Reduction in ovulation or male sex phenotype increases long-term anoxia survival in a daf-16-independent manner in Caenorhabditis elegans

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Mendenhall AR, LeBlanc MG, Mohan DP, Padilla PA. Reduction in ovulation or male sex phenotype increases long-term anoxia survival in a daf-16-independent manner in Caenorhabditis elegans. Physiol Genomics 36: 167–178, 2009. First published December 2, 2008; doi:10.1152/physiolgenomics.90278.2008.—Identifying genotypes and phenotypes that enhance an organism’s ability to survive stress is of interest. We used Caenorhabditis elegans mutants, RNA interference (RNAi), and the chemical 5-fluorodeoxyuridine (FUDR) to test the hypothesis that a reduction in progeny would increase oxygen deprivation (anoxia) survival. In the hermaphrodite gonad, germ line processes such as spermatogenesis and oogenesis can be simultaneously as well as independently disrupted by genetic mutations. We analyzed genetic mutants [glp-1(q158), glp-4(bn2ts), plc-1(rx1), ksr-1(ku68), fog-2(q71), fem-3(q20), spe-9(hc52ts), fer-15(hc15ts)] with reduced progeny production due to various reproductive defects. Furthermore, we used RNAi to inhibit the function of gene products in the RTK/Ras/MAPK signaling pathway, which is known to be involved in a variety of developmental processes including gonad function. We determined that reduced progeny production or complete sterility enhanced anoxia survival except in the case of sterile hermaphrodites [spe-9(hc52ts), fer-15(hc15ts)] undergoing oocyte maturation and ovulation as exhibited by the presence of laid unfertilized oocytes. Furthermore, the fog-2(q71) long-term anoxia survival phenotype was suppressed when oocyte maturation and ovulation were induced by mating with males that have functional or nonfunctional sperm. The mutants with a reduced progeny production survive long-term anoxia in a daf-16- and hif-1-independent manner. Finally, we determined that wild-type males were able to survive long-term anoxia in a daf-16-independent manner. Together, these results suggest that the insulin signaling pathway is not the only mechanism to survive oxygen deprivation and that altering gonad function, in particular oocyte maturation and ovulation, leads to a physiological state conducive for oxygen deprivation survival.

oxygen deprivation; germ line; insulin-like signaling; stress; anoxia

METAZOANS INHABITING aquatic or subterranean ecosystems have evolved mechanisms to survive changes in oxygen levels. For example, repression of energy-requiring processes or reallocation of metabolism increases oxygen deprivation survival (21). Although most terrestrial organisms live in environments where the oxygen tension does not fluctuate much, they do have the capacity to sense and respond to oxygen deprivation. In humans, oxygen deprivation is central to various health conditions including myocardial infarction, stroke, blood loss, pulmonary disorders, and solid tumor progression (37, 62). Thus identifying the molecular mechanisms that govern meta-

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dity and increased egg production later in life) (15, 31, 38, 45, 56). Whether any of these daf-2(e1370) characteristics contribute to oxygen deprivation tolerance is not understood.

There is evidence that reproduction influences various biological processes including stress responses and longevity (19, 48, 58, 72). One study found that D. melanogaster females that were mated and had high levels of reproduction were significantly more resistant to starvation compared with age-matched virgin females. However, these mated females had a significantly lower resistance to oxidative stress compared with virgin females (58), indicating that reproduction influences stress responses. In C. elegans, stressed (heat shock, osmotic shock, and anoxia) or quiescent oocytes have altered germ line morphology. For example, accumulation of large ribonucleoprotein foci occurs in oocytes of both stressed animals, virgin fog-2(q71) females and aged hermaphrodites. It should be noted that spermatogenesis, but not oogenesis, is disrupted in the fog-2(q71) female, leading to quiescent oocytes in the germ line. In stressed animals, virgin fog-2(q71) females and aged hermaphrodites, the accumulation of large ribonucleoprotein foci is regulated by the major sperm protein (MSP) pathway. However, it is not yet understood whether any of these changes affect maintenance or survival of stressed or quiescent oocytes (27).

The relationship between reproduction and longevity has been investigated in C. elegans. It is thought that the germ line sends specific signals to repress longevity, based on the finding that ablation of the germ line precursor cells (Z2 and Z3) leads to an increase in life span (3, 75). The germ-line-ablated animals are also resistant to oxidative damage, but the relationship between stress resistance and longevity is not understood. In regard to life span extension, both the somatic and germ line reproductive tissues send signals that influence longevity (75). However, the extension of life span is not due to complete sterility, given that animals with whole gonad removal by ablation or genetic mutations, resulting in sterility, do not have an increased life span (3, 24).

Several studies have identified sex-specific differences that influence stress responses or longevity. In C. elegans, males, compared with hermaphrodites, are better able to survive exposure to the pathogenic fungus Cryptococcus neoformans (70); the male’s resistance to C. neoformans requires the activity of DAF-16. Another study suggesting that sex may influence stress response and survival involved rodents exposed to ischemic conditions. In this study adult female rats, compared with males, sustained smaller infarcts after experimental stroke (1). It is thought that modulation of estrogen and progesterone affects postischemic outcome (2). In regard to longevity, the average life span is different between the sexes not only in humans but in other animals as well (42, 44). Together, these studies suggest that in several species there are molecular differences between the sexes that affect environmental stress response or life span.

We are interested in identifying genetic and physiological changes associated with surviving the stress of anoxia. In this study we tested the hypothesis that reduced progeny production in C. elegans hermaphrodites increases long-term anoxia survival. We determined that a decrease in progeny production enhanced anoxia survival except in the case of mutant hermaphrodites that are sterile yet laid unfertilized oocytes. The finding that virgin fog-2(q71) but not mated fog-2(q71) animals survive long-term anoxia suggests that decrease in oocyte maturation and ovulation increases anoxia tolerance. Additionally, we found that compared with hermaphrodites males had a significantly higher survival rate when exposed to anoxia. Together, our results indicate that a reduction in oocyte maturation and fertilization or the male sex phenotype correlates with increased survival of long-term anoxia exposure.

