

Glycogen Fuels Survival During Hyposmotic-Anoxic Stress in *Caenorhabditis elegans*

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ABSTRACT Oxygen is an absolute requirement for multicellular life. Animals that are deprived of oxygen for sufficient periods of time eventually become injured and die. This is largely due to the fact that, without oxygen, animals are unable to generate sufficient quantities of energy. In human diseases triggered by oxygen deprivation, such as heart attack and stroke, hyposmotic stress and cell swelling (edema) arise in affected tissues as a direct result of energetic failure. Edema independently enhances tissue injury in these diseases by incompletely understood mechanisms, resulting in poor clinical outcomes. Here, we present investigations into the effects of osmotic stress during complete oxygen deprivation (anoxia) in the genetically tractable nematode *Caenorhabditis elegans*. Our findings demonstrate that nematode survival of a hyposmotic environment during anoxia (hyposmotic anoxia) depends on the nematode's ability to engage in glycogen metabolism. We also present results of a genome-wide screen for genes affecting glycogen content and localization in the nematode, showing that nematode survival of hyposmotic anoxia depends on a large number of these genes. Finally, we show that an inability to engage in glycogen synthesis results in suppression of the enhanced survival phenotype observed in *daf-2* insulin-like pathway mutants, suggesting that alterations in glycogen metabolism may serve as a basis for these mutants' resistance to hyposmotic anoxia.

KEYWORDS glycogen; anoxia; osmotic stress; insulin signaling; *C. elegans*

OXOGEN is an essential and defining element of metazoan life. Nearly all known animal species require oxygen to complete their life cycles, a fact that is easily understood through oxygen's fundamental role in respiration (Ernster and Schatz 1981). In spite of this, however, there is a broad range of tolerance for periods of complete oxygen deprivation (anoxia) within the animal kingdom (Bickler and Buck 2007; Ramirez *et al.* 2007). While most mammals are relatively sensitive to anoxia, displaying brain and heart injury after only minutes or hours following the removal of oxygen (Miller 1949; Jennings *et al.* 1986; Leblond and Krnjevic 1989), many fish and turtle species are capable of surviving anoxia for much longer (Bickler and Buck 2007). The painted turtle *Chrysemys picta*, for example, enters a dormant state during winter and is

capable of surviving in the absence of oxygen for up to 4 months (Ultsch 1985). The crucian carp *Carassius carassius*, another excellent animal facultative anaerobe, sustains cardiac pumping and autonomic cardiovascular regulation during severe hypoxia (<0.01 mg O₂ per liter) for several days (Stecyk *et al.* 2004). Adaptations in these species that are not present in anoxia-sensitive animals should be instructive about specific survival mechanisms during oxygen deprivation. Once characterized, these mechanisms may then be employed as the basis for the development of therapies for human diseases triggered by clinical oxygen deprivation (ischemia) such as stroke and myocardial infarction (Bickler 2004).

During ischemia, injury is often enhanced and even directly triggered by increased intracellular water volume, which leads to cell and tissue swelling (edema). This is a consequence of reduced ATP supply and a direct result of failure of ATP-dependent (active) mechanisms of ion transport such as the Na/K ATPase (Simard *et al.* 2007; Garcia-Dorado *et al.* 2012; Khanna *et al.* 2014). In stroke, for instance, Na/K ATPase failure results in rapid loss of ion homeostasis and subsequent swelling of brain cells in a process that is referred to as cytotoxic cerebral edema (CCE). If allowed to progress, CCE can

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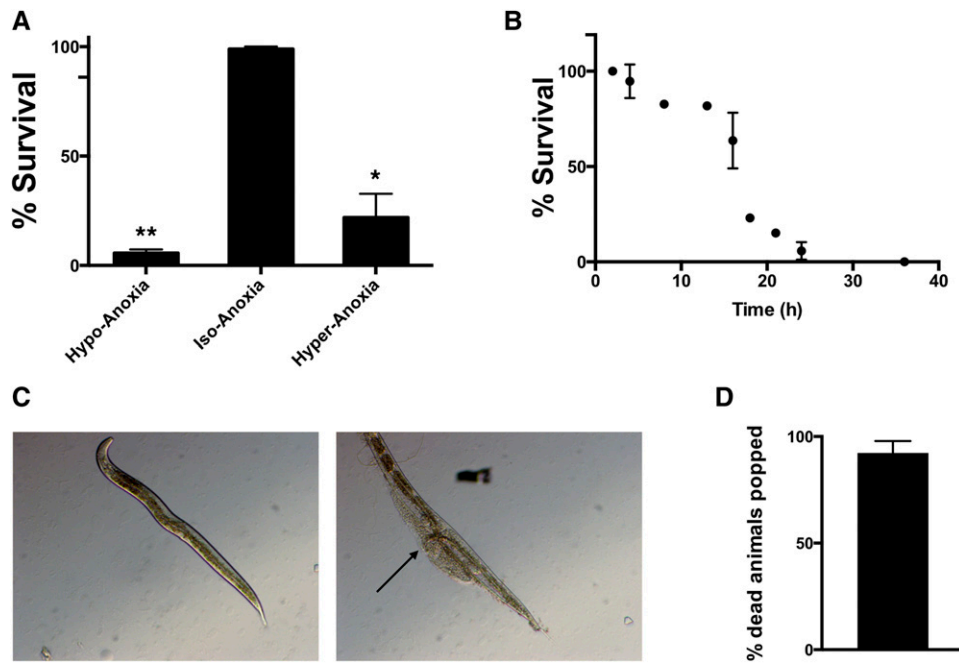


