 24 hours of anoxia revealed dephosphorylation of cell regulated mitotic proteins recognized by antibodies that recognize phosphorylated Histone 3. This suggests dephosphorylation of cell cycle regulated proteins correlates with anoxia-induced cell cycle arrest (Padilla et al., 2002).

An RNA interference (RNAi) screen of genes from chromosome I identified the spindle checkpoint genes, san-1 and mdf-2, as being required for anoxia-induced metaphase arrest (Nystul et al., 2003). mdf-2 is another spindle checkpoint gene required for proper regulation of the anaphase promoting complex (APC) (Kitagawa and Rose 1999). Embryos exposed to 24 hours of anoxia contain increased numbers of blastomeres arrested at metaphase. Anoxic san-1(RNAi) and mdf-2(RNAi) embryos did not contain blastomeres arrested at metaphase but instead accumulate abnormal nuclei and chromosome segregation defects such as anaphase bridging. The transcriptional response of hypoxia inducible factor (HIF1) that plays a major role in hypoxia survival in C. elegans is not required to enter and exit anoxia-induced suspended animation (Jiang
et al., 2001; Padilla et al., 2002). Therefore HIF-1 independent mechanisms exist for anoxia survival.

**Other Models to Study Hypoxia/Anoxia Responses**

Hypoxia

Research with the model organism, *Drosophila*, has been pivotal to understanding the mechanisms involved with development in low oxygen environments. The fly has conserved responses with worms and mammals in response to hypoxia. For example, the HIF signaling pathway is required for development and survival in low oxygen environments (Nagao et al., 1996; Nambu et al., 1996). While the adult fly is capable of withstanding only 3-4 hours of severe hypoxia (<0.1 O₂) the larvae are able to withstand 8 days of moderate hypoxia (1-5% O₂) (Wingrove et al., 1999). Thus, hypoxia sensitivity in *Drosophila* is also affected by developmental stage. It is still not understood if there are lasting affects on animals exposed to hypoxia but recent research has established that different genetic networks are involved in tolerance to intermittent or constant hypoxia (2.5 hours) in *Drosophila* (Azad et al., 2009b).

In addition to conserved genetic responses to oxygen deprivation, the fly has several behaviors associated with oxygen deprivation that allow it to be used as a model for understanding the physiological affects of hypoxia and recovery. Well-fed larvae exposed to hypoxic environments will exhibit exploratory behavior. This includes not feeding and leaving the food source (Wingrove et al., 1999). *Drosophila* adults exposed to severe hypoxia loose coordination, stop moving, and fall and remain motionless (Haddad et al., 1997). Upon return to normoxic conditions flies resume their
normal behavior. At 1% $O_2$ adult flies also do not lay eggs and will start to die at 3 days (Azad et al., 2009a). Consequently, *Drosophila* is not an appropriate model for studying tolerance of chronic hypoxia throughout the life cycle since they require normoxic conditions to finish their life cycle.

Anoxia

Cell cycle arrest in response to low oxygen tension is a conserved mechanism seen in fly embryos, zebrafish embryos, and mammalian fibroblasts (Foe and Alberts 1985; Padilla and Roth 2001;(Green et al., 2001). However, the position of the cell cycle in which arrest occurs differs depending upon the organism. Upon re-exposure to normoxic environment these arrested cells or blastomeres resume cell cycle progression. Blastomeres of flies exposed to 24 hours of anoxia arrest at interphase; and all stages of mitosis except anaphase (Foe and Alberts 1985). *Drosophila* embryos exposed to hypoxia arrest at either interphase or metaphase (DiGregorio et al., 2001; Douglas et al., 2001). Cultured mammalian cells exposed to hypoxia arrest at the G1 stage of cell cycle in response to hypoxia (Green et al., 2001). The mechanism by which reduction in $O_2$ levels signals cell-cycle arrest at specific stages of the cell cycle is not completely understood.

**Genes Required for Oxygen Deprivation Survival in C. elegans**

Insulin Signaling

Previous work characterizing *C. elegans* responses to hypoxia shows hypoxia and increased temperature induces death. This hypoxic death is regulated by the
Insulin/IGF receptor homolog DAF-2. A reduction in function of DAF-2 allows animals to survive 20 hours of <0.3% O₂ at 28°C (Scott et al., 2002). The method used in this study include heat stress, 28°C, and extreme oxygen deprivation, <0.3% O₂, and the results resemble previous work showing high temperature anoxia is lethal to wildtype animals at one day and DAF-2(1f) mutant animals are resistant. Although wildtype animals are sensitive to oxygen deprivation at high temperatures, DAF-2(1f) animals survive both high temperature (25°C) and long term anoxia (>3 days) (Mendenhall et al., 2006). Therefore, a mutation in the C. elegans insulin-like receptor gene promotes survival of hypoxia and anoxia. Long term anoxia and high temperature anoxia survival are also mediated by the glyceraldehyde 3-phosphate dehydrogenases GPD-2 and GPD-3 (Mendenhall et al., 2006). Hypoxic sensitivity at temperatures below 25°C are poorly understood.

*hyl-2* and Ceramide

While mutation in daf-2 promote survival of anoxia, loss of a member of the longevity assurance genes, *hyl-2*, increases sensitivity of C. elegans to anoxia (Menuz et al., 2009). In normoxia, the *hyl-2(gnv1)* mutant appears wildtype and has normal fecundity and lifespan. Yet when *hyl-2(gnv1)* young adults are exposed to anoxia for 48 hours their survival is reduced compared to wildtype animals (Menuz et al., 2009). The gene *hyl-2* is one of three ceramide synthase genes in C. elegans that create bioactive lipids with different fatty acyl chains. Ceramides are precursors to sphingomyelin and are implicated in a variety of physiological functions including apoptosis, cell growth arrest, differentiation, cell senescence, cell migration and adhesion (Hannun et al.,
Menuez et al. found hyl-2-deficient worms have fewer ceramide and sphingomyelin species with C\textsubscript{20} and C\textsubscript{22} fatty acyl chains and more with C\textsubscript{24} and C\textsubscript{26} fatty acyl chains than N2 animals. The association of shorter fatty acyl chains in anoxia resistance is unclear. Analysis of a double mutant for hyl-2 and daf-2 show these two genes are working in parallel pathways in regard to anoxia resistance (Menuez et al., 2009). Ceramides have been reported to be effectors of multiple kinases and phosphatases and may represent key activators during oxygen deprivation.

Other Types of Biological Arrest

Environmentally Induced Arrest

C. elegans has several responses to 24 hours of oxygen deprivation depending on the severity of the hypoxic insult. In hypoxia, around 1% O\textsubscript{2}, some aerobic metabolism is possible through electron transport (Van Voorhies et al., 2000). In anoxia, C. elegans survive by entering a reversible suspended animation, in which all-observable movement, including cell division and developmental progression, stops (Padilla et al., 2002). However, embryos exposed to an intermediate range of oxygen tension (<0.1% O\textsubscript{2}), between hypoxia and anoxia, do not survive (Nystul et al., 2004).

Hydrogen sulfide (80 ppm H\textsubscript{2}S) induces a suspended animation-like state in mice (Blackstone et al., 2007). Suspended animation for this experiment was defined by a reduction in metabolic rate and a drop in core body temperature similar to what is seen in hibernating mammals (Carey et al., 2003). Furthermore, it was shown that mice pretreated with H\textsubscript{2}S could survive oxygen deprivation (5% O\textsubscript{2} for 6.5 hours) better than nontreated animals (Blackstone and Roth 2007). Wildtype C. elegans exposed to
hydrogen sulfide (50 ppm H$_2$S) from embryo to adult are healthy and have increased thermotolerance and lifespan (Miller et al., 2007). Yet hif-1(ia04) animals can not survive a 24 hour exposure to 15 ppm H$_2$S (Budde et al., 2010). In addition to increasing hif-1 transcript levels, it was shown that HIF-1 nuclear localization occurs in a manner indistinguishable from hypoxia (Budde and Roth 2010). These data suggest that H$_2$S can have a protective role during environmentally induced oxygen deprivation.

Carbon monoxide interacts with heme containing proteins as an oxygen mimetic. For example, CO can influence electron transport reactions producing either prooxidant or antioxidant effects (Piantadosi et al., 2008). It was proposed that as a competitive inhibitor of oxygen binding, CO might induce suspended animation and protect against the lethal range of hypoxia (Nystul and Roth 2004). C. elegans embryos can tolerate a wide range of CO tensions and survive 100% CO for 24 for hours with high viability. In addition, CO does protect against hypoxic damage in the presence of lethal oxygen tension by inducing suspended animation (Nystul and Roth 2004). The mechanism of CO-induced suspended animation is unclear.

Developmentally Induced - Germline Arrest

Mammalian oocytes arrest at prophase I from birth until sexual maturity. Hormone signals induce cell cycle progression to meiosis II, the stage where fertilization occurs. This arrest is tightly coupled to the granulose cells surrounding the oocytes. These follicle cells hold the oocytes in prophase arrest and arrest is highly dependent on high cAMP levels within the oocytes (Mehlmann, 2005). This makes studying
prophase arrest in mammalian oocytes difficult since the follicles are within the adult animal and would need to be removed.

Proper regulation of oocyte maturation is an important process in every species. The process of oogenesis in humans is characterized by meiotic diapause until sexual maturity. When the maturation process continues at reproductive maturity these cells are released from diapause and continue through meiosis. If the oocytes do not reenter meiosis correctly nondisjunction, when chromosomes do not properly separate, can occur after exiting diapause. For example, miscarriages and congenital birth defects in humans can result from nondisjunction during meiosis I (Hassold et al., 2001). While defects in both the nucleus and cytoplasm of old-aged oocytes have been linked to causes of fertility problems, defective mechanisms have not been identified (Sherins, 1995).

Genetic pathways affecting the development and maintenance of the C. elegans germ line are known. This provides an advantage for studying signaling processes essential for oocytes growth and differentiation in the germ line. For example, major sperm protein (MSP) was identified as a secreted signal in sperm, which is sufficient to stimulate oocyte maturation, mitogen-activated protein kinase (MAPK) activation, and sheath cell contractions (Miller et al., 2001). The two locations within the gonad regulated by MSP are the somatic germline, containing sheath cells, and the non-somatic gonad of the maturing oocyte. Sheath/oocyte gap junctions connect both locations and require a POU-homeodomane protein, CEH-18, for proper communication (Rose et al., 1997). MSP signals oocyte maturation and MAPK activation by
agonizing both the VAB-1 Eph receptor protein on oocyte membranes and a sheath cell-dependent pathway involving CEH-18 (Miller et al., 2003a).

In *C. elegans* the adult hermaphrodite gonad contains germ cells at various stages of meiosis. The large oocytes proximal to the spermatheca are at Diakinesis of Prophase I. Mammalian oocytes enter into an extended arrest in prophase of meiosis before maturation that can last decades in some species (Masui et al., 1979). In contrast, *C. elegans* hermaphrodites ovulate oocytes every 23 minutes and it is hypothesized there is no arrest since the signal for maturation, sperm, is constitutive (McCarter et al., 1999). Worms lacking sperm, for example, *fem-1(e1965)* and *fog-2(q71)* mutant animals have null mutations in sex determining genes causing feminization, show arrest in diakinesis (Doniach et al., 1984; Schedl et al., 1988a). While this arrest can last for days, the primary oocytes still mature and ovulate at a low rate resulting in unfertilized oocytes in the uterus. *C. elegans* consequently provides a model to study changes in cellular structure and morphology between actively maturing and quiescent meiotic cells.

Reproduction has the potential to influence cellular stress responses and animal longevity (Mukhopadhyay et al., 2007). In *C. elegans*, germ line ablated animals are resistant to oxidative damage, but the relationship between stress resistance and longevity is not understood (Yamawaki et al., 2008). In regard to life span extension, both the somatic and germ line reproductive tissues send signals influencing longevity (Yamawaki et al., 2008). However, the extension of life span is not due to complete sterility, since animals with whole gonad removal using ablation or genetic mutations do not have an increased life span (Hsin et al., 1999). How the germ line maintains itself
during environmental stresses such as starvation and oxygen deprivation when the animal stops laying eggs is not understood.

Research Objectives

Objective 1: Characterize the Affect Oxygen Deprivation has on Germline Structure and Function

- Hypothesis: Cellular changes associated with anoxia-induced germline arrest overlaps with previously identified stress responses.
  - Aim 1.1: Characterize germline chromatin localization
    - Environmental stress
    - Quiescent oocytes
  - Aim 1.2: Determine if nucleoporin function is required for anoxia-induced germline arrest
  - Aim 1.3: Determine if P-bodies or stress granules accumulate in the germline of anoxia exposed animals

Objective 2: Characterize C. elegans Response to Chronic Hypoxia

- Hypothesis: Chronic hypoxia will affect the developmental progression and behavior of C. elegans.
  - Aim 2.1: Characterize C. elegans Development in Chronic Hypoxia
  - Aim 2.2: Characterize C. elegans Behavior Response to Chronic Hypoxia
    - General hypoxic egg lay behavior
    - Genes required for hypoxic egg lay behavior
  - Aim 2.3: Investigate Genetic Mechanisms regulating Responses to Chronic Hypoxia
Figure 1.1. *C. elegans* arrest when exposed to anoxia. A) Two cell embryos were collected and exposed to either a normoxic (21 kPa, 21% O₂) environment or an anoxic (0 kPa, 0% O₂) environment. Embryos are ~50 μm in length. Bar, 50 μm for image showing L1 larva only. (B) L3 larvae were collected and exposed to either normoxic or an anoxic environment for 24 hours. Time 0 is the time nematodes began to arrest development. The second time point is 24 hours after Time 0 (Padilla et al. 2002).
Figure 1.2. Chromosomes of prophase blastomeres exposed to brief periods of anoxia align near the nuclear membrane. Embryos were exposed to either normoxia (A-C), or 24 hours of anoxia (G-I) collected, and stained with DAPI (A,D,G) and the mAb414 to recognize nuclear pore complex (B,E,H). Images were merged (C,F,I) to evaluate prophase chromosome location relative to the nuclear membrane. The letter P is to the left of prophase blastomeres. Scale bar is 10 µm. (Hajeri et al. 2005)
CHARACTERIZATION OF GERMLINE STRUCTURE
AND FUNCTION IN ANOXIA

Introduction

It has been shown that anoxia induces an arrest of oocyte maturation and ovulation in the adult hermaphrodite (Mendenhall et al., 2009). This arrest is reversible suggesting a level of DNA maintenance in meiotic cells exists during environmental stresses such as oxygen deprivation. As the *C. elegans* embryo blastomeres are not the only dividing cells capable of undergoing arrest, I hypothesize meiotic cells should also undergo an arrest when exposed to anoxia. To monitor meiotic cells and germ line function under normoxic and anoxic conditions, I conducted experiments analyzing subcellular changes in the germ line from animals exposed to anoxia.