MATERIALS AND METHODS

Strains and growth conditions. The wild-type Bristol strain (N2) and mutant strains were cultured on nematode growth medium (NGM) plates seeded with Escherichia coli (OP50) and raised at 20°C as described previously (65). Synchronized populations of animals were obtained by collecting embryos from hypochlorite-treated adults. Depending on the strain, anatomic markers such as gonad morphology or the hours after L4-to-adult molt were used to determine the developmental stage of the animal. The following genetic strains were obtained from the Caenorhabditis elegans Genetics Center: RB915 [ksr-1(ok786)], JK987 [tra-2(q276) mma1 dpy-10(e128) unc-52(e444III)], MH734 [ksr-1(ku68)], BA15 [fer-15(hc15ts)], BA708 [spe-9(hc52ts)], CB1370 [daf-2(e1370)], JK1019 [vha-19(e1259); gdp-1(q158); daf-3(III)]; CB4108 [fog-2(q71)], SS104 [gfp-4(bln2s)], JK816 [fer-3(q20)], PS4112 [pcl-1(+); kex-2(pcl-1(+)) + sur-5::GFP], ZG31 [Hif-1(a04)], and CF1038 [daf-16(mu86)]. Standard genetic crosses were conducted to produce the daf-16(mu86); ksr-1(ok786) (PM125) strain. The ksr-1(ok786) animal (RB915 strain) was backcrossed twice with the wild-type N2 strain to produce the PM124 strain [ksr-1(ok786)]. For analysis of temperature-sensitive mutants [fer-15(hc15ts)], gdp-4(bln2s), spe-9(hc52ts), or fer-3(q20)], animals were raised at 15°C and then shifted to 25°C. At the L2 or L3 larval stage, the larvae of which were grown to adulthood at 25°C and then exposed to long-term anoxia at 20°C. The wild-type, daf-16(mu86), and spe-9(hc52ts) hermaphrodites fed 5-fluorodeoxyuridine (FUDR) were fed as described in Ref. 73. Male C. elegans were collected as young adults from a synchronized population of hermaphrodites and males.

Oxygen deprivation experiments. For all experiments, adult nematodes were collected 22–26 h after the L4-to-adult molt and exposed to an anoxic environment at 20°C with either a gas flow-through chamber with 100% nitrogen gas (Air Liquide) or anoxia Bio Bags to an anoxic environment at 20°C with either a gas flow-through chamber with 100% nitrogen gas (Air Liquide). For the purpose of these studies, we refer to anoxia as 3 days of anoxia at 20°C. The wild-type, daf-16(mu86), and spe-9(hc52ts) hermaphrodites fed 5-fluorodeoxyuridine (FUDR) were fed as described in Ref. 73. Male C. elegans were collected as young adults from a synchronized population of hermaphrodites and males.

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placed on 2.5% agarose pads, and the number of eggs within the uterus was quantitated. The oocyte maturation rate, per gonad arm per hour, was determined by measuring total embryo production in individuals as described in Ref. 43.

**RNA interference assays.** We used RNA interference (RNAi) to inhibit the genes glp-1, daf-16, and hif-1 and those genes thought to be involved with the RTK/RAS/MAPK pathway (see Supplemental Fig. S1), as described previously (45). Briefly, synchronized L1 larvae were collected and grown to adulthood on NGM-isopropyl-β-D-thiogalactopyranoside (IPTG) plates (200 μg/ml ampicillin, 12.5 μg/ml tetracycline, and 0.5 mg/ml IPTG) seeded with the E. coli strain for RNAi of a specified gene. The E. coli strains were developed by the J. Ahringer laboratory and obtained from the Medical Research Council Geneservice (Cambridge, UK) (28, 69). As a control, worms were fed the E. coli strain containing the plasmid with no insert.

**Nomarski microscopy analysis.** Microscopy analysis was conducted as previously described (45). Briefly, nematodes were exposed to either a normoxic or an anoxic environment; the anoxia-exposed nematodes were allowed to recover in air at 20°C for the time indicated. The animals were placed on a 2% agarose pad containing 0.2 mM levamisole in M9 (63). Animals were also analyzed without anesthetic to verify that this chemical did not contribute to the observed phenotype. For each experiment at least four independent evaluations were done. Microscopy was conducted with a motorized Zeiss Axioscope with a ×100 objective lens and Openlab imaging software 3.17. Images were prepared with Adobe Photoshop CS (Adobe Systems) and Adobe Illustrator CS.

**Statistical analysis.** Data set distribution was determined as normal or nonnormal with appropriate normality tests. One-way ANOVA or one-way ANOVA on ranks was performed after data set distribution was determined. Depending on the distribution of the data set and the number of values in each data set, Dunn’s or Holm-Sidak’s methods were used to make multiple comparisons against a control group. The data sets found to be significantly different from controls were reevaluated with Student’s t-test or Mann-Whitney U-test.

**RESULTS**

We are interested in determining whether specific physiological states lead to long-term anoxia survival. To address this we chose to examine the role that progeny production has in long-term anoxia survival. Many of the genes involved with gonad development and function are understood in *C. elegans*; thus a variety of genetic mutants with defects in specific gonad processes are available for phenotype analysis (16, 32). For example, in the hermaphrodite gonad, germ line processes such as spermatogenesis and oogenesis can be simultaneously as well as independently disrupted by genetic mutations. To test the hypothesis that hermaphrodites with a reduction in progeny production are long-term anoxia tolerant, we examined genetic mutants with a reduced progeny production or completely sterile phenotype. Table 1 lists the genetic mutants we used in this study; summary and phenotype descriptions are based on published germ line phenotypes and, in the case of *ksr-1* mutants, phenotypes that we observed and documented in the present study. Some of the mutants we examined are completely sterile because of independent dysfunctions. For example, both glp-1(q158) and fog-2(q71) are sterile, but they are distinct in that spermatogenesis and oogenesis are disrupted in the glp-1(q158) animal whereas spermatogenesis, but not oogenesis, is disrupted in the fog-2(q71) animal; we organized Table 1 to denote such distinctions. In addition to the genetic mutants, we used the drug FUDR to reduce progeny production.

**Disruption of germ line proliferation and long-term anoxia tolerance.** In regard to wild-type hermaphrodites’ long-term anoxia tolerance, we determined that despite the various methodologies used to induce anoxia, the use of anaerobic Bio Bags traditionally used to grow anaerobic bacteria or a chamber in which air is replaced with nitrogen, the wild-type hermaphrodites had a decrease in survivorship and normal motility (Table 2). The animals exposed to anoxia with a nitrogen flow-through chamber had a higher average survival rate compared with those grown in an anaerobic Bio Bag chamber; however, the survival rate of wild-type hermaphrodites was still significantly lower compared with what we had previously observed in the long-term anoxia-tolerant animal *daf-2(e1370)* (45). Since several of the mutants we analyzed are temperature sensitive, we needed to determine whether a temperature shift from 15°C to 25°C significantly affected anoxia survival rate. The wild-type hermaphrodites were grown at 15°C, transferred as L3 larvae to 25°C for 24 h, and then exposed to anoxia for 1 or 3 days of anoxia at 20°C. The animals exposed to 1 day of anoxia had high survival rates (100%, n = 100), and the animals exposed to 3 days of anoxia did not survive (Table 2); these survival rates are consistent.