Figure 1 Osmotic stress sensitizes wild-type (*N2*) animals to anoxia. (A) Percent survival of *N2* animals after exposure to 24-hr anoxia while on media that was hyposmotic (hypo; 10 mOsm), isosmotic (iso; 200 mOsm), or hyperosmotic (hyper; 600 mOsm) relative to conventional nematode growth medium (~200 mOsm) on which animals were raised. (B) Percent survival of *N2* animals in hyposmotic anoxia at indicated time points. (C) Representative micrographs of *N2* L4 animals on hyposmotic media before (left) and after (right) lethal hypoxic exposure. Arrow points to rupture of intestinal and gonadal tissue through vulva (“popped” phenotype). (D) Percentage of dead *N2* L4 animals that exhibit popped phenotype following a 24-hr exposure of hyposmotic anoxia. All experimental data represent a minimum of three independent trials with at least 20 animals. Errors bars represent \pm standard error of the mean. * $P < 0.05$, ** $P < 0.01$ (two-tailed *t*-test).

lead to an increase in intracranial pressure that further impedes brain perfusion and can perpetuate a downward cycle of ischemic edema (Kahle *et al.* 2009). Edema is associated with poor clinical outcomes in acute myocardial infarction and other ischemic diseases as well (Garcia-Dorado *et al.* 2012), suggesting that osmotic stress is a general consequence of oxygen and nutrient deprivation.

The genetically tractable nematode *Caenorhabditis elegans* can survive anoxia (<10 ppm O₂) for more than 24 hr with no apparent injury (Van Voorhies and Ward 2000). During anoxia, *C. elegans* enters a reversible state of suspended animation that is characterized by the cessation of all observable life processes, including motility, feeding, and reproduction (Padilla *et al.* 2002). Because the suspended nematode does not feed, it is dependent on preexisting energy stores. Previous work in *C. elegans* has implicated several pathways in the regulation of anoxia survival, including *daf-2* insulin-like signaling and protein translation (Anderson *et al.* 2009; Mabon *et al.* 2009). Whether these pathways converge on a general mechanism for survival is completely unknown. We have previously developed *C. elegans* as a model for studying glycogen storage and metabolism during anoxia, showing that nematode glycogen stores are modulated by environmental cues in a *daf-2*-dependent fashion (Frazier and Roth 2009). In the present study, we sought to model osmotic-anoxic stress in the *C. elegans* for the purpose of identifying mechanisms that regulate survival in this environment. In conjunction with the results from a genome-wide screen for nematode glycogen storage genes (GSGs), we demonstrate that survival in an hyposmotic-anoxic environment requires functional glycogen synthesis and breakdown pathways. Furthermore, we demonstrate that signaling through the *daf-2* insulin/IGF pathway modulates glycogen consumption rates during hyposmotic anoxia in

a manner that is directly correlated with animal survival times.

Materials and Methods

Nematode strains and culture

Nematodes were grown at 20° on nematode growth medium (NGM)-lite agar seeded with *OP50* bacteria and manipulated using standard protocols (Brenner 1974). Worms were used only from populations that had not experienced a starvation event in the last week. The following strains were acquired from the *Caenorhabditis* Genetics center: *N2* (wild-type var. Bristol); *CB1370* (*daf-2(e1370)III*); *DR1309* (*daf-2(e1370) III; daf-16(m26) I*); and *RB1169* (*daf-18(e1375)IV*).

Oxygen deprivation experiments

All oxygen deprivation experiments were performed at an incubation temperature of 20°. Anoxic atmospheres were generated using airtight chambers and 100% nitrogen gas as previously described (Miller and Roth 2009). Immediately prior to anoxia exposure, L4 *C. elegans* were transferred via wire pick to either NGM (~100 mOsm) or anisomotic media containing varying concentrations of NaCl. For hyposmotic-anoxia experiments, animals were transferred via wire pick to media containing 2% agar + 5 mM potassium phosphate buffer (~10 mOsm) seeded with live *OP50 Escherichia coli*. Following anoxia, animals were returned to room air, allowed to recover for at least 6 hr, and scored for survival within 12 hr. Animals exhibiting major trauma (“popped” phenotype) were scored as dead. Animals in which visual trauma was not apparent were scored as dead if they were unresponsive to 30 sec of gentle touch with a platinum wire. For glycogen quantification experiments,

animals were returned to room air and immediately stained with iodine.

Glycogen staining of nematodes with iodine

We previously described a rapid assay for glycogen by exposure of nematodes to iodine (Frazier and Roth 2009). For primary screening and subsequent rescreening, animals were stained for 1 min with 50–100 μ l of diluted (1:15) Lugol's iodine solution (2% I₂ in 4% KI) before viewing. For photographing individual animals, live nematodes on agarose pads were inverted over the opening in a 100-g bottle of iodine crystals (Sigma) and allowed to sit for 40–60 sec before viewing. Photographs were taken with a Zeiss MRc color camera. Quantification of glycogen staining by iodine was performed using mean stain intensity and animal area as previously described (LaMacchia and Roth 2015).

RNA interference experiments

RNA interference (RNAi) experiments were conducted by feeding worms *E. coli* HT115 clones that express double-stranded RNA corresponding to a particular gene. These strains were obtained from a commercially available *C. elegans* RNAi library (MRC Geneservice). The library contains ~17,000 clones that target about 86% of the open reading frames in the nematode. Adult animals were transferred to RNAi feeding plates and allowed to reproduce. F₁ progeny were used for all phenotypic analyses.