In *C. elegans* the CEH-18 signaling pathway controls rearrangement of the microtubule cytoskeleton in response to the presence of major sperm protein (MSP) (Miller et al., 2003b). There is a change in cortical location of microtubules in oocytes as a response to time and depletion of sperm. Young hermaphrodites have an even cytoplasmic distribution of microtubules. Sperm depleted, 4-day old animals, have a reversible cortical enrichment of microtubules reversed by mating (Harris et al., 2006). The significance of cortical microtubule enrichment in oocytes when sperm are absent is not fully understood; however, since microtubules play critical roles during oogenesis in regulating protein trafficking, cell polarity, and RNA localization, the changes in localization are hypothesized to have functional importance for the worm.

* Results from this chapter are reproduced from Developmental Biology, Jud et al. 2008 with permission from Elsevier, and were also published in Molecular Biology of the Cell, Hajeri et al. 2010.
Previous work in our lab showed differences in nuclear localization, phosphorylation of histone H3, and spindle microtubule structure in embryos exposed to anoxia (Hajeri, Trejo et al. 2005). Some germ cell specific cytoplasmic rearrangements in response to stresses include accumulation, rearrangement or loss of P-bodies and stress granules. Germ cells contain germ granules that are characteristically spherical shapes called P-bodies. These granules contain RNA decay factors, decapping enzymes and proteins controlling deadenylation and translation. They do not contain ribosomal subunits or translation factors. These P-bodies have been shown to increase in response to osmotic stress, UV stress and increased cell density in yeast cells (Brengues et al., 2005). Another more irregular shaped structure known as stress granules are also found within germ cells and contain some P-body associated proteins. They also contain unique proteins; for example, stalled initiation complexes, poly A binding proteins and RNA binding proteins that serve as shuttles. Stress granules have mainly been studied in cell culture and how they are affected in an organism responding to environmental stress (heat shock), oxidative stress, O₂ deprivation and osmotic stress are poorly understood.

The main objective of this research study is to characterize the effect oxygen deprivation has on germ line structure and function. Hence, I took the approach of analyzing sub-cellular changes in the germline of animals exposed to anoxia. The general hypothesis of this research is that cellular changes associated with anoxia-induced germline arrest overlap with previously identified stress responses. To test this hypothesis I conducted live cell imaging of animals with a GFP marker that allows for chromosome visualization. I focused my study on analyzing chromatin localization and
movement in quiescent oocytes and oocytes of animals exposed to environmental stresses. To understand how arrest in response to a reduction in O2 affects other subcellular changes in the cytoplasm of meiotic cells we took a cell biological approach. We chose to characterize ribonucleoprotein (RNP) foci dynamics in oxygen-deprived oocytes since this induces the formation of stress granules in mammalian cells (Anderson et al., 2006). This study used live cell imaging of animals with a GFP marker for RNP foci and immunostaining of germlines from animals exposed to anoxia. Overall, this study laid the foundation for understanding germline arrest in response to quiescence and environmental stress.

Results

Germline Chromatin Localization During Stress

Anoxia

To determine if anoxia affects bivalent chromosome location within the nucleus of oocytes we analyzed oocytes in adults exposed to 24 hours of anoxia (using anaerobic biobags) or brief periods of anoxia using an N2 gas flow through chamber. We used the TH32 (tbg-1::GFP; pie-1::GFP::H2B) strain to monitor chromosome location. In control animals exposed to normoxic conditions, the bivalent chromosomes are found to move throughout the nucleoplasm of oocytes in hermaphrodites (Figure 2.1). However, we indeed found the bivalent chromosomes aligned with the nuclear periphery in arrested oocytes of adult hermaphrodites exposed to anoxia (Figure 2.1). This chromosome alignment was observed in all oocytes and not just the primary oocyte (Figure 2.1). Oocytes of adult animals exposed to anoxia are viable and able to be fertilized after
anoxia treatment, as determined by viable offspring produced by post-anoxic hermaphrodites. We did find after longer exposures to anoxia (24 hours) it took approximately 1.5 hours for oocytes to recover in air and proceed with ovulation (N=6 animals observed). To determine how quickly the chromosomes respond to anoxia we used the N$_2$ gas flow-through chamber and found chromosomes aligned with the inner nuclear periphery after exposure to 12 minutes of N$_2$ gas flow. Upon re-exposure to normoxic conditions the chromosomes within the primary oocyte moved off the nuclear envelope just prior to maturation and ovulation, whereas the chromosomes in the other oocytes remained associated with the inner nuclear periphery (N=6 germlines observed). This association suggests the recovery process may be linked with signals involving oocyte maturation and ovulation. Furthermore, these results show that similar to prophase blastomeres, oocytes also contain chromosomes associated with the inner nuclear periphery after exposure to anoxia.

Other Environmental Stress and Quiescent Oocytes

Since anoxia is a stress phenomenon that induces arrest of oocyte fertilization we next wanted to determine if bivalent chromosome alignment with the nuclear periphery is a characteristic also associated with stresses and/or with quiescent oocytes. We found starving adult hermaphrodites for 12 hours, which caused adults to hold embryos in the uterus, did not induce bivalent chromosomes to align with the nuclear periphery (Figure 2.2). To test whether bivalent chromosome alignment with the nuclear periphery was induced by lack of respiratory function, we treated animals with sodium azide, an inhibitor of cytochrome C in the electron transport chain, which is also
known to induce ovulation arrest. As shown in Figure 2.2, not all of the bivalent chromosomes aligned with the nuclear periphery in animals exposed to sodium azide. This phenotype may be associated with the azide-induced ovulation arrest. We found bivalent chromosomes also aligned with the nuclear periphery in quiescent oocytes of animals in which ovulation does not or only rarely occurs due to sperm depletion (aged hermaphrodites) or non-functional sperm (fog-2(q71) mutant) (Figure 2.2) (Schedl et al., 1988b). We did not observe any major difference in the chromosome association with the inner nuclear periphery in anoxia-arrested oocytes compared to the quiescent oocytes due to sperm depletion or dysfunction.

**Role of Nucleoporin Function in Anoxia-induced Germline Arrest**

Others in the lab have shown that prophase arrest in prophase blastomeres of anoxic embryos requires the nucleoporin NPP-16. There are several lines of reasoning that led us to investigate nucleoporins in relation to cell cycle arrest. First, the transition from prophase to prometaphase is characterized by chromosome condensation, nuclear envelope breakdown (NEBD), and the initiation of chromosome movement to the equatorial plate; NEBD is considered a commitment to mitosis. Second, microtubule formation is required for moving from metaphase to anaphase and is thought to signal NEBD (Gorbsky et al., 1998; Muhlhausser et al., 2007; Stein et al., 2007). We have previously shown microtubules depolymerize in embryos exposed to anoxia (Hajeri, Trejo, et al. 2005). In *Drosophila*, *C. elegans* embryos, and starfish oocytes the disassembly of nucleoporins occurs prior to the disassembly of inner nuclear membrane-associated proteins and NEBD (Kiseleva et al., 2001; Lee et al., 2000;
Lénárt et al., 2003). Therefore, we next tested if nucleoporin function was involved in the signaling of anoxia-induced prophase arrest in embryos. In *C. elegans* there are 24 genes predicted to encode nucleoporins (D'Angelo et al., 2008; Galy et al., 2003). Using RNAi to knock-down individual NPC components, we found, consistent with previous reports, most nucleoporin genes serve essential functions, and accordingly, knock-down resulted in various phenotypes including embryo lethality, sterility, larval lethality and larval arrest. However, RNAi of the nucleoporin genes *npp-14*, *npp-16*, or *npp-18* (predicted homologues of mammalian Nup214, Nup50, and Seh1, respectively) did not result in obvious phenotypes (Galy et al., 2003).

These results led us to further investigate NPP-16 function in anoxia-induced germline arrest. To characterize the germ line phenotype in *npp-16(ok1839)* hermaphrodites, I used the TH32 strain backcrossed into the *npp-16(ok1839)* strain or stained the gonad of *npp-16(ok1839)* hermaphrodites with mAb414 and DAPI to recognize the NPC and chromosomes, respectively. I observed several phenotypes associated with the *npp-16(ok1839)* mutation. First, unlike control animals, the *npp-16(ok1839)* hermaphrodites exposed to normoxia contain oocytes in which the bivalent chromosomes associate with the inner nuclear periphery; this was observed in animals analyzed by live imaging analysis or by immunostaining of the gonad (Figure 2.3, Figure 2.4, Figure 2.5). Analysis of the *npp-16(ok1839)* hermaphrodites exposed to anoxia indicates many of the oocytes had an abnormal nucleus as determined by an abnormal shape or if the membrane, as detected by mAb414, was not round/oblong and appeared to have invaginations or folds (Figure 2.3, Figure 2.4). To determine if abnormal nuclear shape was an artifact of fixation and staining, I used live imaging of
npp-16(ok1839) animals. I also observed phenotypes associated with anoxia exposure in live npp-16(ok1839) animals (Figure 2.5). Others have shown, in the wildtype germ line, mAb414 localizes at unique regions in germ cell nuclei (Pitt et al., 2000). I observed in control germ cells mAb414 localized around the nuclear DNA. However, in both wildtype and npp-16(ok1839) animals the localization of mAb414 is more dispersed in the germ cells within the pachytene region of the gonad (Figure 2.3) suggesting the NPC of these germ cells are perturbed in response to anoxia. Second, in oocytes the mAb414 detection accumulates at the cortical region of the oocyte and is not as intense at the nucleus in comparison to normoxic controls (Figure 2.3, compare oocytes noted by * and arrow). Third, the sperm are always visible in the gonad of anoxia exposed animals (Figure 2.3, region noted as SP); this suggests ovulation may arrest in a specific manner but further analysis of the spermatheca would need to be conducted to determine if such is the case. To determine if aged oocytes were viable in npp-16(ok1839) hermaphrodites depleted of sperm, as determined by the lack of offspring and occasional oocytes on NGM plate, the hermaphrodites were mated with adult N2 males; 62.5% of the npp-16(ok1839) hermaphrodites (N=16) were able to produce offspring in which a large frequency of males were observed. In comparison, 83% of sperm-depleted wildtype hermaphrodites produced offspring (N=6). The large frequency of males in the offspring of a hermaphrodite animal is evidence of a successful mating with a male animal. Together our data indicate the gonad of npp-16(ok1839) animals is functional but has some phenotypes associated with the mutation.
*P-bodies and Stress Granules Accumulate in the Germline of Anoxia Exposed Animals:*

In *C. elegans*, ribonucleoprotein (RNP) foci form in oocytes arrested after sperm depletion (Schisa et al., 2001). These foci are dynamic in formation and reversible after mating. Initial characterization of these RNP foci shows an RNA-binding protein, MEX-3, and nuclear pore proteins colocalized with these foci in arrested oocytes (Jud et al., 2007). What function these RNP foci may have is unknown. Our hypothesis for the function of oocyte RNP foci is that they share related functions with the RNA-related functions of processing bodies (P bodies) and stress granules; it has been shown three orthologs of P body proteins, DCP-2, CAR-1 and CGH-1, and two markers of stress granules, poly (A) binding protein (PABP) and TIA-1, appear to be present in the oocyte RNP foci (Jud et al., 2008).

In animals subjected to 4 and 20 hours of anoxia, over 90% of the animals formed small cytoplasmic foci of GFP::MEX-3 (N=11, 22 animals observed respectively) (Figure 2.6). To determine if the foci induced by oxygen deprivation were similar to those formed after heat shock or osmotic stress, we determined if the anoxia-induced foci included a P body protein, DCP-2, or stress granule proteins, TIA-1 and PAB-1. Worms were co-stained after 20 hours of anoxia for DCP-2 and MEX-3; however no large foci containing DCP-2 and MEX-3, which are the hallmarks of heat shock and osmotic stress, were detected (Figure 2.7). In contrast when LAP::TIA-1 worms were subjected to anoxia, the GFP signal was detected in cytoplasmic foci slightly smaller than the foci seen after heat shock or in the absence of sperm (Figure 2.6). While TIA-1 protein localization changed, the other stress granule protein analyzed, PAB-1, showed no change in anoxia exposure (Figure 2.6). These results are the first *in vivo*
demonstration linking components of P bodies and stress granules in the germ line of a metazoan. Taken together, these data suggest the formation of foci in arrested oocytes may play a similar role as P bodies and/or stress granules that increase in size or form in response to multiple environmental stresses.

Conclusion

In this study I characterized the phenomenon of chromosome association with the nuclear periphery in oocytes of hermaphrodites exposed to anoxia as well as oocytes in a quiescent state due to sperm depletion (aged hermaphrodites) or disruption of fertilization (fog-2 mutants). I found that adult hermaphrodite animals exposed to anoxia for 24 hours contain arrested oocytes with bivalent chromosome association with the nuclear periphery. This chromosome docking phenotype could be attributed to meiotic cells undergoing arrest or a general response to environmental stress. To determine if chromosome localization to the nuclear envelope is a response to other stresses or quiescence, I assayed chromosome location in starved adult animals and adult animals with quiescent oocytes. I found that quiescent oocytes have bivalent chromosome association with the nuclear periphery yet starved animals have chromosomes located within the nucleoplasm. These data suggest bivalent chromosome association with the nuclear periphery is not signaled by stress per se but is rather a phenotype associated with stress-induced arrest or quiescence due to absence of functional sperm.

I further characterized chromosome localization to the nuclear envelope in oocytes by investigating the role of the NPC. To determine the role of the NPC, I used
RNAi to knock down candidate NPC genes and assayed chromosome localization to the nuclear envelope during anoxia exposure. This screen isolated \textit{npp-16} as a candidate for a role in anoxia-induced oocyte quiescence. I found that in the oocyte of animals exposed to anoxia, \textit{npp-16} is not required for chromosome association with the nuclear periphery but is required for maintenance of nuclear shape. Furthermore, anoxia affects the localization of NPC in the meiotic cells of both wildtype and \textit{npp-16(ok1839)} animals further supporting the idea the NPC has a role in anoxia responses. In addition to changes in chromatin during anoxia, the cytoplasm also undergoes rearrangement in localization of RNP foci. Taken together these events allow arrested germ cells to maintain structure and function while waiting for signals to begin or resume reproduction.