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1 The online version of this article contains supplemental material.
with previous experiments in which the animals were grown at 20°C before anoxia exposure (45). Our results verify that wild-type hermaphrodites are sensitive to long-term anoxia.

The gonad in *C. elegans* hermaphrodites will develop to produce ~1,200–1,500 germ cells (4, 6). We analyzed the glp-1(q158) and glp-4(bn2ts) mutants, which are completely sterile because of a severe reduction in germ cell production. The glp-1(q158) hermaphrodites produce ~5–7 germ cells that prematurely differentiate into sperm (4). The glp-4(bn2ts) hermaphrodites raised at the nonpermissive temperature have a reduction in the number of germ cells produced, and a functional germ line does not develop (6). We found that glp-1(q158) and glp-4(bn2ts) hermaphrodites survive and display normal motility after long-term anoxia exposure (Table 2).

RTK/Ras/MAPK signaling pathway-reduced mutant progeny and long-term anoxia tolerance. The process of ovulation, which involves the entry of the mature primary oocyte into the spermatheca, requires contraction of gonadal sheath cells, spermatheca dilation, and signals from the maturing oocyte. The spermatheca contains sperm capable of fertilizing the oocyte; the fertilized oocyte will then exit the spermatheca and enter into the uterus and develop for a few hours before being laid (16). Mutations affecting ovulation will result in hermaphrodites with a reduced number of progeny. For example, plc-1(rx12) hermaphrodites have a severe reduction in progeny (7.8 ± 5.0) due to abnormal ovulation in which mechanical defects involving oocyte entry and exit from the spermatheca result in either oocyte tearing or multiple eggs within the distended spermatheca. PLC-1 encodes a phospholipase Cε and is expressed in the spermatheca, which is consistent with the idea that PLC-1 is involved with ovulation (30). To further test the hypothesis that animals with reduced progeny production are long-term anoxia tolerant, we exposed plc-1(rx12) hermaphrodites to anoxia. We determined that the plc-1(rx12) hermaphrodites had a high survival rate and normal motility after exposure to long-term anoxia (Table 2).

Others have shown, using the yeast two-hybrid system, that PLC-1 interacts with LET-60/RAS, suggesting that plc-1 has a functional role is in the RTK/Ras/MAPK signaling pathway (64). Over 56 genes have been identified to be involved in the RTK/Ras/MAPK signaling pathway in *C. elegans*. Many of these genes regulate diverse developmental processes, including vulva development or gonad function (66). We used RNAi to inhibit gene function to determine whether specific genes, thought to function in the RTK/Ras/MAPK signaling pathway, influence anoxia survival. The genes we screened included those with RNAi food available (see MATERIALS AND METHODS) and shown to be core components, regulators, or targets of the RTK/Ras/MAPK signaling pathway (see Table 1 in Ref. 66). We screened hermaphrodites subjected to RNAi for long-term anoxia tolerance and determined that RNAi of 26 genes resulted in a >75% anoxia survival rate phenotype (Supplemental Table S1). However, just four of these strains (mek-2(RNAi), mpk-1(RNAi), sem-5(RNAi), and par-1(RNAi)) had >80% of survivors displaying normal motility after long-term anoxia exposure (Supplemental Table S1), suggesting that these animals not only survived long-term anoxia but also maintained tissue structure and function.

In the RNAi screen of core components, regulators, or targets of the RTK/Ras/MAPK signaling pathway for anoxia-tolerant hermaphrodites, we noted that 14 of the genes subjected to RNAi resulted in animals that had a ≥90% survival rate, yet the majority of the survivors displayed abnormal motility after treatment (Supplemental Table S1). We chose to focus on one of the 14 genes, ksr-1, that when subjected to RNAi led to a high survival rate but abnormal motility (Supplemental Table S1). The ksr-1 (kinase suppressor of Ras) gene was originally discovered as a suppressor of the let-60/ras gain-of-function allele, and many ksr-1 alleles have been identified (34, 67). To determine whether ksr-1 mutants are indeed long-term anoxia tolerant, we exposed ksr-1(ok786) and ksr-1(ku68) hermaphrodites to 3 days of anoxia. Compared with the wild-type hermaphrodites, the ksr-1(ok786) and ksr-1(ku68) hermaphrodites were long-term anoxia tolerant, and the survivors displayed normal motility (Table 2), suggesting that the alleles have a stronger anoxia tolerance phenotype compared with the ksr-1(RNAi) animals. To further characterize the phenotype of the ksr-1 mutants, we counted the total number of progeny produced in 1- to 6-day-old ksr-1 mutants. We determined that the ksr-1 mutants are not sterile but produce less progeny compared with wild-type hermaphrodites (P < 0.02) (Fig. 1). Examination of the ksr-1(ok786) gonad indicates that the 1-day-old hermaphrodite exhibits various phenotypes including an increase in the average number of oocytes present in the gonad resulting in a “stacked oocyte” phenotype, an increase in fluid (likely to be yolk) within the gonad (100%, n = 12), the association of sperm with distal oocytes (25%, n = 12), and abnormal spermatheca morphology (Fig. 2). These results support the idea that ksr-1 has a role in gonad function.

On the basis of genetic analysis, the genes mek-2, mpk-1, sem-5, par-1, and ksr-1 have been shown to be involved with

<table>
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<th>Genotype</th>
<th>Survival, %</th>
<th>Normal Motility, %</th>
<th>n</th>
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<tr>
<td>Wild type*</td>
<td>4.7±6.7</td>
<td>2.8±4.2</td>
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<tr>
<td>Wild type†</td>
<td>39.4±16.1</td>
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<tr>
<td>glp-1(q158)*</td>
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<td>95.4±5.1</td>
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<tr>
<td>glp-4(bn2ts)+</td>
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<td>83.6±13.9</td>
<td>270</td>
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<td>55.9±23.2</td>
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<tr>
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<tr>
<td>fer-15(hc15)+</td>
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<tr>
<td>spe-9(hc52s) fed FUDR‡</td>
<td>99.0±2.0</td>
<td>97.9±2.8</td>
<td>190</td>
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</table>

Values are means ± SD; n = total no. of animals analyzed from at least 4 independent experiments. *Animals grown at 20°C; the methodology used to induce anoxia was a Bio Bag placed at 20°C; †Animals grown at 20°C; the methodology used to induce anoxic environment was a nitrogen flow-through chamber at 20°C; ‡Animals grown to L2 or L3 larval stage at 15°C, shifted to 25°C until they reached adulthood (1-2 days), and then placed into anoxia with the anoxia Bio Bag at 20°C. For the spe-9(hc52s) on 5-fluorodeoxyuridine (5-FDUDR) experiments, the animals were shifted from 15°C to 25°C at the L3 larvae stage, grown at 25°C, and placed on FUDR plates as L4 larvae. For all analyses, adult hermaphrodites were exposed to 3 days of anoxia. For all experimental sets, with the exception of spe-9(hc52s) and fer-15(hc15), P < 0.001 compared with respective control. The viabilities of spe-9(hc52s) and fer-15(hc15) were not significantly different from their respective controls.