Glycogen storage screen

Approximately 50 synchronized L1 worms were grown on RNAi or empty vector control food in blinded 24-well plates as described (Hanover *et al.* 2005) from L1 to young adult (48 hr at room temperature) before being stained with iodine. Animals that did not reach adulthood were excluded from the screen. Targets from the primary screen that produced glycogen staining phenotypes deviant from controls in terms of either density or localization were cloned into a new sublibrary and this library was rescreened three additional times in a blinded manner. The rescreens included four empty-vector control wells in each plate. During the final rescreen, photographs were taken of each well of stained nematodes. Clones that displayed a consistent phenotype in three of four independent, blinded trials were considered for further analysis. We also documented any secondary phenotypes (such as sterility or egg-laying defective) that were observed for each clone.

Enzymatic determination of glycogen content

Approximately 4000 starved L1 larvae hatched overnight from bleached adults were grown 48 hr at room temperature on 15-cm NGM-lite plates. Worms were washed off in M9, centrifuged at 1500 relative centrifugal force (RCF) for 1 min, washed twice with water and centrifuged again. A total of 100 μ l of concentrated worms was moved to a screw-top 1.5-ml centrifuge tube (VWR) and frozen in liquid nitrogen. To improve accuracy of worm transfer, we

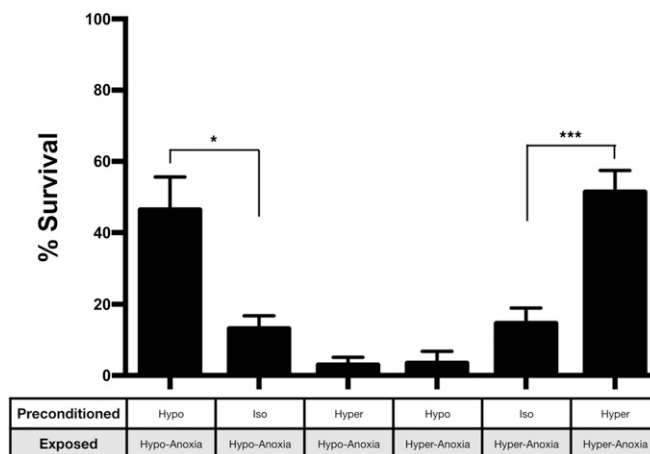


Figure 2 Osmotic preconditioning in normoxia significantly alters survival rates in anoxia. Wild-type (N2) L4 animals were preconditioned for 24 hr in normoxia on hyposmotic (hypo), isosmotic (iso), or hyperosmotic (hyper) media. Shown are percent survival rates for these animals after 24 hr in anoxia while on hyposmotic (hypo), isosmotic (iso), or hyperosmotic (hyper) media. Experimental data represent a minimum of three independent trials, each with at least 10 animals. Errors bars denote \pm standard error of the mean. * P < 0.05, *** P < 0.001 (two-tailed t -test).

used pipette tips with a wide opening that were blocked with 1% BSA before use. Half of the frozen aliquot was added to an equal volume of 2% SDS, boiled, and protein content determined by BCA assay (Pierce). A total of 100 μ l of 30% NaOH was added to the other half of the pellet and boiled for 1 hr. After centrifugation, the supernatant was moved to a new tube and an equimolar amount of HCl was added to neutralize the solution, after which two volumes of ice-cold ethanol was added to precipitate the glycogen. The pellet was washed twice with 66% ethanol and dried. The pellet was resuspended in 400 μ l 50 mM sodium acetate, 5 mM CaCl₂ (pH 5.0) and digested with 30 μ g amyloglucosidase and 2 μ g amylase at 56° for 12 hr. The glucose concentration of the samples was then determined enzymatically by the hexokinase method (Sigma).

Data availability

Strains are available upon request. Table S1 contains detailed phenotypic data from glycogen storage screen.

Results

We found that exposing *C. elegans* to combined osmotic-anoxic stress results in significantly decreased animal survival compared to osmotic stress or anoxia alone. While on isosmotic media, wild-type (N2) animals display >95% survival after 24 hr in anoxia (Figure 1A). Survival decreases by 10- to 20-fold, however, if animals are placed on media for the duration of anoxia that is hyposmotic or hyperosmotic relative to the media on which the animals were raised (Figure 1, A and B). Survival rates in normoxia are not significantly different across these three different osmotic environments (all >95%), suggesting that the lethality

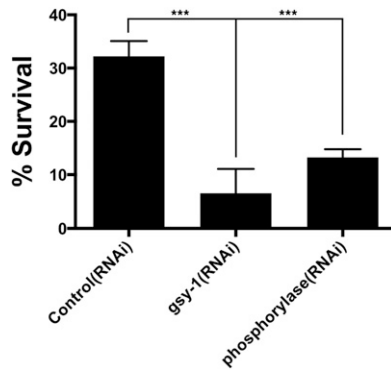


Figure 3 Glycogen synthesis and breakdown are required for survival of a hypotonic-anoxic environment. Survival of control(RNAi), *gsy-1*(RNAi), and *phosphorylase*(RNAi) L4 animals following 18-hr exposure to hypotonic anoxia (hypo-anoxia). Data represent means of at least five independent experiments. Error bars denote \pm standard error of the mean. *** $P < 0.001$ (two-tailed *t*-test).

observed in hypo-/hyperosmotic-anoxia arises as a result of synergy between osmotic and anoxic stresses. Interestingly, in hypotonic anoxia, the vast majority of animals that die (>95%) display a rupturing (“popping”) of gonadal and intestinal tissue through the outer cuticle (Figure 1, C and D). This phenotype is highly indicative of tissue swelling due to water influx. To determine whether adaptation to altered osmotic environments can precondition animals to survive oxygen deprivation in that same environment, we cultured wild type (N2) animals on hypotonic, isosmotic, or hyperosmotic media for 24 hr prior to anoxia exposure. We found that preconditioning animals in hypotonic or hyperosmotic media increased their survival of the same osmotic condition while in anoxia by nearly 10-fold (Figure 2). The same type of osmotic stress (hypotonic or hyperosmotic) was required for effective preconditioning, suggesting that animals were adapting to the direction of the osmotic gradient.