**Materials and Methods**

**Strains and Growth Conditions**

The \textit{C. elegans} wildtype N2 Bristol strain was raised on NGM plates seeded with \textit{E. coli} (OP50) as previously described (Sulston, 1988). For all experiments nematodes were maintained at 20°C. The following strains were obtained from \textit{Caenorhabditis elegans} Genetics Center and raised under similar conditions as the wildtype strain: TH32 (\textit{tbg-1::GFP; pie-1::GFP::H2B}), RB1534 (\textit{npp-16(ok1839)}), CB4108 (\textit{fog-2(q71)}). The \textit{Caenorhabditis elegans} Gene Knockout Consortium (Oklahoma Medical Research Foundation) produced the \textit{npp-16(ok1839)} deletion allele (strain RB1534), which has a 1120 bp deletion resulting in removal of amino acids 175 to 472 of the 512 amino acid nucleoporin NPP-16 protein. \textit{npp-16(ok1839)} was backcrossed three times to wildtype
N2 animals to produce the strain PM118 in which all analyses were conducted. Using standard genetic techniques, the TH32 strain was crossed with the \textit{npp-16(ok1839)} deletion PM118 strain to produce strain PM119 (\textit{npp-16(ok1839); tbg-1::GFP; pie-1::GFP::H2B}) or the CB4108 strain to produce PM122 (\textit{fog-2(q71); tbg-1::GFP; pie-1::GFP::H2B}). The \textit{npp-16(ok1839)} genotype was verified by conducting single worm PCR using \textit{npp-16} forward primer (5'-TGACTCATCGAGCCTGAAAA-3'), and \textit{npp-16} reverse primer (5'-GAGTCGAACTTCCCAAGCAG-3').

\textit{Live Animal Imaging Analysis}

Live animal imaging analysis was conducted using strains TH32 (\textit{tbg-1::GFP; pie-1::GFP::H2B}) and PM119 (\textit{npp-16(ok1839); tbg-1::GFP; pie-1::GFP::H2B}), which are both expressing \textit{γ}-tubulin::GFP and histone2B::GFP, as previously described (Hajeri et al., 2008). These strains allow one to monitor chromosome and mitotic progression by visualization of centriole location and chromosome condensation based on GFP fluorescence detection. Nematodes were grown to adulthood on NGM plates seeded with \textit{E. coli}. Gravid adults were anaesthetized (0.5% tricaine, 0.05% tetramisole in M9 buffer) and mounted on 2% agarose pads placed on cover slips (Warner instruments, USA); this method to anesthetize adults slows their movement during microscopic examinations, but does not interfere with ovulation and fertilization (McCarter et al. 1999). The anaesthetized nematodes were coated with a drop of halocarbon oil to prevent desiccation during exposure to a flow of nitrogen gas or air, and placed in an enclosed chamber (Harvard apparatus, Leiden Closed Perfusion Microincubator). To monitor anoxia-induced prophase arrest, the chamber was perfused
with 100% nitrogen (Air Liquide-Calgaz, Cambridge Maryland, USA) and time-lapse images of embryos within the uterus of the adult nematode were collected using a spinning disc confocal microscope (McBain, CA) (see below). Time-lapse movies were processed by using Image J (NIH) and imported into QuickTime (Apple Inc.) for display.

**Analysis of Bivalent Prophase Chromosomes in Oocytes and Anoxia Assays**

To analyze the nuclear location and movement of bivalent chromosomes in oocytes of animals exposed to stress (anoxia, starvation, sodium azide) or animals with quiescent oocytes (aged animals, fog-2 mutant), time-lapse microscopy of the primary oocytes of TH32 animals (*tgj-1::GFP; pie-1::GFP::H2B*) and PM122 (*fog-2(q71); tgj-1::GFP; pie-1::GFP::H2B*) animals was conducted. For anoxia assays, one-day old TH32 adults were exposed to 24 hours of anoxia using the anaerobic bio-bag Type A environmental chamber as previously described (Padilla et al., 2002). After anoxia exposure, animals were quickly mounted on a 2% agar pad and time-lapse microscopy of oocytes was conducted; all image acquisition was performed within 10 minutes of re-exposure to normoxia. Control oocytes were from 1-day old TH32 animals exposed to a normoxic environment. For analysis of oocytes from starved animals, one-day old hermaphrodites were washed four times in M9 buffer to remove *E. coli* and then placed on an unseeded NGM plate for 12 hours. These starved adults held embryos within the uterus indicating the lack of food was indeed inducing a physiological response. To analyze oocytes of one-day old hermaphrodites with blocked respiratory function, animals were placed in M9 buffer with 10 mM sodium azide for up to 1 hour. Quiescent oocytes were analyzed from aged TH32 animals (6 days post L4 molt) and 1-day old
PM122 (fog-2(q71), pie-1::GFP::H2B) animals. For all assays, time-lapse microscopy of primary oocytes was conducted, to examine the localization and movement of bivalent prophase chromosomes within the nucleus, using a spinning disc confocal microscope (McBain, CA) and processed using Image J software (NIH) and Adobe Photoshop.

**Indirect Immunofluorescence Assays**

Analysis of the germline was conducted in a similar manner as previously described (Jud et al., 2008). Briefly, one-day old hermaphrodites were transferred to M9 and washed twice to remove bacteria. Approximately 20 animals were placed on a cover slip and gonads were extruded using a 27 gauge syringe needle, fixed for 20 minutes in 2% paraformaldehyde in a humid chamber, placed on a poly-lysine coated slide, freeze-cracked and fixed in -20°C methanol for 10 minutes. Slides were incubated with mAb414 for 1 hour and secondary antibody for 30 minutes at room temperature. To stain DNA, DAPI was included in the last rinse prior to mounting for microscopy analysis. The dissected gonads were examined using either a Zeiss Axioscope fluorescence microscope or a spinning disc confocal microscope (McBain, CA); image acquisition was done using Simple PCI (Hamamatsu) and processed using Image J software (NIH) and Adobe Photoshop (Hajeri et al., 2005).

**RNAi Assays**

A synchronous population of TH32 (tbg-1::GFP; pie-1::GFP::H2B) or N2 L1 larvae were grown to adulthood on RNAi plates (NGM supplemented with 200 µg/ml ampicillin, 12.5 µg/ml tetracycline and 2 mM IPTG). The RNAi plates were seeded with
the *E. coli* strain for RNAi of a specified gene of interest. The *E. coli* strains were developed by the J. Ahringer laboratory and obtained from the Medical Research Council Geneservice (Cambridge, UK) (Kamath et al., 2003). As a control, worms were fed the *E. coli* HT115 strain containing the plasmid L4440 without insert. Adult RNAi treated animals, which did not show a phenotype under normoxic conditions were exposed to 24 hours of anoxia using the anaerobic bio-bag Type A environmental chamber (Fisher Scientific) (Padilla *et al.*, 2002; Hajeri *et al.*, 2008) or exposed to hypoxia in an In vivo2 anaerobic chamber (Ruskinn Scientific). After anoxia or hypoxic exposure, the animals were quickly transferred to a 2% agar pad and assayed for oxygen deprivation phenotypes by spinning disc confocal microscopy or by Differential interference microscopy (DIC) on a Zeiss Mot II Plus microscope with a X100 objective lense, Axiocam camera and software.
Figure 2.1. Bivalent chromosomes align with the nuclear periphery in oocytes of animals exposed to anoxia. Live cell imaging, using a tbg-1::GFP; pie-1::GFP::H2B strain (TH32), to analyze the chromosomes in the oocyte of adult control animals exposed to either normoxia or anoxia. Primary oocyte is noted by *. Scale bar, 10 µm.
Figure 2.2. Chromosomes associate with the nuclear periphery in anoxia-arrested quiescent oocytes. Live cell imaging, using a *tbg-1::*GFP; *pie-1::*GFP::*H2B* strain (TH32), to analyze the chromosomes in the oocyte of adult control animals exposed to either normoxia, anoxia, starvation or sodium azide or in a quiescent state due to sperm depletion in aged hermaphrodites or sperm dysfunction in *fog-2(q71)* females. Time-lapse analysis was used to track chromosome location within the nucleus. For each experiment, at least 30 oocytes from three independent experiments were examined. Scale bar, 5 µm. Hajeri et al. 2010.
Figure 2.3. The germline of wild type and npp-16(ok189) adult hermaphrodites exposed to normoxia or anoxia. Shown are representative images of N2 wild type and npp-16(ok1839) adult gonads dissected and stained with DAPI to detect DNA and mAb414 to detect NPC. The NPC is noted by an arrowhead, SP denotes the spermatheca region, and * notes the observation that in anoxia exposed animals the mAb414 detection accumulates at the cortical region of the oocyte. Note that in comparison to control, in anoxia-exposed animals the localization of mAb414 appears more dispersed within the pachytene region of the gonad. Scale bars, 10 µm. Hajeri et al. 2010.
Figure 2.4. The germline of wild type and *npp-16(ok1839)* adult hermaphrodites exposed to normoxia or anoxia. Shown are representative images of N2 wild type and *npp-16(ok1839)* adult gonads dissected and stained with DAPI to detect DNA and mAb414 to detect NPC. This image shows representative enlarged images of the pachytene region, normal oocytes or abnormal oocytes are shown. Note that in comparison to control, in anoxia-exposed animals the localization of mAb414 appears more dispersed within the pachytene region of the gonad. We classified oocytes as abnormal if the bivalent chromosomes associated with the nuclear periphery under normoxic conditions or if the nuclear membrane shape was not round/oblong and appeared to have invaginations or folds. Scale bars, 10 µm. Hajeri et al. 2010.
Figure 2.5. The nucleus is abnormally shaped in arrested \textit{npp-16(ok1839)} oocytes. Live cell imaging, using a \textit{tbg-1::GFP; pie-1::GFP::H2B} strain (TH32), to analyze the chromosomes in the oocyte of adult control and \textit{npp-16(ok1839)} animals exposed to either normoxia or anoxia. Scale bar, 10 \textmu m. Hajeri et al. 2010.
Figure 2.6. GFP::MEX-3 and LAP::TIA-1 expressing gonads show foci formation after 20 hour anoxia exposure. Shown are representative confocal images of MEX-3 and TIA-1 and PAB-1 expression in proximal oocytes of live adult animals. Note foci formation with MEX-3 and TIA-1 shown with arrow. TIA-1 is nuclear and cytoplasmic in normoxia and is only cytoplasmic after anoxia exposure. Nuclear localization in normoxia is shown with the *. Scale bar, 10 µm. Jud et al. 2008.
Figure 2.7. MEX-3 and DCP-2 relocalize after 20 hour anoxia exposure. Shown are representative images of N2 wildtype adult gonads dissected and stained with DAPI to detect DNA and anti MEX-3 and DCP-2 to show P body components. Scale bar, 10 µm. Jud et al. 2008.
CHARACTERIZATION OF DEVELOPMENT IN CHRONIC HYPOXIA

Introduction

Developmental programs, which include the timing of major morphological changes in a developing organism, are influenced by many different environmental factors. Some environmental stresses induce morphological differences such as changes in tail size and muscle structure in response to predation (Kraft et al., 2006). While this form of phenotypic plasticity occurs without changes in developmental trajectory, other stressful environments are known to speed up or slow down development. The ability to speed up or slow development in response to environmental cues is evolutionarily conserved in organisms across several phyla. For example, the invertebrate butterfly, Bicyclus anynana, vertebrate fish, Fundulus heteroclitus, and marsupial, Macropus eugenii all have the ability to change developmental trajectory in response to changes in their environments (Bauerfeind et al., 2009; Fletcher et al., 1988; Tingaud-Sequeira et al., 2009). During the larval development of the amphibians Hyla cinerera and Hyla gratiosa, different environmental effectors such as food and temperature can produce different developmental trajectories. In experiments with H. gratiosa, a decrease in food and temperature resulted in a 74% increase in time to metamorphosis from 36 days to 62 days (Leips and Travis 1994).

Environmental oxygen concentration influences the development and survival of animals at the whole organism and organ level. For example, in the bullfrog (Rana catesbeiana) gill ventilation is influenced by hypoxia (Jia et al., 1997). The effect of decreasing oxygen concentration has on gill ventilation is dependent on developmental stage. Several studies have been conducted on C. elegans that investigate the affects
of brief and intermittent exposure to low oxygen levels. *C. elegans* exposed to a severe hypoxic environment at high temperatures (<0.3% O₂ and 26.5°C) go through cellular death and eventually animal death (Scott et al., 2002). However, *C. elegans* are able to survive and develop in 1 day of hypoxia exposure (0.5%-1%) at standard lab temperature (20°C) (Jiang et al., 2001). Yet the affects of oxygen deprivation on development in this model organism are not known.

The main objective of this research study is to characterize the affect oxygen deprivation has on *C. elegans* developmental progression. Hence, I took the approach of observing anatomical markers in animals exposed to hypoxia (0.5%-1%). The general hypothesis of this research is that at standard lab temperatures (20-22°C) *C. elegans* will develop and reproduce under hypoxic conditions (0.5%-1%). To test this hypothesis, I conducted development assays of embryos exposed to hypoxia at 0.5% O₂ and 1% O₂ in a hypoxia glove box. I focused my study on analyzing germline development and fitness levels of animals exposed to hypoxia. I then took a targeted approach to identify genetic mutations that might have an effect on viability and developmental progression in mutants exposed to hypoxia. These studies included phenotype analysis of animals with mutations in genes involved in oxygen sensing (*hif-1(ia04), egl-9(sa307)*) or development, such as TGF-β (*daf-7(e1372)*). Overall, this study laid the foundation for understanding the affects of hypoxia on development in *C. elegans*. 
Results

_Hypoxia Exposure Methods Differentially Influence_  
_Hypoxia Induced Stress Phenotypes_

We used a hypoxia glove box chamber to expose wildtype _C. elegans_ to severe hypoxia (0.5% and 1% O₂) for acute (< one day) or chronic (> one day) exposure and analyzed the responses to this stress. Previously it had been shown that wildtype embryos are able to survive 0.5% and 1% O₂ exposure with a high survival rate (Jiang et al., 2001; Padilla et al., 2002). We found using our hypoxia glove box chamber that eggs laid on both OP50 and HT115 food and exposed to 1% O₂ survive at a high rate (Table 3.1). However, embryos laid on OP50 and exposed to 0.5% O₂ had a decrease in survival rate. The hypoxia chamber we use is preset to the noted oxygen level before the embryos are exposed to the hypoxic environment. Consequently, the embryos have a quick environmental change from normoxia to the severe hypoxic environment, likely resulting in a minimal transition time from normoxia to 0.5% O₂. In the past (Jiang et al., 2001; Padilla et al., 2002), methods used to induce hypoxia involved placing embryos into a chamber and then flowing gas through the chamber, likely resulting in a long transition time from normoxia to 0.5% O₂. We exposed embryos using a similar hypoxia gas flow chamber (0.5% O₂) and found that embryos survive at the previously established rates (Table 3.1). These results demonstrate that methodology used to induce a hypoxic environment affects survival rate. It is not known specifically why there are differences in viability but a variable such as the transition time from normoxia to hypoxia may be a factor in survival rate.
C. elegans Development in Chronic Hypoxia

To determine how prolonged exposure to hypoxia affects C. elegans developmental trajectory, wildtype embryos were collected by egg lay and exposed to chronic hypoxia (O₂ deprivation beginning in embryogenesis). These embryos were exposed to 1% and 0.5% O₂ at 23°C for 72 hours and developmental trajectory was analyzed at 12-hour increments. Results show hypoxia slows developmental progression and that an environment of 0.5% O₂ slows development more than a 1% O₂ environment (Figure 3.1). Wildtype embryos exposed for 72 hours in 0.5% O₂ develop to the L4 stage as determined by gonad morphology and distal tip location. After 120 hours in 0.5% O₂ wild-type embryos were reproductively viable adults (Figure 3.2). These results provide evidence C. elegans has mechanisms that allow proper development and reproduction in lower oxygen concentrations.