Table 2. Genotypes or conditions that enhance long-term anoxia survival
long-term anoxia survival

Various developmental processes. For example, mek-2 and mpk-1 are thought to have a functional role in meiosis and vulva development (9, 33, 36, 39, 74). The gene sem-5 is involved with vulva induction and sex myoblast migration (10), whereas par-1 is required for cytoplasmic localization in embryos and vulva development and morphogenesis (25). Finally, others have shown that ksr-1 is involved with vulva development, and our results suggest a role in gonad function (67). Others have shown that RNAi of mek-2, mpk-1, sem-5, or par-1 results in a sterility phenotype (12, 29, 40). To further analyze gonad function in mek-2(RNAi), mpk-1(RNAi), sem-5(RNAi), par-1(RNAi), and ksr-1(ok786) hermaphrodites, we calculated the oocyte maturation rate, the number of eggs observed in the uterus of 1-day-old adults. We determined that, compared with wild-type animals, the mek-2(RNAi), mpk-1(RNAi), sem-5(RNAi), par-1(RNAi), and ksr-1(ok786) hermaphrodites had a reduction in both oocyte maturation rate and the number of eggs laid per hour (Table 3). The mek-2(RNAi), mpk-1(RNAi), and sem-5(RNAi) animals had the most severe reduction in oocyte maturation rate. Compared with wild-type animals, the mek-2(RNAi) animals had a higher than calculated number of embryos in the uterus given the oocyte maturation rate, which may be due to egg-laying defects. These data are consistent with the idea that mpk-1 and mek-2 are involved with vulva function. Only par-1(RNAi) animals had an increased number of eggs, many of which appeared dead, in the uterus as observed by differential interference contrast (DIC) microscopy, suggesting that an increase in egg load within the uterus does not affect long-term anoxia tolerance. Together, these results further support the idea that hermaphrodites with reduced progeny production are long-term anoxia tolerant.

We previously showed (52) that adult C. elegans exposed to anoxia enter into a state of suspended animation in which developmental and behavioral processes such as feeding and egg laying arrest. We determined that wild-type adult hermaphrodites exposed to anoxia arrest ovulation (Table 3). This suggests that the ability of sterile adults to survive long-term anoxia is not due to an overall reduction in ovulation during anoxia exposure.

Spermatogenesis and fertilization mutations and long-term anoxia tolerance. To further test the hypothesis that hermaphrodites with a reduction in progeny production are long-term anoxia tolerant, we examined mutants that are sterile because of spermatogenesis or fertilization defects. The fog-2(q71) hermaphrodite has a feminized gonad in that all the germ cells...
develop into oocytes. The unmated fog-2(q71) female accumulates unfertilized oocytes in the proximal gonad, is non-egg bearing, and rarely lays unfertilized oocytes (59). We determined that the fog-2(q71) females are long-term anoxia tolerant and the animals that survive have normal motility (Table 2). The fem-3(q20) mutant, when grown at the non-permissive temperature, makes only sperm and not oocytes. We determined that these fem-3(q20) animals are long-term anoxia tolerant (Table 2). The spe-9(hc52ts) and fer-15(hc15ts) mutants are also sterile as a result of abnormal spermatogenesis, yet these mutants do not survive long-term anoxia (Table 2).

The methodology required to observe the sterility phenotype (a shift from 15°C to 25°C) probably does not affect the spe-9(hc52ts) or fer-15(hc15ts) animals such that they cannot survive long-term anoxia, because the glp-4(bn2) animals were subjected to the same methodologies and are long-term anoxia tolerant. Additionally, a temperature shift from 15°C to 25°C does not alter the ability of wild-type animals to survive 1 day of anoxia (see Table 2).

To gain a greater understanding as to why fog-2(q71), but not spe-9(hc52ts) or fer-15(hc15ts) animals are long-term anoxia tolerant, we considered the phenotypes associated with these three mutants. The fog-2(q71) female rarely lays unfertilized oocytes, whereas the spe-9(hc52ts) and fer-15(hc15ts) animals do lay unfertilized oocytes at a significant rate; therefore, it is possible that an increase in oocyte maturation and ovulation phenotype will suppress the long-term anoxia survival phenotype observed in the fog-2(q71) female (35, 57). To test this, we analyzed mated fog-2(q71) animals and determined that mating the fog-2(q71) female with males that have functional sperm [fog-2(q71) or wild-type males], before anoxia exposure, suppressed the long-term anoxia survival phenotype (Table 4 and data not shown, respectively). Oocyte maturation, ovulation, and fertilization, resulting in viable offspring, occurs in the fog-2(q71) female mated with either wild-type or fog-2(q71) males. To delineate whether an increase in oocyte maturation, ovulation, and fertilization, or just an increase in oocyte maturation and ovulation, will suppress the fog-2(q71) long-term anoxia survival phenotype, we mated the fog-2(q71) animal with spe-9(hc52ts) males. The spe-9(hc52ts) males have sperm that can signal oocyte maturation and ovulation, but the sperm cannot fertilize the oocyte; thus no viable offspring are produced. We determined that female fog-2(q71) mated with spe-9(hc52ts) males laid unfertilized oocytes and did not survive long-term anoxia (Table 4). The act of mating per se likely does not suppress long-term anoxia tolerance because mated daf-2(e1370) animals remain anoxia tolerant. Furthermore, mated wild-type hermaphrodites survive 1 day of anoxia exposure (Table 4). Together, these results suggest that an increase in oocyte maturation and ovulation suppresses the long-term anoxia survival phenotype observed in fog-2(q71) females.

Table 4. Decreases in oocyte maturation and ovulation increase long-term anoxia survival

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Survival, %</th>
<th>Motility, %</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2 mated; 1-day anoxia</td>
<td>100</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>daf-2(e1370) mated with N2</td>
<td>95.3 ± 15.2</td>
<td>86.1 ± 17.0</td>
<td>185</td>
</tr>
<tr>
<td>fog-2(q71) virgins</td>
<td>2.2 ± 3.5</td>
<td>0.6 ± 1.6</td>
<td>165</td>
</tr>
<tr>
<td>fog-2(q71) mated with spe-9(hc52ts) males*</td>
<td>3.7 ± 6.4</td>
<td>0.0 ± 0.0</td>
<td>25</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = total no. of animals analyzed from at least 4 independent experiments. Animals were grown at 20°C, and the methodology used to induce anoxia was a Bio Bag placed at 20°C. *For these experiments, each individual fog-2(q71) animal was mated with spe-9(hc52ts) males and placed as an individual on a plate to verify the laying of unfertilized oocytes before long-term anoxia treatment.