We next hypothesized that glycogen metabolism might play an important role in nematode survival during hypotonic anoxia. To test this, we treated wild type (N2) *C. elegans* with RNAi targeting the nematode homologs of glycogen synthase (*gsy-1*) or phosphorylase (T22F3.3). Previous work in our lab has demonstrated that knockdown of the glycogen synthase homolog *gsy-1* in *C. elegans* significantly reduces animal glycogen content (Frazier and Roth 2009). Consistent with this, *gsy-1*(RNAi) animals exhibit dramatically reduced glycogen staining by iodine compared to controls (Supporting Information, Figure S1A). In contrast, T22F3.3(RNAi) animals exhibit staining patterns indistinguishable from controls (Figure S1B). We found that, after 18 hr in hypotonic anoxia, the survival rates of both *gsy-1*(RNAi) and T22F3.3(RNAi) animals were significantly reduced compared to RNAi controls (Figure 3). These data suggest that nematode survival in hypotonic anoxia requires functional glycogen synthesis and breakdown.

In *C. elegans*, *daf-2* codes for an insulin/IGF receptor homolog that signals through a conserved PI-3 kinase pathway

to regulate gene expression (Kenyon *et al.* 1993; Morris *et al.* 1996; Ogg *et al.* 1997; Lin *et al.* 2001). The PTEN homolog *daf-18* negatively regulates *daf-2* activity (Ogg and Ruvkun 1998; Gil *et al.* 1999). Reductions in *daf-2* signaling result in nuclear translocation of the forkhead transcription factor *daf-16* and have been associated with resistance to a variety of stresses, including long-term (>3 days) anoxia (Mendenhall *et al.* 2006) and high-temperature hypoxia (Scott *et al.* 2002). While mutations in *daf-16* have been shown to suppress the effects of *daf-2* mutations in certain contexts (Lin *et al.* 1997; Ogg *et al.* 1997; Lamitina and Strange 2005), this is not always the case (Mendenhall *et al.* 2009), and distinct physiologic mechanisms for *daf-2*-mediated stress resistance have remained elusive (Mabon *et al.* 2009). We found that hypomorphic *daf-2*(*e1370*) mutants exhibit an enhanced survival rate in hypotonic anoxia, such that survival of *e1370* animals is >15 times greater than that of N2 animals after 24 hr (Figure 4A). We also found that this enhanced survival phenotype is suppressed in the presence of the *daf-16*(*m26*) loss-of-function mutation (Figure 4A). Conversely, loss-of-function *daf-18*(*e1375*) mutants display significantly reduced survival relative to wild-type animals following 16-hr exposure to hypotonic anoxia (Figure 4B). These data strongly suggest that reductions in *daf-2* signaling promote survival in hypotonic anoxia. Because previous studies have shown that *daf-2*(*e1370*) animals exhibit increased body glycogen content (Frazier and Roth 2009), we hypothesized that the *daf-2* enhanced survival phenotype hypotonic anoxia depends on intact glycogen metabolism. To test this, we performed RNAi on *gsy-1* and glycogen phosphorylase in *daf-2*(*e1370*) animals and measured the survival rates of these knockdowns in hypotonic anoxia. We found that while *gsy-1* knockdown reduced *daf-2*(*e1370*) survival to that of wild type, glycogen phosphorylase knockdown produced no changes in *daf-2*(*e1370*) survival (Figure 5). These data suggest that, in the context of reduced *daf-2* signaling, glycogen synthesis is relatively more important than glycogen consumption for survival of hypotonic anoxia.

A possible explanation for why phosphorylase knockdown produced no survival rate changes in the *e1370* mutant is that glycogen breakdown rates have already been reduced to baseline levels. To test this hypothesis, we employed the previously described technique of quantitative iodine staining (LaMacchia and Roth 2015) to measure glycogen content changes over the course of hypotonic anoxia in N2 and *daf-2* pathway mutant animals. Interestingly, we found that rates of glycogen utilization during hypotonic anoxia are significantly decreased for *daf-2* mutant animals relative to wild type in a manner that is dependent on *daf-16* (Figure 6). Conversely, *daf-18* mutant animals displayed a significantly increased glycogen consumption rate relative to wild type (Figure 6). This suggests that insulin-like signaling in the nematode regulates glycogen demand during hypotonic anoxia and that this demand is a major determinant of nematode survival limits in this environment.