Chronic Hypoxia Reduces Fecundity and Increases Longevity

To further examine the lasting affects of chronic hypoxia on wildtype C. elegans we measured other determinants of animal fitness after exposure to hypoxia (1% O₂). One major determinant of fitness is the number of offspring that an animal produces. To determine if exposure to hypoxia during development has an affect on fecundity I quantified the number of offspring that reach adulthood from animals raised in hypoxia. I determined that C. elegans exposed to chronic (72 hours) and acute (24 hour exposure at late L4 stage) hypoxia have a reduced number of offspring that reach adulthood compared to animals raised in normoxia (Table 3.2). Hypoxia also induces sterility and increases the incidence of males in the offspring of animals raised in hypoxia (Table
It is possible that chronic hypoxia exposure during development leads to changes in lifespan after removal from hypoxia. To test this hypothesis we conducted life span assays on animals after exposure to hypoxia (1% O₂). We determined that lifespan was not reduced by hypoxia (Figure 3.3). We actually observed a slight increase in lifespan when animals are exposed to hypoxia (1% O₂ at 22°C) for 72 hours or 96 hours (Figure 3.3). These results show that hypoxia exposure has an effect on fitness in C. elegans.

**Genetic Mechanisms Regulating Responses to Chronic Hypoxia**

**HIF-1 Signaling**

A null mutation in hif-1, hif-1(ia04), causes embryos to be sensitive to hypoxia. Of the hypoxia exposed hif-1(ia04) embryos that hatch, the majority arrest development and few reach adulthood (Jiang et al., 2001; Padilla et al., 2002). It has not been determined how hif-1(ia04) L1 larvae respond in chronic (>3 days) hypoxia. To determine this I placed hif-1(ia04) L1 larvae into hypoxia (0.5% O₂) and determined that 100% of the hif-1(ia04) animals do not develop normally and never reach the L4 larval stage (N=132 animals from 3 independent trials).

EGL-9 is a prolyl-hydroxylase that regulates HIF-1 accumulation in normoxia. In the presence of sufficient O₂ levels HIF-1 is hydroxylated by EGL-9 leading to the degradation of HIF-1 by the VHL-1/Protease pathway (Epstein et al., 2001a). In hypoxic environments this modification of HIF-1 is decreased leading to an accumulation and thus increased activation of HIF-1. Therefore, in an egl-9 null mutant such as egl-9(sa307) HIF-1 levels are increased (Epstein et al., 2001a). In C. elegans the egl-9(sa307) mutant has been extensively studied in respect to the signaling pathway
involving *hif-1* (See Chapter 1 for more detail). However, the phenotype of *egl-9(sa307)* when exposed to chronic hypoxia has not been analyzed. We exposed *egl-9(sa307)* embryos to hypoxia and found, similar to wildtype embryos, that the *egl-9(sa307)* embryos were viable and capable of hatching (Table 3.3). These results demonstrate that unlike *hif-1*, *egl-9* is not required for embryos that have been laid on a plate to survive hypoxia exposure. Mutations in *vhl-1* also lead to an accumulation of HIF-1 (Epstein et al., 2001a). We determined that *vhl-1(ok161)* embryos exposed to 1% O$_2$ had a small, yet significantly different decrease in viability in comparison to wildtype embryos (Table 3.3).

Wildtype L1 larvae exposed to 0.5% O$_2$ for 168 hours develop at a slower rate than in 1% O$_2$ but are capable of reaching gravid adulthood (N=90 animals from three independent trials). The embryos from these hypoxia-exposed parents are viable and will hatch and develop. As expected *hif-1(RNAi)* L1 larvae exposed to hypoxia will arrest development and not reach adulthood. Similar to wildtype animals, *vhl-1(ok161)* L1 larvae exposed to 0.5% O$_2$ for 168 hours will develop into gravid adults (N=90 animals from three independent trials). However, *egl-9(sa307)* L1 larvae exposed to 0.5% O$_2$ for 168 hours arrest development (N=150 from 5 independent trials). It is known that in *egl-9* and *vhl-1* mutants HIF-1 accumulates (Epstein et al., 2001a). However, this accumulation of HIF-1 is not likely the reason why *egl-9(sa307)* animals arrest when exposed to hypoxia since *hif-1(RNAi)* does not suppress the hypoxia induced arrest observed in *egl-9(sa307)* larvae (N=90 animals from three independent trials). Furthermore, *vhl-1(ok161)* animals that have HIF-1 accumulation do not exhibit a
hypoxia induced larval arrest. These results show that EGL-9 may have additional functions besides HIF-1 regulation in hypoxia.

Wildtype embryos exposed to 1% $O_2$ for 96 hours will develop into gravid adults (Figure 3.4). The embryos produced by adults that have developed in hypoxia are viable as indicated by their hatching and progression through development (N=90 embryos, three independent trials). If $egl-9(sa307)$ embryos are exposed to 1% $O_2$ (instead of 0.5% $O_2$) development occurs, although at a slower rate than in wildtype animals (Figure 3.4). 100% of these animals become gravid adults (N=60 animals, three independent trials). However, the embryos within these adults appear abnormal. To determine if the embryos within the $egl-9(sa307)$ exposed to chronic hypoxia were viable, the adults were removed from hypoxia and allowed to lay these eggs in normoxia. The eggs laid were given 24 hours to hatch. I determined that unlike wildtype embryos that have a hatch rate of 100% (N=90 embryos, three independent trials), 100% of the $egl-9(sa307)$ embryos were not viable (N=378, four independent trials). Together these results indicate that $egl-9(sa307)$ embryos placed into hypoxia (1% $O_2$) develop and hatch (Table 3.3). However, embryos produced from a parent raised in hypoxia are not viable.

It is not clear whether development in chronic hypoxia (>8 days) causes abnormal germline development in $egl-9(sa307)$ animals that leads to lethality to $egl-9(sa307)$ embryos or if embryos being in utero causes embryo lethality. To determine if $egl-9(Sa307)$ embryos die in utero from acute (48 hours) hypoxia exposure, I placed adult $egl-9(sa307)$ animals into hypoxia (1% $O_2$) and quantified embryo viability after 48 hours of hypoxia. 100% of embryos in utero died and were not viable after 24 hours.
recovery in normoxia (N=21 animals, three independent trials). *egl-9(sa307)* animals are egg laying defective and in normoxia die when the animal stops laying eggs and the developing offspring hatch and eat through the adult, referred to as bagging out (Trent et al., 1983). The *egl-9(sa307)* adults placed in hypoxia for 48 hours survive the hypoxia exposure and maintain their egg load for another 5 days without bagging out. The embryos within the adults are late stage embryos. This suggests that embryo lethality of *egl-9(sa307)* embryos *in utero* is not dependent on hermaphrodite germline development in hypoxia.

*Mutations Causing Hypoxia Sensitivity Identified Through Targeted Screen*

Wildtype embryos grown on OP50 and exposed to 0.5% O₂ have a decreased survival rate in comparison to embryos exposed to 1% O₂ (Table 3.1). Therefore, 1% O₂ instead of 0.5% O₂ was used for the embryo viability assays of specific genetic mutants of interest.

TGF-β Signaling

Aerotaxis assays have shown that wildtype animals migrate to oxygen tensions between 5-12% O₂ and avoid O₂ levels above 12% (termed hyperoxia for *C. elegans*) (Gray et al., 2004). Wildtype animals do not avoid hyperoxia in the presence of bacterial food (Chang et al., 2006). The *egl-9(sa307)* mutant avoids hyperoxia in the presence or absence of food. This oxygen specific behavioral response is dependent on *hif-1* (Chang et al., 2006).
Hyperoxia avoidance is also regulated by the neuronal TGF-β homolog, DAF-7, which is a secreted mediator of crowding and stress responses. *daf-7* activity suppresses hyperoxia avoidance on food like *egl-9(sa307)* (Chang et al., 2006). To determine if genes required for hyperoxia avoidance are involved in hypoxia survival, I investigated if TGF-β ligands are required for embryogenesis and development in hypoxia. To understand the effect of loss of TGF-β ligand function on the survivability of animals exposed to hypoxia, I analyzed available mutants in four different *C. elegans* TGF-β ligands under hypoxic conditions. *daf-7(e1372)* mutant animals do not show an embryo viability phenotype (Table 3.3). Phenotypic characterization of the *daf-7(e1372)* mutant exposed to chronic hypoxia at 22°C is a challenge because the mutation causes a constitutive dauer phenotype at 22°C. Mutants for two other genes encoding TGF-β ligands, *tig-2(ok3416)* and *unc-129(ev544)*, do not show embryo viability phenotypes and develop like wildtype animals.

A fourth gene encoding a TGF-β ligand, *dbl-1*, shows a hypoxia phenotype. *dbl-1* encodes a member of the transforming growth factor beta (TGF-β) superfamily, which includes *Drosophila* decapentaplegic (Dpp) and the vertebrate bone morphogenetic proteins (BMPs). Analysis of the null mutant *dbl-1(wk70)* shows a 65.4 ± 1.1 % survival rate of embryos exposed to hypoxia (Table 3.3). It is not understood why this BMP would be required for embryogenesis in hypoxia but not required for normoxia.

NPR-1 is required for embryogenesis in *C. elegans*

The neuropeptide receptor NPR-1 influences the way *C. elegans* responds socially to different oxygen concentrations (12% to 21%). NPR-1 is a G protein-coupled
receptor related to mammalian neuropeptide Y receptors. \textit{npr-1(lf)} animals avoid high oxygen and aggregate in the presence of food; animals bearing \textit{npr-1} alleles with high activity do not aggregate (Chang et al., 2006). Natural polymorphisms between \textit{C. elegans} strains has led to a better understanding of NPR-1 signaling in response to higher oxygen concentrations (Cheung et al., 2005). A polymorphism exists in the \textit{npr-1} gene causes a difference in a single amino acid, NPR-1(215) (de Bono et al., 1998). The 215V allele, found in the laboratory Bristol strain (N2), has high NPR-1 activity and the 215F allele, found in the Hawaiian isolate (CB4856), has low NPR-1 activity. These results with \textit{npr-1} mutants suggest NPR-1 signals different behavior in response to higher oxygen concentrations while factoring in presence of food. It has not been determined if NPR-1 is required for survival and development in hypoxia.

To determine if low levels of NPR-1 have an effect on development I exposed \textit{npr-1(ad609,lf)} and CB4856 embryos to hypoxia (1% O$_2$). These embryos survive at 65.4 ± 1.1 % and 62.7 ± 16.1% respectively (Table 3.3). These data suggest that high levels of NPR-1 are required for embryogenesis in hypoxia. It is not understood why loss of function in \textit{npr-1} can lead to embryo lethality in animals exposed to hypoxia.

**Conclusion**

In this study I have established new methods to understand how oxygen deprivation affects development in \textit{C. elegans}. These experiments determined that methodology used when subjecting \textit{C. elegans} to low oxygen has an effect on survival capabilities of wildtype animals. Analysis of developmental progression in hypoxic conditions shows that developmental trajectory in \textit{C. elegans} is affected by oxygen
deprivation in a concentration dependent manner. For example, animals at 0.5% O₂ develop slower than animals at 1% O₂. This suggests that *C. elegans* can be used as a model for studying development under hypoxic conditions.

My data also suggest that development in hypoxia has a lasting affect on the animals' fitness even after being removed from the stress. Exposure to hypoxia during development reduces fecundity and increases lifespan. Others have shown using mutants and laser ablation experiments that reduced fecundity can also increase longevity in *C. elegans* (Hsin and Kenyon 1999). It is of interest to test the relationship between reduced fecundity and increased longevity induced by hypoxia.

It is not clear how environmental oxygen deprivation could induce sterility. *C. elegans* produce males at a low frequency through meiotic nondisjunction of the X chromosome (Hodgkin et al., 1979). More work will be required to determine if the increase in males from hypoxia exposed animals is the result of an environmentally induced meiotic nondisjunction or another form of DNA damage that occurs to the animals either in hypoxia and/or after hypoxia exposure has ended.

Through a targeted screen of mutants based on normoxic (7-21% O₂) oxygen sensing phenotypes, I have determined two genes with previously unknown phenotypes in hypoxia, *npr-1* and *dbl-1*, to be hypoxia sensitive. Furthermore, I have characterized new functional relevance for *egl-9* during development in hypoxia in *C. elegans*. In another invertebrate model organism, *Drosophila*, loss of the *egl-9* homolog, Fatiga, is lethal in normoxia (Lavista-Llanos et al., 2002). It is interesting that mutations in the *C. elegans* homolog for *egl-9* are not embryo lethal under normal lab conditions but does affect development and offspring viability in animals raised in hypoxia. This suggests an
unknown function for *egl-9* in development, since it should not be required for degrading HIF-1 in hypoxic conditions. If this developmental defect were due to misregulation of HIF-1 in hypoxia I would expect a similar phenotype in *vhl-1(lf)* animals. *vhl-1(lf)* mutants also have high HIF-1 levels, since it works with EGL-9 in degrading HIF-1 during normoxia (Epstein et al., 2001b). A *vhl-1* null mutant develops to a reproductively viable adult suggesting EGL-9 has a role in severe hypoxic development independent of HIF-1 regulation in *C. elegans*. This phenotype is not understood and will need to be further characterized.