Chemical inhibition of germ line function and long-term anoxia tolerance. As a final means to reduce progeny production, we subjected hermaphrodites to FUDR. FUDR is known to inhibit DNA synthesis, which decreases the number of offspring in hermaphrodites fed FUDR from L4 larvae to adulthood. The sterility phenotype could be due to effects on embryogenesis and germ line proliferation (23). In yeast, FUDR also induced growth defects, presumably by interfering with the rRNA processing complex (41); thus sterility in C. elegans could be due to the combination of DNA synthesis inhibition and a decrease in protein production. We observed that the L4 hermaphrodite fed FUDR for 1 day displayed a reduction in ovulation and the number of eggs laid per hour (Table 3). The FUDR hermaphrodites held some embryos within the uterus; however, the embryos were not viable, as indicated by dead eggs in the uterus, the absence of eggs laid on the plate, and no larvae hatching. Using DIC microscopy, we noted that animals fed FUDR had a reduced number of oocytes, consistent with the idea that germ line proliferation was being affected (data not shown). To determine whether FUDR could enhance anoxia survival in animals that do not survive 3 days of anoxia, we fed FUDR to wild-type and spe-9(hc52ts) hermaphrodites. We determined that the FUDR-fed hermaphrodites were long-term anoxia tolerant at a significantly higher rate compared with hermaphrodites fed control food (Table 2).

Long-term anoxia tolerance due to progeny reduction is independent of daf-16 or hif-1 function and not suppressed by starvation. Previously, we showed (45) that the daf-2(e1370) long-term anoxia tolerance phenotype is dependent on DAF-16. Thus we wanted to determine whether DAF-16 function is required for long-term anoxia tolerance in animals with reduced progeny. We reduced the function of DAF-16 by using RNAi or the null mutation daf-16(mu86). The glp-1(q158); daf-16(RNAi), glp-4(bn2);daf-16(RNAi), and fog-2(q71); daf-16(RNAi) animals were long-term anoxia tolerant (Table 5). We also analyzed glp-1(RNAi) and glp-1(RNAi);daf-16(mu86) animals and determined that, similar to the glp-1(q158) animals, the glp-1(RNAi) and glp-1(RNAi);daf-16(mu86) animals were long-term anoxia tolerant. Similar to the N2 hermaphrodite fed FUDR (Table 2), the daf-16(mu86) hermaphrodite fed FUDR survived long-term anoxia (Table 5; P < 0.002), suggesting that FUDR increases anoxia tolerance independent of genotype. As a control, we assayed daf-2(e1370) and daf-2(e1370);daf-16(RNAi) animals; RNAi of daf-16 was sufficient to suppress the long-term anoxia phenotype in daf-2(e1370); daf-16 animals (Table 5). Together, our results suggest that the long-term anoxia tolerance observed in glp-1(q158), glp-4(bn2), fog-2(q71), and wild-type hermaphrodites fed FUDR is independent of daf-16 function.

Studies suggest that daf-2 may act upstream of let-60/raz (50). Thus the functional role that daf-16 has in the long-term anoxia tolerance of plc-1(rxl), sem-5(RNAi), mek-2(RNAi), mkp-1(RNAi), par-1(RNAi), and ksr-1(ok786) animals may be
We produced a double mutant for phenotype analysis. The two genes are genetically interacting and are synergistically required for survival of long-term anoxia but not significantly different from wild-type hermaphrodites (Table 6). To determine whether hif-1 is required for long-term anoxia survival in animals with reduced progeny production, we used RNAi to reduce hif-1 in seven animals. We determined that hif-1(RNAi) did not suppress the phenotype of long-term anoxia tolerance (Table 6). Together, these findings suggest that hif-1 function is not required for long-term anoxia survival in C. elegans.