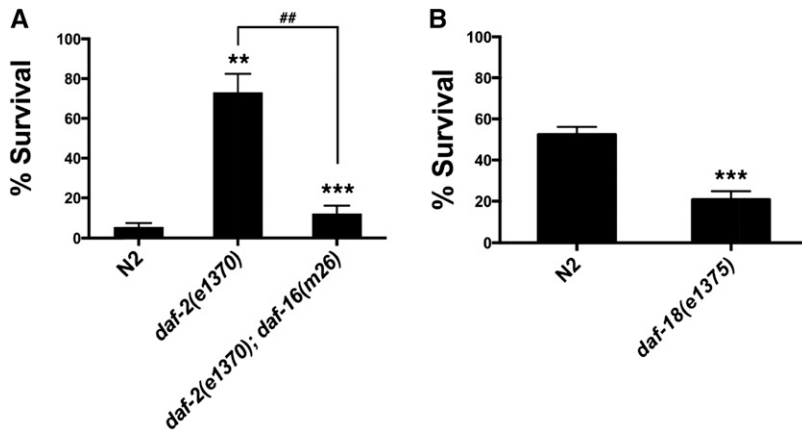


Figure 4 Nematode insulin-IGF signaling suppresses survival in hyposmotic-anoxia. (A) Percent survival of *daf-2(e1370)* and *daf-2(e1370);daf-16(m26)* mutant L4 animals following 24 h exposure to hyposmotic-anoxia. (B) Percent survival of *daf-18(e1375)* L4 mutant animals following 16 h exposure to hyposmotic-anoxia. All experimental data represent a minimum of four independent trials with at least 20 animals. Errors bars represent \pm standard error of the mean. ** $P < 0.01$ vs. N2, ### $P < 0.01$ *daf-2(e1370)* vs. *daf-2(e1370);daf-16(m26)*, *** $P < 0.001$ vs. N2 (two-tailed *t* test).

Given the apparent dependence of nematode hyposmotic-anoxia survival on glycogen metabolism, we performed a genome-wide RNAi screen for genetic knockdowns that altered animal glycogen content. We used an RNAi library that covers $\sim 86\%$ of the genes in the *C. elegans* genome to assess the influence of these genes on the ability of young adult nematodes to accumulate glycogen. The primary screen identified 640 RNAi targets that had a possible effect on glycogen storage. These were classified into four major phenotypes: more glycogen and normal localization (+), less glycogen and normal localization (-), more glycogen, including prominent glycogen in hypodermal or gut cells (G/H), and abnormal localization of glycogen (Ab). Figure 7 A–D shows examples of the range of glycogen storage phenotypes observed. Of the 640 phenotypic knockdowns, 264 maintained their phenotype in three of four trials (Table S1). We chose to use this set for further analysis. We designed this screen to minimize false positive results. Subscreens conducted >1 year after the primary screen indicates that the false positive rate is $<5\%$. Due to the fact that the entire library was not screened more than once, the false negative rate is likely to be substantial. As additional verification of the validity of the iodine stain for identifying changes in glycogen accumulation, we used an enzymatic assay to determine the glycogen level in several of the RNAi-treated strains from this screen (Figure S1). These results verify iodine as a stain for glycogen and sug-

gest that it can detect differences in glycogen storage that are less than twofold altered from wild type.

To draw conclusions regarding the impact of particular biological processes on glycogen storage in the worm, we categorized the genes that showed effects by their biological function and phenotype (Figure 7E and Table S1). The single largest class of genes (19%) identified in the screen are involved in metabolism. Of these, 92% have a (-) phenotype, indicating that, in general, glycogen is decreased when metabolic gene expression is knocked down (z -test $P < 0.001$). Of the metabolic genes, 51% are components of the electron transport chain, including 10 RNAi clones each against subunits of complex I and V. This category also contains several genes that directly influence glycogen synthesis, such as glycogen synthase, glycogen branching enzyme, and UDP-glucose pyrophosphorylase. The phenotype observed upon knockdown of glycogen branching enzyme (a blue shift in the color of the iodine–glycogen complex) is indicative of an decrease in glycogen branch length (Bailey and Whelan 1961) and may be relevant to human glycogen branching enzyme (*GBE1*) deficiency, in which disease phenotypes (progressive liver cirrhosis leading to liver failure, muscular atrophy, hypotonia, nervous system dysfunction) are correlated with the degree of deposition of an insoluble, poorly branched form of glycogen in tissues (Bao *et al.* 1996; Raju *et al.* 2008). Protein homeostasis also appears to have a major influence over glycogen accumulation in the

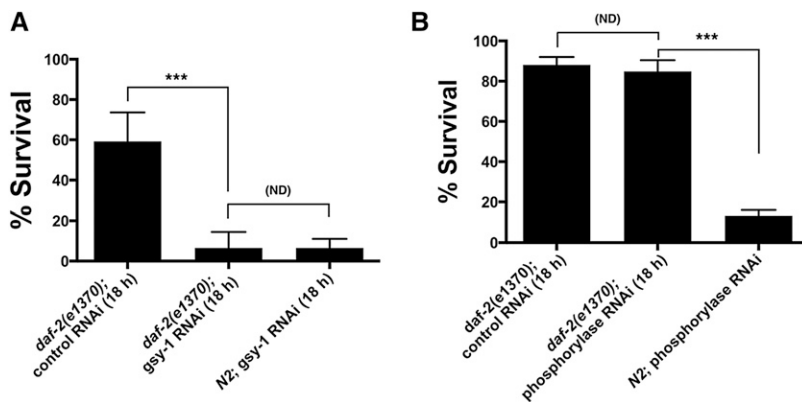


Figure 5 The *daf-2(e1370)* enhanced survival phenotype in hyposmotic anoxia depends on glycogen synthesis but not glycogen breakdown. (A) Survival of *daf-2(e1370); control(RNAi)*, *daf-2(e1370); gsy-1(RNAi)*, and N2 *gsy-1(RNAi)* L4 animals following 18-hr exposure to hyposmotic anoxia. (B) Survival of *daf-2(e1370); control(RNAi)*, *daf-2(e1370); phosphorylase(RNAi)*, and N2 *phosphorylase(RNAi)* L4 animals following 18-hr exposure to hyposmotic anoxia. Data represent means of at least five independent experiments. Error bars denote \pm standard error of the mean. *** $P < 0.001$ (two-tailed *t*-test).