Others have shown that wildtype *C. elegans* embryos exposed directly to 0.1% O₂ die but embryos *in utero* while exposed to 0.1% O₂ are able to survive by entering suspended animation (Miller and Roth 2009). This is similar to how embryos survive anoxia by entering into suspended animation. One marker of anoxia-induced suspended animation is the requirement for SAN-1, a spindle checkpoint protein (Hajeri et al., 2005). The ability of wildtype animals *in utero* to survive severe hypoxia requires SAN-1, a component of the spindle checkpoint that is required for anoxia-induced suspended animation, but not HIF-1 (Miller et al., 2009; Nystul et al., 2003). Therefore, wildtype animals protect their offspring *in utero* from lethal hypoxic conditions. If *egl-9(sa307)* animals were missing the ability for *in utero* protection then animals exposed to hypoxia might create a lethal environment *in utero*. This could explain the F1 abnormal phenotype seen in *egl-9(sa307)* animals. *egl-9(sa307)* mutants survive embryogenesis when exposed to hypoxia outside of the hermaphrodite. Animals *in utero* die and do not survive hypoxia at 1% O₂. While *in utero* embryos might be exposed to lower oxygen concentrations than embryos outside of the adult, my data
suggest that misregulation of the cell cycle is not completely responsible for the egl-9 mutant phenotype in hypoxia. If misregulation of cell cycle control is the reason for embryo death in egl-9(sa307) embryos in utero, I would have expected only early stage embryos and chromosome abnormalities as seen in cell cycle mutants (Hajeri et al., 2008). Since other phenotypes including late stage embryos with abnormal tissue differentiation are visible, further characterization of the dead embryos will be needed to determine the cause of the F1 abnormal phenotype of these animals. I hypothesize that novel genes are required for development and reproductive fitness in hypoxia. Further genetic screens and further characterization of mutants will be required to understand how C. elegans responds to hypoxia.

Materials and Methods

Strains and Growth Conditions

The C. elegans wildtype N2 Bristol strain was raised on NGM plates seeded with E. coli (OP50) as previously described (Sulston, 1988). For all hypoxia experiments the nematodes were maintained at 22-23°C as described. For all other experiments except those on ts mutants nematodes were maintained at 22°C. The following strains were obtained from Caenorhabditis Genetics Center and raised under similar conditions as the wildtype strain: ZG31 hif-1(ia04), CB5602 vhl-1(ok161), JT307 egl-9(sa307), CB4856, DA609 npr-1(ad609), CX6448 gcy-35(ok769), AX1297 gcy-36(db66), LT121 dbl-1(wk70), RB2476 tig-2(ok3416), NW987 unc-129(ev544), CB1372 daf-7(e1372).
The Gonad as a Development Marker

Although several tissue structures of the worm contain cells that divide after hatching, most divisions are distinguished by counting cells or nuclei and require compound microscopy to distinguish morphological age. Most tissues are fully developed by the second or third molt and are not useful as developmental markers beyond those stages. One tissue with characteristics that are visible and which continue to develop from L1 to adult is the gonad. The germline is visible with a dissecting scope and has several characteristics that make it useful as a marker for developmental progression. First, it is easily identified upon hatching within the animal. Second, as this structure continues to develop it increases in size and changes morphologically. Third, gonad development correlates with several molts allowing for timing of development. These features make it ideal for measuring developmental trajectory.

Hypoxia Assay

Hypoxia assays were conducted in an Invivo2-200 hypoxia workstation (Ruskinn) or an oxygen gas flowthrough chamber as previously described (Padilla et al. 2002). Assays were conducted by initial egg lay and exposing synchronized embryos to hypoxia. Briefly, five 1-day old adults were allowed to lay eggs on a plate for 1 hour and then removed. Embryos were then placed into the chamber. Monitoring development was accomplished by using multiple replicates of plates placed into hypoxia at the same time and removing sample populations at mentioned time points. Animals assayed were removed, anaesthetized (0.5% tricaine, 0.05% tetramisole in M9 buffer) and mounted on 2% agarose pads placed on cover slips (Warner instruments, USA) and imaged within 5
minutes of hypoxia using a Zeiss Axioscope fluorescence microscope; image acquisition was done using Simple PCI (Hamamatsu) and processed using Image J software (NIH) and Adobe Photoshop (Hajeri et al., 2005).

**Embryo Viability Assay**

Embryo viability assays were conducted by exposing embryos to hypoxia (0.5% or 1.0% O₂) for 36 hours. Embryos were collected by egg lay and exposed to hypoxia. Five 1-day old adults were allowed to lay eggs on a plate for 1 hour and then removed. Embryos laid were counted then placed immediately into the hypoxia chamber (0.5% or 1.0% O₂). Following hypoxia exposure (36 hours) embryo viability was quantified after removal from hypoxia. Animals were removed from hypoxia and counted as hatched or dead eggs after 24-hour in normoxia.

**Fecundity Assay**

Fecundity assays were conducted on animals exposed to acute (24 hours starting as an L4) or chronic (72 hours hypoxia from embryo egg lay) hypoxia. For acute exposure, five 1-day old adults were allowed to lay eggs on a plate for 1 hour and then removed. Embryos were allowed to develop to an L4 larvae in normoxia (21% O₂, 22°C) then placed on individual plates, one worm per plate and exposed to 24 hours of hypoxia (1% O₂, 22°C). After 24 hours of hypoxia plates were removed from hypoxia and all progeny counted for each individual worm. All offspring were counted after reaching adulthood. Hypoxia exposed animals were considered done producing offspring after two days passed without living offspring on the plate. For chronic hypoxia
exposure, five 1-day old adults were allowed to lay eggs on a plate for 1 hour and then removed. Embryos were placed into hypoxia (1% O₂, 22°C) to develop for 72 hours. These animals were removed from hypoxia and progeny counted as described above.

**Longevity Assay**

Adult animals were allowed to lay eggs on a plate prior to removal from the plate. The plates containing embryos were placed either in a normoxia (22°C) or hypoxia (1% O₂, 22°C) and allowed to develop for 3 or 4 days prior to lifespan analysis. Animals were transferred daily to fresh plates and live adult animals that responded to touch with the pick were counted. Lifespan analysis was conducted at the same temperature in which the hypoxia chamber is maintained (22°C). Censoring in the lifespan analysis included animals that crawled off the plate.

**Table 3.1. Survival rate of wildtype embryos exposed to hypoxia**

<table>
<thead>
<tr>
<th>E. coli Strain</th>
<th>Normoxia Control</th>
<th>0.5% O₂, hypoxia box chamber</th>
<th>0.5% O₂, hypoxia flow chamber</th>
<th>1% O₂, hypoxia box chamber</th>
</tr>
</thead>
<tbody>
<tr>
<td>OP50</td>
<td>100 ± 0.0</td>
<td>51.5 ± 2.9*</td>
<td>87.1 ± 7.7*</td>
<td>95.7 ± 1.5</td>
</tr>
<tr>
<td>HT115</td>
<td>100 ± 0.0</td>
<td>99.6 ± 0.6</td>
<td>97.6 ± 0.5</td>
<td>99.2 ± 0.8</td>
</tr>
</tbody>
</table>

For above experiments temperature was 22°C. Data presented is average survival rate ± SD, from four independent experiments with a total of at least 500 animals. *P<0.05 compared to normoxia controls, Student’s t-test.
Figure 3.1. Developmental trajectory is influenced by oxygen concentration. Wild type embryos are exposed to hypoxia from egg to adult at 23°C. Multiple plates of ~25 animals are placed into hypoxia and each image at the indicated time point is a representative animal from one individual plate imaged within 30 minutes post hypoxia. Gonad development is used as a marker for developmental progression. Arrows represent location of distal tip and V denotes vulva. Scale bar, 50 µm.

Figure 3.2. Animals exposed to 0.5% oxygen reach adulthood within 120 hours. Gonad development is used as a marker for developmental progression. At 122 hours oocytes have begun maturation and by 140 hours several eggs are visible in the uterus. Scale bar, 50 µm.
Figure 3.3. Chronic hypoxia exposure during development leads to a small increase in lifespan. Survival curve for wildtype animals that developed, from embryos to adulthood, in either normoxic or hypoxic environments (22°C) is shown. Animals were moved daily and kept at 22°C so as to not experience a change in temperature after hypoxia treatment. Adults were moved daily to fresh NGM plates and live adult animals were counted. This curve contains data from two experiments with a total of >70 animals. A replicate experiment was also conducted.

Table 3.2 Fecundity of wildtype animals exposed to hypoxia

<table>
<thead>
<tr>
<th>Environment</th>
<th>Sterile (&lt;5 progeny)</th>
<th>Offspring that reach adulthood ± SD</th>
<th>Male offspring (%) (n)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>0</td>
<td>333.0 ± 50.6</td>
<td>0.01 % (1 of 22)</td>
<td>22</td>
</tr>
<tr>
<td>24 hrs Hypoxia&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td>299.1 ± 63.2 *</td>
<td>0.12 % (5 of 36)</td>
<td>36</td>
</tr>
<tr>
<td>72 hrs Hypoxia&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6</td>
<td>194.4 ± 91.4 **</td>
<td>0.33 % (8 of 35)</td>
<td>35</td>
</tr>
</tbody>
</table>

Experiments conducted at 22°C.
N = total number of animals, n = number of animals that showed phenotype
<sup>a</sup> Animals exposed to 24 hours of hypoxia from L4 to adulthood
<sup>b</sup> Animals exposed to 72 hours of hypoxia from embryos to adulthood
* P < 0.05, **P < 0.001 compared to normoxic controls, Student’s t-test
Table 3.3. Embryo survival rate for genetic mutants exposed to hypoxia

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Normoxia (N)</th>
<th>1% O₂ (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wildtype</td>
<td>100 ± 0.0 (201)</td>
<td>97.9 ± 0.8 (441)</td>
</tr>
<tr>
<td>hif-1(ia04)</td>
<td>100 ± 0.0 (172)</td>
<td>6.2 ± 3.2 (568) *</td>
</tr>
<tr>
<td>egl-9(sa307)</td>
<td>100 ± 0.0 (112)</td>
<td>100.0 ± 0.0 (211)</td>
</tr>
<tr>
<td>vhl-1(ok161)</td>
<td>100 ± 0.0 (135)</td>
<td>87.1 ± 4.8 (283) *</td>
</tr>
<tr>
<td>CB4856 Hawaii</td>
<td>100 ± 0.0 (244)</td>
<td>62.7 ± 16.1 (587) *</td>
</tr>
<tr>
<td>npr-1(ad609)</td>
<td>100 ± 0.0 (124)</td>
<td>65.4 ± 1.1 (261) *</td>
</tr>
<tr>
<td>gcy-35(ok769)</td>
<td>100 ± 0.0 (136)</td>
<td>98.4 ± 1.5 (250)</td>
</tr>
<tr>
<td>gcy-36(db66)</td>
<td>100 ± 0.0 (166)</td>
<td>99.7 ± 0.5 (325)</td>
</tr>
<tr>
<td>dbl-1(wk70)</td>
<td>100 ± 0.0 (256)</td>
<td>59.4 ± 9.0 (400) *</td>
</tr>
<tr>
<td>tig-2(ok3416)</td>
<td>100 ± 0.0 (292)</td>
<td>99.3 ± 0.7 (464)</td>
</tr>
<tr>
<td>unc-129(ev544)</td>
<td>100 ± 0.0 (283)</td>
<td>99.1 ± 1.1 (428)</td>
</tr>
<tr>
<td>daf-7(e1372)</td>
<td>100 ± 0.0 (132)</td>
<td>96.7 ± 2.9 (155)</td>
</tr>
</tbody>
</table>

Numbers represent the average animals that hatch ± SD
N = number of embryos quantified over 4 independent experiments
*P<0.05 compared to wildtype hypoxia controls, Student’s t-test
Figure 3.4. EGL-9 is required for producing viable offspring in hypoxia. Representative images from three independent experiments with over 90 animals per experimental condition shows arrested egl-9(sa307) larvae at 0.5% O₂ and surviving egl-9(sa307) animals with dead eggs from 8 days of hypoxia 1.0% O₂ at 22°C. egl-9(sa307) mutants take twice as long to develop in hypoxia (1.0% O₂ at 22°C). After 8 days of exposure the adult animals contain dead eggs and 100% of laid eggs are dead (n=378 from 3 independent trials). Scale bar, 20 µm.
CHARACTERIZATION OF BEHAVIOR IN CHRONIC HYPOXIA

Introduction

Studying behavior in model organisms help scientists understand the genetic mechanisms that modulate complex behaviors. One such behavior in *C. elegans* is thermotaxis. Thermotaxis is a behavioral response to temperature in which wildtype animals change their preferred temperature on a thermal gradient. After exposure to food at a uniform temperature animals will migrate to the cultivation temperature when placed on a temperature gradient without food (Hedgecock et al., 1975; Mohri et al., 2005). By isolating mutants (tax mutants) defective for this behavior and using laser ablation experiments of specific neurons researchers were able to determine specific neurons that are required for thermotaxis (Hedgecock et al., 1975; Mori et al., 1995). Through studying mutant behavior several lab groups have shown that animals are able to sense temperature, remember and associate that sense with food, and adjust behavior when different environments are encountered. This is just one example of how behavior has been studied in the lab to identify genetic mechanisms.

*C. elegans* has additional behaviors influenced by environment. For example, animals adjust locomotion and feeding behavior when they encounter food. When N2 adult animals encounter bacteria they reduce locomotion and are solitary feeders under normal lab conditions (de Bono and Bargmann 1998). If animals are removed from food they begin roaming, which is characterized by high speed and low turning rates (Fujiwara et al., 2002). As mentioned previously, the cGMP pathway mediated by *gcy-35* is involved in aggregation and bordering behavior on bacteria (Coates et al., 2002). Genetic mutation of *gcy-35* in an *npr-1* mutant suppresses aggregation and bordering
seen in \textit{npr-1} mutants and GCY-35 is also required for avoidance of hyperoxia (>12% \textit{O}_2.) (Gray et al., 2004). As GCY-35 is an oxygen sensor, it was hypothesized that these behaviors were associated with a lower \textit{O}_2 microenvironment created by the bacterial lawn. Reducing oxygen levels to 7\% \textit{O}_2 is sufficient to suppress aggregation and bordering in \textit{npr-1(ad609)} animals and supports this hypothesis (Gray et al., 2004). How other behaviors such as egg laying are influenced by oxygen deprivation is uncharacterized.

The main objective of this research study is to characterize the effect oxygen deprivation has on \textit{C. elegans} behavior. The general hypothesis of this research is that at standard lab temperatures (20-22\(^\circ\)C) \textit{C. elegans} will exhibit behaviors under hypoxic conditions (0.5\%-1\%) that are different than animals in normoxia. To test this hypothesis I conducted assays of egg laying behaviors of adult animals exposed to chronic hypoxia (1\% \textit{O}_2 from embryo to adult) in a hypoxia glove box. I focused my study on location of eggs laid relative to the bacterial lawn from animals, while they were exposed to hypoxia. I then took a targeted approach to identify genetic mutations that might have an effect on egg laying behavior in hypoxia. This approach includes known mutants in oxygen sensing, for example mutations in the globin gene family, and mutants in aerotaxis, such as guanylyl cyclases. Overall, this study laid the foundation for understanding how specific behaviors found in animals exposed to hypoxia might be used to discover unknown mechanisms for hypoxia tolerance in \textit{C. elegans}.
Results

*Characterization of C. elegans Behavioral Response to Chronic Hypoxia*

General Hypoxic Egg Laying Behavior

In normal laboratory conditions, wildtype adult hermaphrodite animals are found randomly on the bacterial lawn on which they feed. They also lay their eggs on the bacterial lawn and rarely lay their eggs off food unless the animals are crowded or food source is diminishing (Figure 4.1). Wildtype animals analyzed after chronic hypoxia exposure (1% O₂ beginning in embryogenesis) are found randomly on the agar of the petri dish and are typically not found on the bacterial lawn they use as a food source. Animals raised in hypoxia (0.5% or 1% O₂) from embryo to adulthood lay the majority of their eggs off the bacterial lawn regardless of bacterial strain used for food (OP50 or HT115) (Figure 4.2). I refer to the shift from laying eggs on the bacterial lawn to off the bacterial lawn during exposure to hypoxia as hypoxia-induced egg laying behavior.