Table 5. Long-term anoxia tolerance is independent of daf-16 function

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Survival, %</th>
<th>Normal Motility, %</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (control food)</td>
<td>9.1 ± 7.9</td>
<td>5.1 ± 6.3</td>
<td>430</td>
</tr>
<tr>
<td>daf-2(e1370)</td>
<td>99.8 ± 0.2</td>
<td>98.0 ± 2.8</td>
<td>100</td>
</tr>
<tr>
<td>daf-2(e1370);daf-16(RNAi)</td>
<td>52.0 ± 23.2</td>
<td>38.0 ± 19.1</td>
<td>150</td>
</tr>
<tr>
<td>daf-16(mu86) (control food)</td>
<td>7.2 ± 1.9</td>
<td>3.2 ± 2.8</td>
<td>165</td>
</tr>
<tr>
<td>glp-1(q158) (control food)</td>
<td>96.8 ± 3.4</td>
<td>97.3 ± 4.1</td>
<td>150</td>
</tr>
<tr>
<td>glp-1(q158);daf-16(mu86)</td>
<td>96.9 ± 3.6</td>
<td>92.9 ± 9.4</td>
<td>275</td>
</tr>
<tr>
<td>glp-1(RNAi)</td>
<td>99.0 ± 1.4</td>
<td>96.0 ± 2.8</td>
<td>100</td>
</tr>
<tr>
<td>glp-1(RNAi);daf-16(mu86)</td>
<td>93.2 ± 4.8</td>
<td>89.8 ± 4.2</td>
<td>330</td>
</tr>
<tr>
<td>glp-4(hub2) (control food)</td>
<td>100.0 ± 0.0</td>
<td>100.0 ± 0.0</td>
<td>115</td>
</tr>
<tr>
<td>glp-4(hub2);daf-16(mu86)</td>
<td>99.7 ± 0.8</td>
<td>98.7 ± 1.6</td>
<td>275</td>
</tr>
<tr>
<td>fog-2(q71) (control food)</td>
<td>98.9 ± 2.2</td>
<td>96.7 ± 6.5</td>
<td>164</td>
</tr>
<tr>
<td>fog-2(q71);daf-16(mu86)</td>
<td>98.4 ± 3.6</td>
<td>98.0 ± 3.5</td>
<td>225</td>
</tr>
<tr>
<td>Wild-type fed FUDR</td>
<td>98.0 ± 2.4</td>
<td>94.0 ± 2.8</td>
<td>200</td>
</tr>
<tr>
<td>FUDR daf-16(mu86)</td>
<td>97.0 ± 2.6</td>
<td>90.0 ± 6.7</td>
<td>200</td>
</tr>
<tr>
<td>ksr-1(ok786)</td>
<td>78.2 ± 6.6</td>
<td>51.9 ± 25.2</td>
<td>400</td>
</tr>
<tr>
<td>ksr-1(ok786);daf-16(mu86)</td>
<td>74.1 ± 13.0</td>
<td>46.8 ± 22.9</td>
<td>341</td>
</tr>
<tr>
<td>plc-1(rx1) (control food)</td>
<td>96.6 ± 4.0</td>
<td>87.8 ± 15.0</td>
<td>145</td>
</tr>
<tr>
<td>plc-1(rx1);daf-16(mu86)</td>
<td>73.3 ± 25.2</td>
<td>56.4 ± 37.8</td>
<td>130</td>
</tr>
<tr>
<td>sem-5(RNAi)</td>
<td>92.5 ± 5.0</td>
<td>89.1 ± 7.7</td>
<td>600</td>
</tr>
<tr>
<td>sem-5(RNAi);daf-16(mu86)</td>
<td>75.9 ± 23.8</td>
<td>63.5 ± 27.1</td>
<td>224</td>
</tr>
<tr>
<td>mek-2(RNAi)</td>
<td>68.3 ± 14.5</td>
<td>45.7 ± 28.5</td>
<td>303</td>
</tr>
<tr>
<td>mek-2(RNAi);daf-16(mu86)</td>
<td>55.3 ± 17.2</td>
<td>34.9 ± 22.7</td>
<td>230</td>
</tr>
<tr>
<td>mph-1(RNAi)</td>
<td>81.0 ± 19.8</td>
<td>70.0 ± 30.8</td>
<td>190</td>
</tr>
<tr>
<td>mph-1(RNAi);daf-16(mu86)</td>
<td>63.1 ± 16.4</td>
<td>47.6 ± 27.6</td>
<td>261</td>
</tr>
<tr>
<td>par-1(RNAi)</td>
<td>70.5 ± 16.0</td>
<td>62.0 ± 20.8</td>
<td>290</td>
</tr>
<tr>
<td>par-1(RNAi);daf-16(mu86)</td>
<td>58.4 ± 6.8</td>
<td>32.8 ± 16.6</td>
<td>200</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = total no. of animals analyzed from at least 4 independent experiments. Unless otherwise noted, animals were grown at 20°C and the methodology used to induce anoxia was a Bio Bag placed at 20°C.

For all analyses, adult hermaphrodites were exposed to 3 days of anoxia. †A reduction of daf-16 function did not significantly decrease the long-term anoxia survival phenotype.

Wild-type males are long-term anoxia tolerant. There are several physiological differences between a hermaphrodite and a male C. elegans, including the production of oocytes and a self-fertilization event that occurs in the hermaphrodite. In wild-type fertile adult hermaphrodites the mature proximal oocyte enters into the spermatheca at ovulation and is fertilized; this cycle of oocyte ovulation and fertilization repeats about every 23 min for each gonad arm (43). The process of oocyte maturation, ovulation, and fertilization could be energetically costly to the hermaphrodite, whereas the C. elegans males do not have the same physiological constraint. To test the hypothesis that sex could affect long-term anoxia tolerance, we analyzed the survival rate of wild-type adult males exposed to long-term anoxia. We determined that wild-type adult males more difficult to interpret, because of the possible interactions between the insulin-like signaling pathway and the let-60/ras signaling pathway. To determine whether the anoxia tolerance phenotype observed in ksr-1(ok786) is dependent on daf-16, we produced a double mutant for phenotype analysis. The daf-16(mu86);ksr-1(ok786) double mutant had a larval arrest and larval lethality phenotype, suggesting that ksr-1 and daf-16 are genetically interacting and are synergistically required for larval development. Of the daf-16(mu86);ksr-1(ok786) animals that developed to adulthood, there was a slight decrease in ability to survive long-term anoxia; however, this decrease was not significantly different from ksr-1(ok786) animals. The plc-1(rx1);daf-16(mu86) animals were somewhat more sensitive to long-term anoxia but not significantly different from their respective controls (Table 5).

In metazoans the response to oxygen deprivation involves hypoxia-inducing factor (HIF)-1. Previously, it was shown that hif-1(la04) embryos do not survive hypoxia (1% or 0.5% O₂) but are able to survive 1 day of anoxia. Furthermore, hif-1(la04) larvae exposed to hypoxia do not have as a severe lethality phenotype as hif-1(la04) embryos exposed to hypoxia (26, 52). These data suggest that in C. elegans HIF-1 is specific for hypoxia viability and that additional factors play a role in anoxic and hypoxic survival in postembryonic animals. Similar to wild-type hermaphrodites, the hif-1(la04) adult hermaphrodite survives 1 day of anoxia (98.8% ± 1.4 survival rate, n = 168) but not 3 days of anoxia (Table 6). To determine whether hif-1 is required for long-term anoxia survival in animals with reduced progeny production, we used RNAi to reduce hif-1 in seven animals. We determined that hif-1(RNAi) did not suppress the phenotype of long-term anoxia tolerance (Table 6). Together, these findings suggest that hif-1 function is not required for long-term anoxia survival in C. elegans.

Table 6. Long-term anoxia tolerance is independent of hif-1 function or starvation

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Survival, %</th>
<th>Normal Motility, %</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2 (control food)</td>
<td>28.3 ± 4.2</td>
<td>20.8 ± 10.4</td>
<td>98</td>
</tr>
<tr>
<td>fog-2(q71)</td>
<td>97.9 ± 3.6</td>
<td>99.0 ± 1.2</td>
<td>200</td>
</tr>
<tr>
<td>glp-1(q158)</td>
<td>96.5 ± 3.0</td>
<td>92.0 ± 7.0</td>
<td>49</td>
</tr>
<tr>
<td>hif-1(RNAi)</td>
<td>17.9 ± 15.2</td>
<td>3.0 ± 2.9</td>
<td>99</td>
</tr>
<tr>
<td>fog-2(q71);hif-1(RNAi)</td>
<td>96.2 ± 4.9</td>
<td>93.3 ± 8.1</td>
<td>108</td>
</tr>
<tr>
<td>glp-1(q158);hif-1(RNAi)</td>
<td>98.3 ± 3.0</td>
<td>91.7 ± 17.4</td>
<td>47</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = total no. of animals analyzed from at least 3 independent experiments. Animals were grown at 20°C and the methodology used to induce anoxia was a Bio Bag placed at 20°C. Unless noted, for all experiments adult hermaphrodites were exposed to 3 days of anoxia. *Reduction in hif-1 function or starvation did not significantly decrease the long-term anoxia survival phenotype (P > 0.4).
exposed to long-term anoxia survived and maintained normal motility (Table 7). These males were grown in the presence of hermaphrodites and likely had an opportunity to mate, during their first day of adulthood, before being exposed to anoxia. Thus mating does not seem to affect the long-term anoxia survival in males. To determine whether long-term anoxia survival in males is dependant on male genotype (XO) or male phenotype, we exposed tra-2(q276) animals to long-term anoxia. Briefly, the tra-2(q276) males are morphologically male but genetically hermaphrodites (XX) (22). We determined that the tra-2(q276) adult males survive and have normal motility after long-term anoxia treatment (Table 7). Several malespecific phenotypes including longevity and resistance to pathogenic fungus are dependent on daf-16 (13, 70). We found that, similar to wild-type males, daf-16(mu86) males survive and have normal motility after anoxia treatment (Table 7), indicating that male long-term anoxia survival is daf-16 independent. Together our previous results and results presented in the present study suggest that long-term anoxia survival can be enhanced by daf-16-dependent and -independent mechanisms.