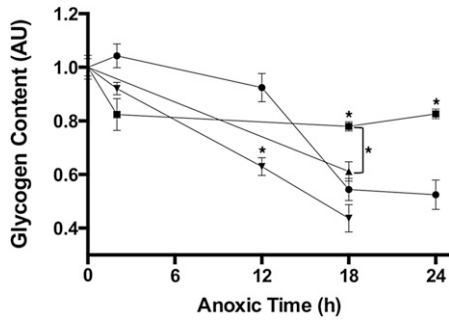


Figure 6 Activity of nematode insulin/IGF signaling pathway influences rate of glycogen consumption in hyposmotic anoxia. Quantification of glycogen staining in N2 (circles), *daf-2(e1370)* (squares), *daf-2(e1370); daf-16(m26)* (upright triangles), and *daf-18(e1375)* (inverted triangles) animals at indicated time points during hyposmotic-anoxia (HA) exposure. Each data point represents measurements of at least five HA-exposed animals normalized to normoxic controls. Error bars denote \pm standard error of the mean. * $P < 0.05$ (two-tailed *t*-test) for *daf-2(e1370)* or *daf-18(e1375)* vs. N2, or *daf-2(e1370); daf-16(m26)* vs. *daf-2(e1370)* (bracket).

nematode. Of the genes identified in the screen, 10% are annotated as being involved in protein synthesis (Figure 7E and Table S1). This group includes seven tRNA charging enzymes, 14 genes involved with ribosomal biogenesis or

ribosomal components themselves, and four translation initiation factors. A total of 73% of these knockdowns resulted in an increase in glycogen storage, which is a significant enrichment of this phenotype in this class of genes (z -test $P < 0.001$). Another 11% of genes were classified as contributing to protein stability. This group included 17 proteasomal subunits, all of which had a (-) phenotype, and five subunits of the chaperonin complex, all of which were characterized as having abnormal glycogen storage. The trafficking category contains several groups of RNAi clones that influence the same biological process. We identified 10 subunits of the vacuolar V-ATPase, all of which had less glycogen. This is consistent with a previous screen for glycogen storage in yeast (Wilson *et al.* 2002) and highlights the importance of autophagy as a means of glycogen generation. Finally, we also identified a group of 10 genes involved with nuclear import/export, which had a range of phenotypes, and 14 genes involved with the budding and transport of vesicles, all of which had a (-) phenotype.

We hypothesized that GSG knockdowns would also exhibit survivorship differences in hyposmotic anoxia relative to controls. To test this, we performed RNAi in wild-type (N2) *C. elegans*, targeting each of the 33 (+) GSGs as well as a randomly selected subset of the (-) GSGs, and then