I then asked if exposure to hypoxia during development is required for hypoxia-induced egg laying behavior in wildtype animals. To determine if the phenotype of laying eggs off the *E. coli* lawn during exposure to hypoxia is dependent on the animal being exposed to hypoxia during a specific window of development I began chronic hypoxia exposure at various stages of development (embryo, L1 or L4 larvae, and 1 day-old adult). Animals were placed into 1% O₂ at the specified developmental stage, raised to adulthood and the location of the eggs laid relative to *E. coli* lawn was quantified. I determined that, regardless of what developmental stage the animals were first exposed to hypoxia, as gravid adults wildtype animals lay the majority of eggs off the *E. coli* lawn (Figure 4.3). L4 animals placed into hypoxia and raised to adulthood lay slightly, yet
statistically significant, more eggs on the bacterial lawn than embryos raised to adulthood. (P<0.05, Student’s t-test) This result suggests that the hypoxia-induced behavior of laying eggs off the *E. coli* lawn is not due to a neurological change induced by hypoxia exposure during development.

Whether egg-laying behavior of animals exposed to hypoxia is signaled by reduced oxygen concentration or presence of an altered bacterial lawn due to hypoxia is unclear. To determine if live OP50 is affecting egg-laying behavior I used heat-killed OP50 bacteria as a food source during hypoxia exposure. I placed 1-day-old adult animals into hypoxia (1% O₂) for 24 hours on heat-killed OP50 and found that animals will lay statistically more eggs on the heat-killed bacteria lawn than if the OP50 was not heat-killed (P<0.01 Student’s t-test, N=9 plates from three independent trials). This result suggests live OP50 has an affect on hypoxia-induced egg laying behavior.

It is still not clear if *C. elegans* exposed to hypoxia can sense live OP50, and whether live OP50 is a deterrent to egg laying behavior, or if the animal is foraging and searching for a more oxygen rich environment. To address if the hypoxia-exposed animals could sense live bacteria while in hypoxia (and thus lay their eggs away from the bacterial lawn) we assayed where animals lay their eggs on a plate that contains OP50 that is alive and heat-killed (Figure 4.4). We put heat-killed OP50 on one side of the plate and live OP50 on the opposite and allowed wildtype 1-day-old adults to lay eggs for 24 hours in hypoxia. In normoxic conditions *C. elegans* will lay the majority of their eggs on OP50 that is alive as opposed to OP50 that has been heat killed. The majority of hypoxia-exposed animals continue to lay their eggs on the agar (off the bacterial lawn) yet on the region of the plate that has live OP50 and not the region of the
plate that has heat-killed OP50. This indicates that the hypoxia-exposed animals can sense the OP50 lawns and are likely attracted to the live OP50 lawn. If animals lay eggs off the bacterial lawn because of dispersion or foraging, eggs would be located on the agar on the side of the plate with heat-killed OP50. Yet these results suggest that animals prefer live OP50 to heat-killed OP50 and that the environment close to the live OP50 bacterial lawn supports egg-laying behavior.

Oxygen Deprivation Avoidance

While hif-1 function is required to survive embryogenesis and development in hypoxia (discussed previously in Chapter 3), it is not required for 1-day-old adults to survive in hypoxia for 24 hours (N=60 animals, three independent experiments) (1% O₂). I wanted to know if hif-1 is required for hypoxia-induced egg laying behavior. I exposed 1 day old adult hif-1(ia04) mutants to hypoxia and found that hif-1(ia04) adults lay 100% of eggs off food (N=77 embryos from three independent experiments). vhl-1(ok161) animals exposed to hypoxia from embryo to adult lay eggs on food more often than wildtype animals (Figure 4.5). Since vhl-1(ok161) animals have been shown to have a high level of HIF-1, it cannot be ruled out that HIF-1 regulation is not required for this specific behavior in hypoxia.

C. elegans wildtype animals avoid low and high (<2% and >12%) levels of oxygen and prefer oxygen tensions of 5-12% (Gray et al., 2004). gcy-35, a gene encoding a soluble guanylate cyclase (sGC) in specific sensory neurons, is required for the avoidance of high levels of oxygen >12% (Gray et al., 2004). I hypothesized that gcy-35 could potentially mediate egg laying behavior in hypoxia. Using the experimental
protocol for chronic hypoxia at 1% O<sub>2</sub> (from embryo to adult), I placed animals with a gcy-35 null mutation, gcy-35(ok769), into hypoxia and quantified number of eggs laid on the agar versus bacterial lawn. Similar to N2 animals, the gcy-35(ok769) embryos reach reproductively viable adults when exposed to chronic hypoxia (N=90 animals, three independent trials). Furthermore, the gcy-35(ok769) mutation did not suppress hypoxia induced egg laying behavior (Figure 4.5).

Another gene known to affect how C. elegans responds behaviorally to changes in oxygen is npr-1. Wildtype animal are solitary feeders, animals are not found in groups on the bacterial lawn used for food. Animals with low expression or loss of function of npr-1 exhibit social feeding (aggregation) and bordering at the edge of the bacterial lawn (de Bono and Bargmann 1998). The behaviors of npr-1 mutants are observed in a normoxic environment of 21% O<sub>2</sub>. The npr-1(ad609) animals exposed to 1% O<sub>2</sub> do not aggregate but resume aggregation when returned to normoxia (Cheung et al., 2005). These experiments were done with a gas flow chamber containing 1% O<sub>2</sub> mixed with 99% nitrogen. I confirmed, using the hypoxia glove box, that hypoxia (1% O<sub>2</sub>, 22°C) suppresses aggregation in mutant npr-1(ad609) animals (N=39 aggregates counted, three independent trials). I quantified aggregation as greater than 10 animals found together on the plate. Although mutant npr-1 animals exhibit some oxygen sensitive behaviors, they have wildtype egg laying behavior when exposed to chronic hypoxia (Figure 4.5).

Genes Required for Hypoxic Egg Laying Behavior

Variations in several genes affect C. elegans behavior in response to oxygen
deprivation. One of these genes encodes a globin, GLB-5, that interacts with NPR-1 and cGMP to signal changes in locomotory activity in response to changes in oxygen concentration (Persson et al., 2009b). The C. elegans genome has a globin gene family composed of 33 putative members with large variations in globin protein structure beyond the globin motif (Hoogewijs et al., 2007). The function of the globin-like genes is unknown beyond the initial characterization of glb-5. To determine if members of the globin-like gene family have a role in egg laying behavior, I used RNA interference to screen through the globin genes and assay hypoxic egg laying behavior in hypoxia. Results from the RNAi screen of globin-like genes show egg-laying behavior can be suppressed (Figure 4.6). RNAi of glb-2, glb-13, glb-16 and glb-29 produced a statistically different increase in eggs found on food after exposure to hypoxia (P<0.01 Student’s t-test N=12 plates per genotype).

To further address if hypoxia-induced egg laying behavior can be genetically suppressed, I screened through putative globin genes with loss of function mutations. The globin mutants, glb-1(ok2747), glb-33(th3656), glb-6(tm3795) and glb-13(tm2825), are putative null mutants with deletions of 2.3kb, 308bp, 638bp, and 305bp respectively. These mutants were isolated through mutagenesis screens by the National Bioresource Project and have uncharacterized function (Gengyo-Ando et al., 2000). I exposed the globin mutants to chronic hypoxia (1% O₂ from embryogenesis to adulthood) and analyzed the adult egg laying behavior. glb-13(tm2825), which suppresses hypoxia egg laying behavior (via RNAi experiments) showed the strongest suppression of egg laying behavior (Figure 4.6). To determine if deleting multiple globin genes can produce an additive affect I mated two globin mutants to produce the double mutant glb-
13(tm2825);glb-1(ok2747). The double mutant has a slight increase in suppression, but is not statistically different from the single glb-13(tm2825) mutant (Figure 4.7).

If a mutation causes suppression of the hypoxia-induced egg laying phenotype then animals will lay eggs uniformly across the agar surface regardless of food area. I quantified the bacterial lawn area for the petri plates used in hypoxia egg laying experiments and found that approximately 25% of the plate area is covered by bacterial (N=6 plates). I hypothesize that the number of eggs laid on the bacterial surface in hypoxia will depend on the surface area of the bacterial lawn. To determine if food area affects the quantity of eggs laid on food in hypoxia, I increased the food area and quantified egg-laying behavior in hypoxia. Wildtype animals will lay the majority of eggs off the bacterial lawn regardless of bacteria surface area (Figure 4.8). I then tested the glb-13(tm2825) mutant for egg laying behavior in hypoxia and found that increasing food area does have an effect on the number of eggs laid on food (Figure 4.8). Therefore when hypoxia induced egg-laying behavior is suppressed by mutation the percentage of eggs off or on food is determined by the surface area of the bacterial lawn. This suggests that the hypoxia egg laying behavior is regulated in part by the globin-like gene glb-13.

Stochastic Variation in Hypoxic Egg Laying Behavior

Locomotion and movement behavior are affected by hypoxia so I asked how uncoordinated movement might affect egg laying in hypoxia (Cheung et al., 2005). To test this I determined if mutants with uncoordinated reverse movement have altered egg-laying behavior in hypoxia. Wildtype animals in hypoxia lay eggs with a stochastic
distribution from the bacterial lawn border to the edge of the plate with a slight increase in eggs at the edge of the plate (Figure 4.9). I divided the plate into three zones: food area, inner zone (from food boarder to 4mm from food), and outer zone (from edge of inner zone to edge of plate), and quantified the location of eggs laid in hypoxia from specific uncoordinated mutants. Two mutants with established mutations that cause uncoordinated reverse movement are pag-3 and unc-129 (Colavita et al., 1998; Jia et al., 1996). pag-3(n3098), pag-3(Is20) and unc-129(ev544) null mutants all cause a shift in egg laying towards the inner zone of the bacterial lawn (Figure 4.9, Figure 4.10). The mean percentages of eggs laid by C. elegans on the 4 mm food border (black bar) were found to be highly significantly different (One-way parametric ANOVA, p < 0.0001). A Dunnett’s Multiple Range Test (α=0.05) showed that the mean percentages of eggs laid by the genotypes pag-3(n3098), pag-3(Is20), and unc-129(ev544) were significantly different from N2, while the genotype unc-130(oy107) was not significantly different from N2. It is unclear how uncoordinated reverse movement might be correlated with the laying of eggs off the bacterial lawn in hypoxia.

I previously found that a null mutation in thedbl-1 gene, encoding a TGF-β ligand, DBL-1, induces an embryo lethal phenotype in 1% O₂. (See Chapter 3, Table 3.3) Since unc-129 also encodes a TGF-β ligand I wanted to determine if the suppression of stochastic variation seen in unc-129(ev544) animals is because of the reverse uncoordinated phenotype. I chose to assay the unc-130(oy170) mutant, which has ectopic expression of the wildtype UNC-129 TGF-β ligand (Nash et al. 2000). This mutant overexpresses UNC-129 and does not have a reverse uncoordinated movement in normoxic conditions. The unc-130(oy107) mutant does not suppress stochastic
variation of hypoxia-induced egg laying as seen in reverse uncoordinated mutants (Figure 4.10). Since this mutant has forward and reverse uncoordinated movement this suggests that animals being uncoordinated alone is not sufficient for changes in stochastic egg laying.

Conclusion

In this study, I characterized a behavior exhibited by C. elegans exposed to hypoxia. These experiments show that egg-laying behavior is altered when wildtype animals are in a hypoxic environment. Analysis of egg location relative to the bacterial lawn that C. elegans uses for food can be used to understand genes regulating the behavior involved in oxygen sensing and foraging. For example, animals at 1% O₂ laid the majority of embryos off the bacterial lawn in a stochastic manner dependent on genotype and coordinated movement. This study suggests that C. elegans can be used as a model for studying novel behaviors under hypoxic conditions.

Through a targeted screen of mutants based on normoxic (7-21% O₂) oxygen sensing phenotypes, I have described globin mutants, specifically glb-13(tm2825), with wildtype normoxic phenotypes yet with a hypoxia specific behavior. This is confirmed with RNAi experiments and a putative null mutation. Furthermore, I provide data that genes involved in regulating oxygen-sensing behaviors at oxygen concentrations between 7-21% O₂ (gcy-35 and npr-1) are not involved in egg laying behavior in hypoxia.

To determine how movement affects egg-laying behavior in hypoxia, I analyzed mutants with uncoordinated phenotypes. Reverse uncoordinated movement reduces
stochastic variation of egg location off the bacterial lawn. If all uncoordinated mutants displayed this phenotype then uncoordinated mutants should have also shared the same phenotype as the reverse uncoordinated mutants pag-3(n308) or unc-129(ev544). While uncoordinated is uncoordinated, this mutant does not exhibit uncoordinated movement similar to pag-3(n308) or unc-129(ev544). So variation in egg laying may be specific to different types of movement or the underlying neuronal defects that cause the movement phenotypes.

Whether hypoxia induced egg-laying behavior is a byproduct of a foraging strategy to escape hypoxia or an actual change in egg laying behavior remains unanswered. I hypothesize that additional genes are required for sensing and responding to hypoxia that are not associated with oxygen sensing at higher oxygen concentrations. Furthermore, if C. elegans altered egg-laying behavior in hypoxia is beneficial to developing embryos or adult reproduction is unclear. Further genetic screens and further characterization of mutants will be required to understand how C. elegans responds to hypoxia.