**Tissue morphology of long-term anoxia survivors.** Previously we showed that wild-type hermaphrodites exposed to 1 day of anoxia do not accumulate significant tissue damage. However, the few wild-type hermaphrodites that survived 3 days of anoxia accumulated significant tissue damage in the form of abnormal tissue morphology and an increase in cavities (45). Thus tissue morphology can be used as an additional parameter to distinguish the phenotype of animals that survive or those that are sensitive to long-term anoxia. We used DIC microscopy to analyze animals exposed to long-term anoxia. We examined the entire worm but focused on the head region of the animal, since this region has a distinct organ structure (pharynx) and diverse cell types. Figures 3 and 4 show that animals with high long-term anoxia survival rates [glp-4(bn2ts), fog-2(q71), FUDR treated, plc-1(rx1), ksr-1(ok786), mek-2(RNAi), mpk-1(RNAi), par-1(RNAi), sem-5(RNAi), and males] do not have severe tissue morphology abnormalities. In comparison, the few fer-15(hc15ts) animals that survive anoxia accumulate tissue morphology defects, including a bent pharynx structure and an increase in cavities (Fig. 3). Together, our results indicate that the animals with long-term anoxia survival phenotypes also have a greater capacity to maintain tissue structure and function.

**DISCUSSION**

*C. elegans* is tolerant to 1 day of anoxia, but prolonged anoxic exposure results in decreased survival and tissue abnormalities. In *C. elegans* there are a variety of known genetic mutations that disrupt reproduction. These attributes make *C. elegans* an ideal model system to test the hypothesis that a reduction in progeny enhances anoxia tolerance. In this study we used genetic mutants, RNAi, and the chemical FUDR to reduce the number of progeny produced.

**Oocyte maturation and ovulation influence anoxia survival.** We found that hermaphrodites with decreased progeny production, due to genetic mutation or feeding with the chemical FUDR, survive long-term anoxia better than wild-type animals. The exception to this includes sterile animals in which ovulation occurs and unfertilized oocytes are laid [spe-9(hc52ts), fer-15(hc15), and 5-fluorodeoxyuridine (FUDR)-fed wild-type adult hermaphrodites and wild-type adult males exposed to long-term anoxia, fer-15(hc15) animals, examined 24 h after long-term anoxia treatment, contained the most morphological abnormalities and an increase in cavities within the head region (arrows). Scale bar = 25 μm.

![Fig. 3. Tissue morphology of wild-type, glp-4(bn2ts), fog-2(q71), fer-15(hc15), and 5-fluorodeoxyuridine (FUDR)-fed wild-type adult hermaphrodites and wild-type adult males exposed to long-term anoxia. fer-15(hc15) animals, examined 24 h after long-term anoxia treatment, contained the most morphological abnormalities and an increase in cavities within the head region (arrows). Scale bar = 25 μm.](image-url)

**Table 7. Males survive long-term anoxia**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Survival, %</th>
<th>Normal Motility, %</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type male</td>
<td>98.8±1.6</td>
<td>97.1±3.0</td>
<td>666</td>
</tr>
<tr>
<td>tra-2(q276)</td>
<td>94.9±5.3</td>
<td>82.9±10.8</td>
<td>154</td>
</tr>
<tr>
<td>daf-16(mu86)</td>
<td>98.4±3.3</td>
<td>97.4±3.9</td>
<td>111</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = no. of animals analyzed from at least 4 independent experiments. Animals were grown at 20°C, and the methodology used to induce an anoxic environment was a nitrogen flow-through chamber at 20°C.
sterile mutants that survive long-term anoxia do so by independent mechanisms. That is, the molecular changes that allow a fog-2(q71) female to survive anoxia may be different from the mechanisms that regulate glp-1(q158) mutants' ability to survive anoxia. However, it is clear that there is a relationship between decreased progeny production and long-term anoxia survival.

In the wild-type hermaphrodite, the process of oocyte maturation and ovulation is likely to be energetically expensive. Oocyte maturation involves cellular changes within the oocyte, including nuclear envelope breakdown. The process of ovulation, which immediately follows oocyte maturation, involves the movement of the oocyte so that fertilization can occur. Specifically, contraction of gonadal sheath cells, dilation of the spermatheca, and signals from the maturing oocyte allow entry of the oocyte into the spermatheca so fertilization can occur. In wild-type animals, as ovulation and fertilization proceed in an assembly-like fashion, additional germ cells must progress through meiosis and differentiate into oocytes, and yolk must be transported from the intestines to be eventually taken up during oogenesis. All of these processes require energy and alter the physiology of the animal.

If the sperm can initiate ovulation but not result in fertilization, as in spe-9(hc52ts) or fer-15(hc15ts) hermaphrodites, the unfertilized oocyte will leave the spermatheca and be laid; germ cells will continue to differentiate into oocytes, which can then go through ovulation. These sterile mutants [spe-9(hc52ts) or fer-15(hc15ts)] that lay unfertilized oocytes do not survive long-term anoxia. Furthermore, we found that if sperm, whether functional sperm [wild-type or fog-2(q71) males] or sperm that cannot fertilize oocytes but signals ovulation to occur [spe-9(hc52ts) males], is mated into the fog-2(q71) female, the fog-2(q71) female will not be able to survive long-term anoxia. These results indicate that the physiological change of increasing oocyte maturation and ovulation suppresses long-term anoxia tolerance.