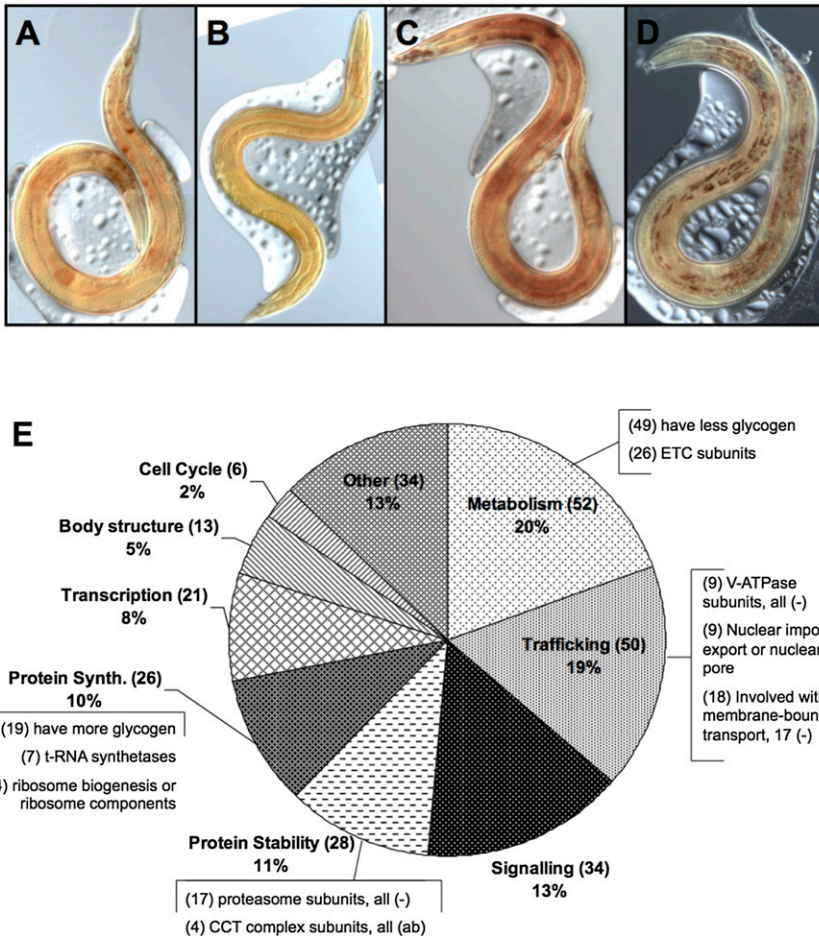


Figure 7 (A–D) Examples of several of the phenotypes observed in an RNAi screen for genes that alter glycogen storage in *C. elegans*. (A) An animal fed empty vector bacteria as a control. (B) Glycogen synthase (*gsy-1*) knockdown with a low glycogen (-) phenotype. (C) Tryptophan tRNA-synthetase (*wrs-1*) with a high glycogen/gut glycogen phenotype. (D) Glycogen branching enzyme (T04A8.7) knockdown with an abnormal glycogen phenotype. (E) Categorization of the target genes identified in the screen by biological process and phenotype. There is notable enrichment of (-) phenotypes in the “metabolism” category, and (+/G) phenotypes in the “protein synthesis” category ($P < 0.001$ for each). Other clusters of targets included genes involved with proteasome function, vesicle and vacuole function, and nuclear import/export.

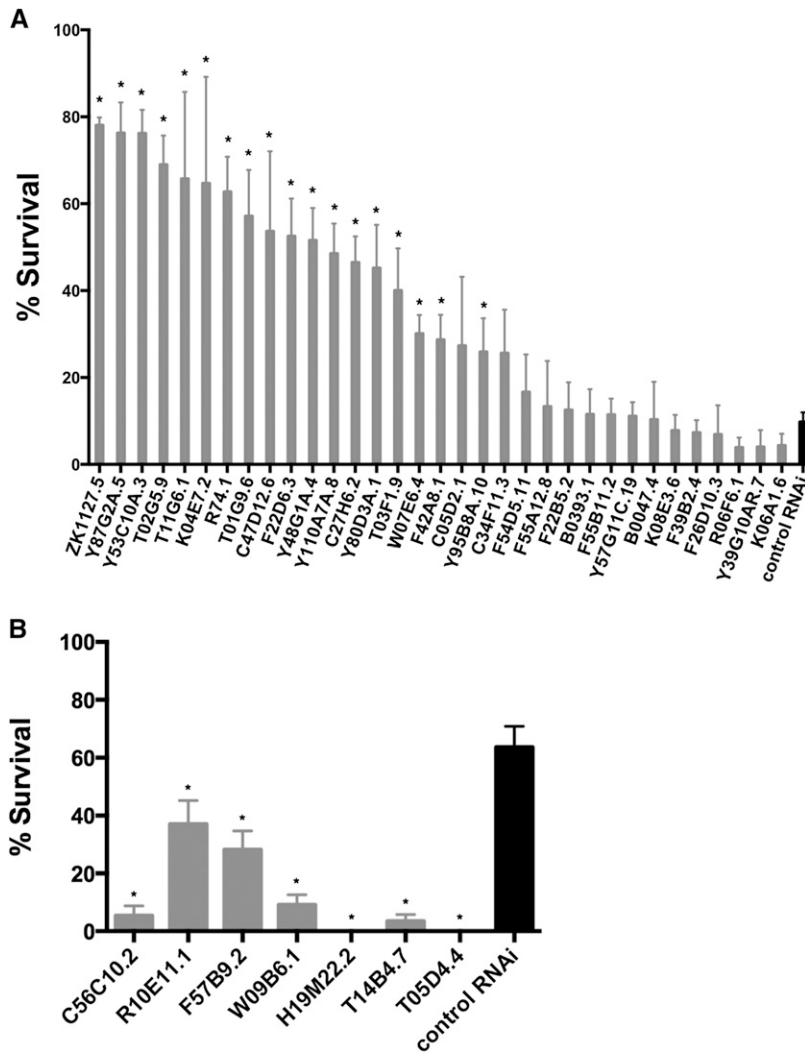


Figure 8 Animal glycogen content is directly correlated with survival of hypotonic anoxia. (A) Survival after 24-hr hypotonic anoxia of wild-type (N2) L4 animals that had been treated with RNAi targeting (+) glycogen storage genes. (B) Survival after 16-hr hypotonic anoxia of wild-type (N2) L4 animals that had been treated with RNAi targeting a randomly selected subset of (-) glycogen storage genes. Each bar represents at least three independent experiments, each with >10 animals. Error bars denote \pm SEM. * $P < 0.05$ vs. vector control (two-tailed t-test).

assayed these knockdowns for survival in hypotonic anoxia. We found a strong correlation between survival and gene activity that alters glycogen content (* $P < 0.001$ vs. control RNAi, one-way ANOVA with Dunnett's multiple comparison correction), such that 18 of the 33 (+) GSG knockdowns displayed significant increases in survival relative to controls (Figure 8A), and all seven of the (-) GSG knockdowns that were tested displayed significant survival decreases (Figure 8B). Because the GSGs represent a functionally diverse set of genes (Table S1), it is unlikely that they converge on another unknown process in parallel to glycogen storage. Therefore, these survival data strongly suggest that nematode glycogen content is a major determinant of survival in hypotonic anoxia. We also found that when the seven (-) GSG knockdowns mentioned above were performed in *daf-2(e1370)* animals, five of them significantly decreased 24-hr hypotonic-anoxia survival relative to a *daf-2(e1370)* control RNAi knockdown (Figure 9). In all cases, however, survival rates of (-) GSG knockdowns were higher in *daf-2(e1370)* animals than in wild type, suggesting that while glycogen content is an important aspect of the *daf-2(e1370)* mutant's resistance in hypotonic anoxia, there likely exist

other mechanisms by which reduced insulin/IGF signaling leads to increased survival in this environment.