Materials and Methods

Strains and Growth Conditions:

The C. elegans wildtype N2 Bristol strain was raised on NGM plates seeded with E. coli (OP50) as previously described (Sulston, 1988). OP50 is grown according to standard laboratory procedures for living food source. Heat Killed OP50 is prepared from stationary phase OP50 heat killed by a 30 minute exposure to 80°F. For all hypoxia experiments the nematodes were maintained at 22-23°C as described. For all
other experiments except ts mutants nematodes were maintained at 22°C. The following strains were obtained from the Caenorhabditis Genetics Center and raised under similar conditions as the wildtype strain: ZG31 hif-1(ia04), CB5602 vhl-1(ok161), JT307 egl-9(sa307), CB4856, DA609 npr-1(ad609), CX6448 gcy-35(ok769), AX1297 gcy-36(db66), LT121 dbl-1(wk70), RB2476 tig-2(ok3416), NW987 unc-129(ev544), CB1372 daf-7(e1372). The Caenorhabditis elegans Gene Knockout Consortium (Oklahoma Medical Research Foundation) produced the glb-1(ok2747) allele (strain RB2081). The alleles, glb-33(th3656), glb-6(tm3795) and glb-13(tm2825,) were produced by the National Bioresource Project for the nematode (Tokyo Women’s Medical University School of Medicine). These four strains have been backcrossed three times and their genotypes confirmed using PCR.

**Hypoxia Egg Lay Assay**

To characterize an egg-laying phenotype during exposure to hypoxia I quantified the location of eggs laid in hypoxia compared to location of eggs laid in normoxia. I quantified the embryos found off the *E. coli* lawn for animals exposed to chronic hypoxia exposure at various stages of development (embryos, L1 or L4 larvae, adult). Animals were placed into 1% O₂ at the specified developmental stage and reached adulthood (as determined by development assays previously described). Animals were given 24 hours to lay eggs in hypoxia after the approximate time the animal reaches adulthood in hypoxia. The plates were then removed from hypoxia and the location of eggs laid relative to the *E. coli* lawn was quantified. The assay occurred in normoxia immediately after hypoxia exposure for all developmental stages. For measurement of stochastic
variation in egg-laying behavior, plates were partitioned into three locations, on the food, inner zone (from the food lawn border out 4mm) and outer zone (from 4mm of lawn boarder to the edge of the plate). Plates with animals were placed into hypoxia as described above and after removal, partitioned, and then location of eggs in the three locations was quantified.

*RNAi Assays*

A synchronous population of N2 larvae is grown to adulthood on RNAi plates (NGM supplemented with 200 µg/ml ampicillin, 12.5 µg/ml tetracycline and 2 mM IPTG). The RNAi plates were seeded with the *E. coli* strain for RNAi of a specified gene of interest. The *E. coli* strains were developed by the J. Ahringer laboratory and obtained from the Medical Research Council Geneservice (Cambridge, UK) (Kamath et al., 2003). As a control, worms were fed the *E. coli* HT115 strain containing the plasmid L4440 without insert. Adult RNAi treated animals, which did not show a phenotype under normoxic conditions, were exposed to hypoxia as outlined previously. Five globin RNAi food strains not included in the Ahringer Library, *glb*-7, 14, 26, 28 and 32, were obtained from Open Biosystems RNAi library.

*Wildtype Hermaphrodites Exposed to Hypoxia on NGM Plates Containing Both Live and Heat Killed Bacteria*

Approximately 10 wildtype L4 larvae were placed on an NGM plate which was divided into four quadrants, two containing live OP50 (150µl) and two containing heat killed OP50 (150 µl). The efficiency of heat killing the bacteria was determined by plating of the bacteria onto LB plates and checking for no growth. The animals were
placed in the middle of the plate and exposed to one day of hypoxia (1% O₂, 22°C) or normoxia (22°C). Adult animals were than removed from the plate and the location of the embryos on the plate was assayed. Embryos were classified as being located on either the live OP50 lawn, on the heat-killed (HK) OP50 lawn, on the agar near the live OP50 or the agar near the heat-killed OP50. Data are from four experiments with a total of >500 embryos assayed.

Figure 4.1. Chronic hypoxia exposure affects egg-laying behavior. NGM plates were seeded with OP50 such that there were locations with and without food. Adult animals were put on the plate and allowed to lay ~ 25 eggs prior to removal. Plates containing embryos were placed into either hypoxia (1.0% O₂ at 22°C) or normoxia (control, 22°C) and embryos were allowed to develop. Wild-type embryos exposed to hypoxia took approximately 96 hours to reach adulthood. Plates were removed from the specified environment and the location of eggs on the plate was scored as being either on or off the food. Above shows a representative image of the plates and egg location (white arrow). We refer to this phenotype as “hypoxia-induced egg lay behavior”.

Normoxia 1% O₂
Figure 4.2. The hypoxia-induced egg laying behavior is influenced by food at 1% O₂. Embryos from an adult egg lay were placed into hypoxia (1.0% O₂ at 22°C) or normoxia (control, 22°C) and embryos were allowed to develop on either OP50 or HT115. Wild-type embryos exposed to hypoxia took approximately 96 hours to reach adulthood. Plates were removed and the location of eggs on the plate was scored as being either on or off the food. N= 12 plates for each condition. Data represent three independent trials. Animals grown on HT115 at 1% O₂ laid statistically more eggs on food than animals grown on OP50 at 1% O₂. * P<0.05, Student’s t-test.
Figure 4.3. Hypoxia-induced egg laying behavior is not influenced by the stage of development while exposed. Time of development at which animals were exposed to hypoxia (1.0% O₂ at 22°C) is abbreviated as follows: Embryogenesis to adulthood (E to A), L1 larvae to adulthood (L1 to A), L4 larvae to adulthood (L4 to A). N=12 plates for each condition. Data represent three independent trials. L4 to A animals laid more eggs on food at a slightly statistically different percentage than E to A animals. * P<0.05 Student’s t-test.
Figure 4.4. Wildtype animals exposed to hypoxia lay their eggs off but near the live bacterial lawn. Wildtype L4 larvae were placed on an NGM plate that was divided into four quadrants, two containing live OP50 and two containing heat killed OP50. The animals were placed in the middle portion of the plate and exposed to one day of hypoxia (1% O₂) or normoxia. Adult animals were than removed from the plate and the embryo location on the plate was assayed. Embryos were classified as being located on either the live OP50 lawn, on the heat-killed (HK) OP50 lawn, on the agar near the live OP50 or the agar near the heat-killed OP50. Data are from four experiments with a total of >500 embryos assayed.
Figure 4.5. Hypoxia-induced egg laying behavior is not dependent on aerotaxis genes. Embryos from an adult egg lay were placed into hypoxia (1.0% O₂ at 22°C). Embryos were allowed to develop on OP50 in chronic hypoxia. Wildtype and mutant embryos exposed to hypoxia took approximately 96 hours to reach adulthood. Plates were removed from hypoxia and the location of eggs on the plate was scored as being either on or off the food. N=12 plates for each genotype. Data represents three independent trials. * P<0.5 compared to N2 control Student's t-test.
Figure 4.6. RNAi of specific globin genes suppresses hypoxia-induced egg laying behavior. Adult animals raised on the specified RNAi food from embryogenesis were allowed to lay eggs on new RNAi plates. Adults were removed and embryos were exposed to chronic hypoxia (0.5% O₂ at 23°C) After exposure to hypoxia for 144 hours plates were removed from the environment and location of laid eggs were scored as being on or off food. N=12 plates for each genotype. Error bars represent SD. Data represent three independent trials. *P<0.01 compared to Control(RNAi), Student’s t-test.
Figure 4.7. Specific globin genes are required for hypoxia-induced egg laying behavior. Adult animals with the specified genotypes were allowed to lay eggs on an NGM plate. Adults were removed and embryos were exposed to chronic hypoxia (1% O₂ at 22°C). After exposure to hypoxia for 96 hours plates were removed from the environment and location of laid eggs was scored as being on or off food. N=12 for each genotype. Data represent three independent trials. *P<0.01 compared to N2, Student's t-test.
Figure 4.8. Penetrance of suppressed hypoxia-induced egg laying behavior is dependent on food area. One-day-old adult animals with the specified genotypes were exposed to hypoxia (1% O₂ at 22°C) for 24 hours and allowed to lay eggs on an NGM plate with the average food areas noted. Plates were removed from hypoxia and location of laid eggs was scored as being on or off food. N=12 for each genotype and condition. Data represent three independent trials. *P<0.01 compared to N2 on 25% Area of Food. **P<0.01 compared to N2 on 40% Area of Food and glb-13(tm2825) on 25% Area of Food, Student’s t-test.
Figure 4.9. Reverse uncoordinated movement influences stochastic variation in hypoxia-induced egg laying behavior. NGM plates were seeded with OP50 such that there were locations with and without food. Embryos from egg lay were placed into hypoxia (1.0% O₂ at 22°C) and allowed to develop. Wildtype and mutant embryos exposed to hypoxia took approximately 96 hours to reach adulthood. Plates were removed from hypoxia and partitioned into three zones. The location of eggs on the plate was scored as being on the food, in the inner zone or in the outer zone. Above shows a representative image of the plates and egg location for wildtype and unc-129(ev554). F= Food; I= Inner zone; O= Outer zone.
Figure 4.10. Reverse uncoordinated movement influences stochastic variation in hypoxia-induced egg laying behavior. NGM plates were seeded with OP50 such that there were locations with and without food. Embryos from egg lay were placed into hypoxia (1.0% O\textsubscript{2} at 22°C) and allowed to develop. Wildtype and mutant embryos exposed to hypoxia took approximately 96 hours to reach adulthood. Plates were removed from hypoxia and partitioned into three zones. The location of eggs on the plate was scored as being on the food, in the inner zone or outer zone. N=12 plates for each genotype. Data represents three independent experiments. The mean percentages of eggs laid by *C. elegans* on the 4 mm food border (black bar) were found to be highly significantly different (One-way parametric ANOVA, p < 0.0001). A Dunnett’s Multiple Range Test (α=0.05) showed that the mean percentages of eggs laid by the genotypes *pag-3(n3098)*, *pag-3(Is20)*, and *unc-129(ev544)* were significantly different from N2, while the genotype *unc-130(oy107)* was not significantly different from N2.
DISCUSSION

Introduction

The natural habitat populated by *C. elegans* is hypothesized to be nutrient- and bacteria-rich organic material, such as compost and organic topsoil (Kiontke et al., 2006). This creates optimal conditions for fast growth and reproduction under ideal environmental conditions. But the presence of alternate stress resistant life stages that are triggered by crowding and starvation suggest that *C. elegans* found in nature develop and reproduce under less than ideal conditions (Albert et al., 1988).

The organic topsoil environment does not protect the animals within from ephemeral stresses such as oxygen deprivation. For example, flooded topsoil is rapidly cut off from the oxygen in the atmosphere by a layer of water. Therefore, *C. elegans* has probably been exposed to low oxygen levels in its natural habitat and has adaptations to sense and respond to oxygen deprivation during its life history.

To gain a better understanding of how *C. elegans* responds to oxygen deprivation I first characterized how meiotic cells within the germline respond to anoxia. To address this question, I analyzed chromatin localization and changes in cytoplasmic ribonucleoprotein (RNP) foci. I found that bivalent chromosomes associate with the nuclear envelope in stress-induced arrest such as anoxia or quiescence due to absence of functional sperm. I also determined that RNP foci in arrested oocytes might play a similar role to P bodies and/or stress granules that increase in size or form in response to multiple environmental stresses.

Second, I characterized how *C. elegans* developmental trajectory is affected by chronic hypoxia (0.5-1% O$_2$ from embryo to adult). I used a hypoxia glove box chamber
to expose an isogenic population of animals to hypoxia at standard laboratory temperatures (22-23°C) and analyze representatives of the population at different time points. I found that *C. elegans* develops at a slower rate when exposed to chronic hypoxia. Through a targeted mutant screen of genes required for oxygen sensing, I determined that the prolyl hydroxylase, *egl-9*, is required for normal development in hypoxia. The stress of chronic hypoxia affects the fitness of the adult animal after removal from hypoxia by reducing offspring that reach adulthood and increasing longevity.

Using the methods I established above for raising *C. elegans* in hypoxia, I asked if hypoxia affects behavior. By analyzing egg-laying behavior, I characterized a behavioral response to hypoxia (0.5-1% O₂) at standard laboratory temperatures (22-23°C). In normoxia wildtype animals lay eggs on the bacterial lawn they use for food. I found that adult animals exposed to hypoxia shift egg laying behavior off the bacterial lawn. This behavior requires *glb-13* and is independent of other known oxygen sensing pathways required for behavior and oxygen sensing at 21% O₂.

While *C. elegans* is tolerant to oxygen deprivation, prolonged anoxia exposure results in decreased survival and tissue abnormalities (Mendenhall et al., 2009). I have shown that *C. elegans* is well suited to hypoxia exposure at standard laboratory temperatures. Although reproductive fitness is reduced in animals exposed to hypoxia, the stress does not cause reduction in adult survival and wildtype animals appear healthy even when exposed to hypoxia for 96 hours from embryogenesis. With the new techniques used here to explore chronic hypoxia I have increased our understanding of how *C. elegans* responds to oxygen deprivation. I have also further established *C.
elegans as a model for studying how chronic hypoxia affects development and behavior.

Germline Responses to Anoxia

I conducted cellular and genetic analysis of prophase oocytes exposed to anoxia to gain a greater understanding of mechanisms regulating arrest of prophase cells. Using time-lapse microscopy I found that chromosomes associate with the inner nuclear periphery in anoxia-induced arrested prophase oocytes. Others have shown chromosomes associate with the nuclear periphery in Drosophila prophase cells exposed to anoxia indicating this response to oxygen deprivation is conserved (Foe and Alberts 1985). I found oocytes, of anoxia exposed adults, aged adults, or adults that have dysfunctional sperm (fog-2(q71) mutant), contain bivalent chromosomes associated with the nuclear periphery. However, I did not observe this in the oocytes of starved adults. Others have shown that brief, acute and chronic starvation induce a variety of cellular and behavioral responses. However, the specific response to starvation often depends upon the stage at which starvation was induced, and how long the animals were exposed to starvation. Known germline responses to starvation include an increase in programmed cell death, arrest of germ cell proliferation, and reproductive diapause. Furthermore, it was shown that after 5 days of starvation L4 larvae will either arrest as L4 larvae, arrest as adults with the germline in reproductive diapause or become adults in which embryos in the uterus develop and cause death of the adult due to matricide (bagging out) (Angelo et al., 2009). In my experiments I found many of the adults were holding embryos in the uterus after 12 hours without a
food source, and longer periods of starvation led to the bagging out phenotype indicating the lack of food was indeed inducing a physiological response. These results demonstrate that chromosome association with the nuclear periphery occurs in both mitotic and meiotic cells and may be a characteristic of arrested or quiescent cells rather than stressed cells.