MSP stimulates oocyte maturation, ovulation, and contraction of gonadal sheath cells and reorganizes oocyte microtubules (18, 46). Thus it is possible that suppression of MSP function could be involved in the signals that enhance anoxia response. It is thought that the MSP signal can induce somatic sheath cell contraction by acting as a VAB-1 agonist and promote oocyte maturation and ovulation by antagonizing ephrin/Eph receptor (EPN-2/VAB-1) and CEH-18 (47). In several of the mutants [glp-1(q158), glp4(bn2ts), fog-2(q71)] that survive long-term anoxia MSP is likely to be absent, given that no sperm are present. The spe-9(hc52ts) and fer-15(hc15) mutants, which do not survive long-term anoxia, lay unfertilized oocytes likely triggered by MSP signal. Thus there may be a relationship between long-term anoxia survival and a suppression of MSP activity. However, the fem-3(q20) animals, which have sperm but no oocytes, survive long-term anoxia, suggesting that it is not the mere absence of MSP that correlates with long-term anoxia survival.

In a study by others, it was shown that in wild-type adult hermaphrodites the rate of sheath contraction cycles and peaks when the spermatheca dilates before ovulation (43). The rate of sheath contraction significantly decreases and does not peak in fog-2(q70) females, whereas in the fem-3(q20) animal sheath contraction is constant and higher than that of fog-2(q70) females but, unlike wild-type hermaphrodites, sheath contraction does not peak (43). These preliminary studies suggest that long-term anoxia survival does not correlate with a significant decrease in gonadal sheath contraction, but there may be a relationship between long-term anoxia survival and animals that have either no or an infrequent peak in sheath contraction.

We found that the oocyte maturation and ovulation rate of wild-type animals is reduced during anoxia exposure. Thus it is probably not a reduction in ovulation during exposure to anoxia that is having an effect on long-term anoxia viability. Rather, it is possible that the physiological state of the animal before anoxia exposure has a role in how well the animal survives. One interpretation of our findings is that animals with a reduction in energetically requiring processes, such as ovu-
lation, have altered energetic or metabolic states and are thus better able to survive the stress of long-term anoxia. Furthermore, the genetic mutants that survive long-term anoxia may have more available resources to maintain tissue structure and function when challenged with anoxia. However, our findings suggest that starvation will not alter the physiology such that suppression of long-term anoxia survival occurs.

Long-term anoxia survival observed in sterile mutants is independent of daf-16. Our previous studies (45), and studies described here, indicate that there are daf-16-dependent and -independent mechanisms that enhance long-term anoxia survival. We previously showed that daf-16 is required for the daf-2(e1370) long-term anoxia survival phenotype. However, in the present studies we show that daf-16 is not required for sterile mutants [e.g., glp-1(q158)] to survive long-term anoxia. The functional role that daf-16 has in regulating long-term anoxia survival in mutants that are known to be involved in the RTK/Ras/MAPK signaling pathway (e.g., mkp-1, ksr-1) may be more difficult to interpret. First, we observed developmental phenotypes (larval arrest and larval lethality) in the ksr-1(ok786);daf-16(mu86) hermaphrodite, indicating that both of these genes are required for normal development. The developmental phenotype observed in ksr-1(ok786);daf-16(mu86) hermaphrodites is not completely penetrant, and there were some hermaphrodites that reached adulthood and were able to survive 3 days of anoxia, supporting the idea that mechanisms independent of insulin-like signaling can enhance anoxia survival. It is possible that several factors contribute to the long-term anoxia survival phenotype observed in sterile mutants known to be involved in the RTK/Ras/MAPK signaling pathway. That is, the tolerance to long-term anoxia observed in the ksr-1 and plc-1 mutants may be due to a decrease in oocyte maturation and ovulation. However, it is possible that these mutations affect other processes, independent of or in addition to the decreased reproductive phenotype, that make the animals less susceptible to long-term anoxia.

There are several possible reasons why a reduction in oocyte maturation and ovulation would be conducive for long-term anoxia survival. First, the metabolism of these animals may be different from that of wild-type animals, and thus specific resources may be available to the hermaphrodite when challenged with the stress of anoxia. Second, it is possible that a decrease in oocyte maturation and ovulation changes the physiology of tissues such that they are less susceptible to long-term anoxia. Third, the availability of specific molecules within cells and tissues may be altered, resulting in long-term anoxia survival. For example, hermaphrodites that have reduced oocyte maturation and ovulation tend to have an increase in available egg yolk. Others have shown that an increase in a yolk precursor gene activity, vitellogenin, protects the honeybee against oxidative damage (61), suggesting that yolk precursor or yolk levels may influence long-term anoxia response and survival in hermaphrodites. Regardless, to gain a greater understanding of oxygen deprivation survival it will be of interest to determine the molecular mechanism that regulates long-term anoxia tolerance in animals with reduced oocyte maturation and ovulation phenotypes.

Long-term anoxia survival and male phenotype. In this study we showed that males, compared with hermaphrodites, are better able to survive long-term anoxia. The mechanism regulating long-term anoxia survival in males is independent of daf-16 function but is dependent on male phenotype and not male genotype. This further supports the idea that there are molecular mechanisms, independent of the insulin-like signaling pathway, that can enhance anoxia survival. Furthermore, this provides more evidence that sex can affect stress response and survival. The underlying mechanism of male anoxia survival is not understood, but there are several possible reasons why males may survive long-term anoxia better than hermaphrodites. First, perhaps the metabolism in hermaphrodites and males is different, such that males have more energy stores available for surviving the stress of long-term anoxia. The male may have more energy stores available because energetically expensive processes such as oogenesis, oocyte maturation, ovulation, and fertilization do not occur in this sex. Second, the male has a different gene expression profile compared with hermaphrodites, which may contribute to enhanced anoxia survival. It will be of interest to further understand the molecular mechanism regulating long-term anoxia survival in male C. elegans.

Reproduction function, longevity, and stress resistance. Research shows that reproduction and sex are linked to aging and stress resistance (14, 42, 48, 70). Genetic analysis in C. elegans, including phenotype analysis of daf-2 mutants, indicates that longevity and stress resistance are regulated by similar mechanisms. In this study we show that reproduction and sex also affect long-term anoxia survival. The mechanism(s) regulating long-term anoxia survival, in both animals with reduced progeny and males, is independent of daf-16 function. Therefore, the insulin-like signaling pathway is probably just one of several pathways that can be modulated to enhance anoxia survival. Mutants with reproductive phenotypes, such as the fog-2(q71) animals, are not long lived, but we show that they have an enhanced ability to survive long-term anoxia. Conversely, some sterile mutants such as glp-1(q158) are both long-lived and have a long-term anoxia survival phenotype. Together, these findings suggest that the mechanisms that regulate longevity and long-term anoxia overlap but are not identical.

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