The relevance of chromosome association with the nuclear periphery in anoxia-exposed oocytes is not understood. Many studies have shown that in interphase cells, transcriptional regulation and silencing of genes is associated with the localization of chromatin to the nuclear periphery (Shaklai et al., 2007). In *Drosophila* and yeast it has been shown that chromosomal loci at the nuclear periphery interact with nuclear pores and that nuclear pores can interact with both transcriptionally inactive and active loci (Akhtar et al., 2007; Casolari et al., 2004). Several nucleoporins as well as nucleoporin-associated proteins (MLP1, MLP2) are required for full repression of specific loci indicating that the nuclear pore complex (NPC) has a role in gene regulation (Feuerbach et al., 2002; Galy et al., 2000). An alternative reason for prophase chromosome association with the inner nuclear periphery in arrested cells may be to maintain genome stability. It has been shown that stabilization of repetitive DNA sequences, which are prone to homologous recombination, occurs by interactions between inner nuclear membrane proteins and chromosomal proteins (Mekhail et al., 2008). In anoxia arrested prophase cells and oocytes the chromosomes are highly condensed and thus likely to not be transcriptionally active. Furthermore, I found that the quiescent oocytes in the sperm-dysfunctional *fog-2(q71)* mutant or sperm-depleted aged hermaphrodite also contain oocytes with bivalent chromosomes associated with
the inner nuclear periphery. These quiescent oocytes are viable and have similar phenotypes to oocytes exposed to anoxia (Jud et al., 2008). The relevance of chromosome association with the nuclear periphery in these arrested cells may not be to regulate gene expression but rather to maintain genome stability and/or chromosome structure in times of arrest. Thus, it may be possible that the mechanisms for maintaining oocytes in an arrested or quiescent state are overlapping, regardless if the arrest is induced by anoxia exposure, sperm depletion or sperm dysfunction. It will be of interest to determine if anoxia-induced chromosome association with the nuclear periphery is important for genome stability and if this phenomenon is an essential component for arresting prophase cells.

The nuclear pore complex (NPC) is a large protein channel associated with the nuclear envelope and made up of nucleoporins. This highly conserved protein structure is important for the transport of macromolecules between the cytoplasm and nucleoplasm. There is increasing evidence that the nucleoporins are involved with a variety of cellular processes including chromatin organization, gene regulation and spindle orientation (Capelson et al., 2009; Schetter et al., 2006). Furthermore, it has been shown that some nucleoporins (NUP107) not only localize to the NPC but, upon NPC disassembly, relocalize to chromatin during mitosis (Galy et al., 2006). Analysis of npp-16(ok1839) oocytes suggests npp-16 is also required for maintenance of oocytes. This is supported by the finding that npp-16(ok1839) hermaphrodites exposed to normoxia contain bivalent chromosomes associated with the nuclear periphery. The npp-16(ok1839) hermaphrodites exposed to anoxia have oocytes with chromosomes associated with the nuclear periphery, yet many of these oocytes have an abnormally
shaped nucleus. These results suggest that in the oocyte *npp-16* is not required for chromosome association with the nuclear periphery but is required for maintenance of nuclear shape in oocytes of animals exposed to anoxia.

Along with the NPC involvement with chromosome docking it has been shown the NPC also clusters with RNP foci within the cytoplasm (Jud et al., 2007). This connection between nuclear and cytoplasmic events under anoxic stress and quiescence are an interesting discovery in how cellular events occur. The small foci that form in response to anoxia have fewer components in common with RNP foci from arrested oocytes or after heat shock (Jud et al., 2008). One possible explanation for these differences is that oocytes do not respond identically to different stresses. In many studies of stress granules performed in cell culture, variable responses were observed. Depending on the type of cell line, different stresses are effective in inducing stress granule formation. Furthermore, different components are observed within stress granules in different cell lines (Tourrière et al., 2003). These data reveal novel connections between cellular responses to stress and delayed fertilization, where cells appear quiescent.

**Effects of Chronic Hypoxia on *C. elegans***

*Development and Fitness*

At standard laboratory conditions *C. elegans* is very sensitive to environment conditions. For example, starvation and temperature have effects on the development and fecundity of the animal (Hirsh et al., 1976). There is a gap in research knowledge on the effects that oxygen deprivation has on the life cycle and reproductive success of
C. elegans. This is due to the challenges of studying the effects of oxygen deprivation. Applying one stress to the animal causes additive affects from other stresses if experiments do not control for multiple variables. This makes interpretation of results difficult. For example, while high temperature and brief exposure to hypoxia are lethal to adults, longer exposure to hypoxia at lower temperatures is tolerated (Jiang et al., 2001; Scott et al., 2002). It is not understood how chronic hypoxia affects development or if chronic hypoxia during development has any affect on adult processes. Consequently, it is difficult to analyze and compare results from experiments with intermittent acute (<24 hours) oxygen deprivation versus chronic (>24 hours) oxygen deprivation.

C. elegans have adapted mechanisms to survive severe oxygen deprivation. However, a decrease in oxygen availability is certainly a stress on the animal. I used a hypoxia glove box chamber to expose wildtype C. elegans to acute (<24 hours) or chronic (>24 hours) hypoxia (0.5% and 1% O₂). Previously it had been shown that embryos have a relatively high survival rate when exposed to hypoxia (0.5% and 1% O₂) (Jiang et al., 2001; Padilla et al., 2002). I found, using our hypoxia glove box chamber, that eggs laid on both live OP50 and live HT115 food and exposed to 1% O₂ survive at a high rate. However, embryos laid on OP50 food and exposed to 0.5% O₂ had a lower survival rate than previously reported (Jiang et al., 2001; Padilla et al., 2002). The hypoxia chamber we used is preset to the noted oxygen level before the embryos were exposed to the hypoxic environment. Thus, the embryos had a quick environmental change from normoxia to the severe hypoxic environment, likely resulting in a minimal transition time from normoxia to 0.5% O₂. In the past, methods used to induce hypoxia involved placing embryos into a chamber and then flowing gas through
the chamber, likely resulting in a longer and more variable transition time from a normoxia to 0.5% O₂. I exposed embryos using a similar hypoxia gas flow chamber (0.5% O₂) and found that embryos had a higher survival rate in comparison to those exposed to the hypoxia using the hypoxia glove box (0.5% O₂). These results indicate that the methodology used to induce a hypoxic environment can affect survival rate. It is not known specifically why there are differences in viability but a variable such as the time it takes to transition from normoxia to hypoxia may be a factor in survival rate.

I conducted chronic hypoxia studies (0.5-1% O₂ at 22-23°C from embryo to adult) to gain a better understanding of how C. elegans is adapted to surviving oxygen deprivation. I determined that developmental trajectory is slowed during exposure to hypoxia. This result may be due to metabolic depression since the experimental hypoxic conditions in these studies reduce metabolic rates by 50% (Van Voorhies and Ward 2000). Another gene within the C. elegans genome that affects development trajectory in normoxia is gro-1. This gene encodes a mitochondrial tRNA transferase that is required for proper development. gro-1(e2400) mutants have down regulated development and reproductive rates and increased lifespan and a maternal effect (Lemieux et al., 2001). Many of these phenotypes are observed in wildtype animals and mutants unrelated to gro-1 when exposed to hypoxia. It is of interest in the future to determine if metabolic genes are required for development in hypoxia.

I found that chronic and acute exposure to hypoxia during development extends lifespan and decreases fecundity. I expect that chronic hypoxia exposure (from embryo to adult) increases and maintains HIF-1 levels throughout development. So increased lifespan after development in hypoxia is surprising, since other researchers have shown
that a deletion mutation in hif-1 increases lifespan in standard laboratory conditions (normoxia) and overexpression of HIF-1 decreases lifespan in wildtype animals (Chen et al., 2009; Zhang et al., 2009). It is not understood how HIF-1 might be changing animal physiology differentially in hypoxia versus normoxia. I have not analyzed wildtype animals with an overexpression of HIF-1. Thus, if overexpression of HIF-1 in wildtype animals affects lifespan in hypoxia will need to be determined.

In C. elegans increased lifespan and reduced fecundity are also observed in animals exposed to dietary restriction during adulthood (Chen et al., 2009). In nutrient rich environments animals with low HIF-1 shift to a dietary restricted state. This is supported by results showing overexpression of HIF-1 partially inhibits lifespan extension of DR (Chen et al., 2009). This result suggests that HIF-1 controls lifespan extension through dietary restriction. I have not analyzed the nutritional state of animals in chronic hypoxia. It is of interest to determine if chronic hypoxia exposure throughout development induces a physiological change in the animal through HIF-1 to alter adult lifespan and fecundity.

The relevance of egl-9 function during development and in utero embryogenesis, in hypoxia is not understood. I found that in severe hypoxia (0.5% O$_2$) egl-9(sa307) animals arrest as larvae. While development to adulthood occurs in less severe hypoxia (1% O$_2$), egl-9(sa307) animals do not produce viable offspring. EGL-9/PHD is a prolyl hydroxylase that uses oxygen, 2-oxogluterate, and Fe$^{+2}$ as substrates for catalytic activity (Kaelin et al., 2008). EGL-9 inactivation in hypoxia leads to the stabilization of molecules such as HIF-1 that would be hydroxylated and degraded when environmental oxygen is available. It has been shown previously that the egl-9 hydroxylase domain is
not required for all mediation of HIF-1 transcriptional activity (Shao et al., 2009). Since *vhl-1(ok161)* animals develop normally and produce viable offspring in chronic hypoxia (0.5% or 1% O₂ from embryo to adult), it is not likely that up-regulation of HIF-1 is the sole cause of development and embryo abnormality. Yet the hydroxylase independent activity of EGL-9 is also VHL-1 independent, so it cannot be ruled out that a misregulation of HIF-1 or HIF-1 target genes are not the cause of larval arrest or embryo lethality. Another study using *Pseudomonas aeruginosa* infection to understand stress and pathogenicity in *C. elegans* found that the SWAN-1 protein binds directly to EGL-9 and controls HIF-1 activity in normoxia (Shao et al., 2010). *C. elegans* SWAN-1 belongs to a family of scaffolding proteins that serve as platforms for larger protein assemblies (de Vetten et al., 1997; Dressel et al., 2009). This result suggests that other mechanisms could exist in hypoxia that requires EGL-9 function for wildtype development. It is of interest to investigate the hydroxylase-independent functions of EGL-9 under hypoxic conditions.

**Behavior**

There are various potential reasons why the adult hermaphrodite may lay eggs off the bacterial lawn when exposed to hypoxia. One possibility is that the animal senses the difference between the microenvironment of food and no food on the plate and somehow chooses to lay their eggs on the area of the plate where no food is located. Within this possibility, the signal distinguishing these microenvironments may be several things such as oxygen level (perhaps more oxygen is available on the area of the plate with no bacteria) or that the *E. coli* produces a repellant in hypoxia or is no
longer an attractant that the animal can sense. Whether *C. elegans* altered egg-laying behavior in hypoxia is beneficial to developing embryos or adult reproduction is unclear. It is of interest to find other mutants with altered egg laying behavior in hypoxia to understand the mechanism controlling this phenotype.

Oxygen interacts with a variety of molecules including heme-containing globin proteins, such as hemoglobin and myoglobin, which are well understood in terms of physiological function, biochemical properties, genetic regulation and expression. The identification and analysis of additional globin molecules (neuroglobin and cytoglobin) indicates the globin family of proteins is evolutionarily conserved, expressed in specific tissues, and binds oxygen. The functional role of globin-like proteins in *C. elegans* is not well understood (Hoogewijs et al., 2008). In vertebrates, neuroglobin is expressed in neurons but not glia cells and may have a protective role against hypoxia (Nienhaus et al., 2007; Venis, 2001; Wystub et al., 2003). However the physiological role of neuroglobin is not understood. Possible functions include oxygen storage and transport, detoxification of reactive oxygen species or nitric oxide, or oxygen sensing and signaling (Yu et al., 2009).

Although *Drosophila* contains 5 globin-like molecules, *C. elegans* contains a large globin-like gene family of 33 putative globin-like genes (Hoogewijs et al., 2004). This may reflect the environmental constraints *C. elegans* has adapted to. Transcriptional analysis of this globin family has been conducted and it has been shown many of the globin genes are transcribed in neuronal cells in the head and tail of the body and nerve cord (Hoogewijs et al., 2008). A recent publication demonstrates one of these globin molecules (GLB-5) is important for *C. elegans* (non-N2 strains carrying the
npr-1 215F allele) to sense the levels of oxygen within the environment. These experiments were not conducted in a hypoxic environment (McGrath et al., 2009). I have described that the wildtype hypoxia-induced egg laying behavior is regulated in part by globin-like proteins. It is of interest to determine if the globin-like proteins identified in my assay bind oxygen. When the potential role of globin-like proteins is identified the mechanism through which they signal in the animal can be elucidated.

Conclusion

In summary, my graduate research studies involved characterization of meiotic oocyte arrest during anoxia induced suspended animation and development and behavior in C. elegans exposed to hypoxia. Specifically I have characterized cellular changes in meiotic cells exposed to anoxia. I conducted genetic analysis of genes important for oxygen deprivation survival and their role in chromosome maintenance in meiotic cells. Furthermore, I characterized developmental trajectory in C. elegans exposed to chronic hypoxia (from embryo to adult). I conducted a targeted genetic screen and found undocumented requirements for egl-9, npr-1, and dbl-1 in hypoxia. My studies also document a hypoxia specific behavior termed hypoxia-induced egg laying. Wildtype animals switch from laying eggs on the bacterial lawn used for food in normoxia to laying off the bacterial lawn when exposed to hypoxia. Hypoxia exposed animals require a wildtype glb-13 gene to exhibit this behavior.

These research projects led to a greater understanding of biological processes, such as how quiescent cells are affected by oxygen deprivation; and how development, fitness and behavior are influenced by hypoxia exposure. First, the significance of
findings published in Hajeri et al. 2010 and Jud et al. 2008 has been cited several times in reviews and primary literature. For example, see reviews by Buchan and Barker 2009 and Powell-Coffman 2010. These reviews describe how this research is invaluable to the understanding of cardiovascular disease and cell biology. Second, I have conducted research to help discover new ways to investigate adaptations to hypoxia. I establish that *C. elegans* is a model for studying unknown mechanisms for sensing and responding to hypoxia. These findings suggest experiments that may be used to understand the affect of oxygen deprivation on animals with long-term exposure to hypoxia. For example, conducting genetic screens to identify mechanisms required for cell migration and differentiation when oxygen is limiting. Discovering mechanisms is important in cancer model systems to treat and diagnose severity of the cancer progression. Since signaling pathways in *C. elegans* such as HIF-1 regulation are highly conserved to mammalian systems, we may find future experiments are translatable to understand hypoxia in mammalian systems.

A long-term goal for this research will be to understand how hypoxia (chronic and acute) exposure induces changes in gene expression that affect fitness and survival. Wildtype *C. elegans* can survive and reproduce under physiological conditions that kill mammals. Therefore, the worm provides a model to identify at the molecular level how cells respond to and survive oxygen deprivation. My studies have contributed to the field of oxygen deprivation and have many practical applications.
REFERENCES