Discussion

Previous studies in other model systems have shown that a decrease in tissue glycogen stores is correlated with the length of time of exposure to oxygen deprivation (Rose *et al.* 1965; Uniacke and Hill 1972; Hems and Whitton 1980). Apart from canonical pathways directly involved in glycogen synthesis and destruction, however, the genetic regulation of glycogen metabolism in metazoans has remained relatively unexplored. We present evidence in *C. elegans* that functional glycogen synthesis and breakdown pathways are necessary and sufficient for promoting animal survival during oxygen deprivation in an environment that combines anoxia with osmotic stress. We also show that RNAi targeting of several genes whose activity increases tissue glycogen content, including the glycogen synthase homolog *gsy-1*, results in a suppression of the *daf-2(e1370)* mutant's enhanced survival phenotype in hypotonic anoxia. Finally, we show that nematode survival is dependent on the rate at which

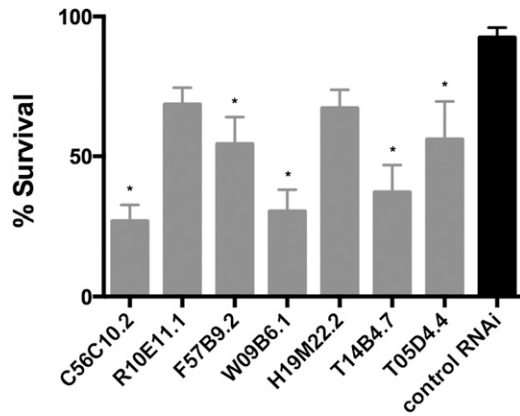


Figure 9 Survival after 24-hr hyposmotic anoxia of *daf-2(e1370)* animals that had been treated with RNAi targeting several (–) glycogen storage genes. For each RNAi target, data represent at least five independent experiments, each with >10 animals. Error bars denote \pm standard error of the mean. * $P < 0.05$ vs. RNAi control (two-tailed *t*-test).

glycogen is consumed during hyposmotic anoxia, such that decreases in rate [as seen in the *daf-2(e1370)* mutant] are associated with increased survival. Prior studies have suggested that *daf-2(e1370)* mutants possess a metabolic profile that is different from that of wild type (Murphy *et al.* 2003; Houthoofd *et al.* 2005; Mendenhall *et al.* 2006). However, specific *daf-2*-regulated processes most relevant to nematode survival in anoxia, metabolic or otherwise, have remained unclear. For instance, while knockdown of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (*gpd-2/3*) was shown to partially suppress *daf-2*-dependent hypoxia survival, knockdown of other glycolytic enzymes did not produce altered survival phenotypes (Mendenhall *et al.* 2006). By showing that the increased survival observed in *daf-2(e1370)* animals depends on functional glycogen synthase (*gsy-1*), we suggest a potential mechanism for *daf-2*-mediated survival during hyposmotic anoxia that requires preexisting glycogen stores and involves down-regulation of glycogen consumption.

We provide results from the first genome-wide screen for genes affecting glycogen storage in a metazoan, showing that individual knockdown of >250 genes modulates glycogen content and/or localization in *C. elegans*. Although several of the genes identified in our screen are homologs for canonical glycogen synthesis/breakdown enzymes, the vast majority have not been previously reported to impact carbohydrate metabolism. Among these are several genes, such as *sca-1* and *prp-31*, whose human homologs are known to be involved in diseases not previously thought to be related to glycogen metabolism (Sakuntabhai *et al.* 1999; Vithana *et al.* 2001). At present, the contribution of glycogen metabolism to human disease is poorly understood; however, recent studies implicate glycogen synthesis and breakdown in the progression of cancer and neurodegeneration (Favaro *et al.* 2012; Tiberia *et al.* 2012). Genes identified in our screen provide leads for the further investigation of glycogen metabolism in these and other pathologic processes.

Our data indicate that several biological processes have the ability to influence glycogen metabolism in a consistent manner. First, knockdown of genes involved in translation and ribosome biogenesis is significantly associated with increased glycogen content. A possible explanation for this is that translation requires ATP and therefore may deplete cellular energy pools that are otherwise stored as glycogen. Interestingly, mutations or RNAi that decrease protein synthesis rates have previously been shown to increase lifespan in the nematode (Hansen *et al.* 2007; Pan *et al.* 2007). We identified several genes that are known to increase lifespan yet do not have increased glycogen, implying that these two processes are not inexorably linked. Second, knockdown of genes involved with protein turnover, such as the proteasome subunits, generally resulted in decreased glycogen storage. We find it interesting to note the reciprocal relationship between glycogen storage and the presumed rate of protein synthesis and destruction in these knockdowns. It may be interesting in the future to investigate possible feedback between the glycolytic/glycogenesis pathway and protein synthesis, especially given that there is evidence for the interaction of these two pathways in diabetes (Barazzoni *et al.* 2000; Ahlman *et al.* 2001). Third, in the area of metabolism the most obvious conclusion of our screen is that knockdown of gene products involved with the electron transport chain results in lower glycogen storage. A trivial explanation for this effect could be that decreased respiration will naturally result in reduced glycogen storage, due to the inefficient substrate use of fermentative metabolism.

Several knockdowns from our screen, all of which possess increased glycogen content, have previously been reported to increase nematode survival during oxygen deprivation (Anderson *et al.* 2009; Mabon *et al.* 2009). We show that, in addition to these, the majority of knockdowns we identified as having increased glycogen also displayed increased survival in hyposmotic anoxia. Consistently, all knockdowns with decreased glycogen that were assayed displayed reduced survival in hyposmotic anoxia. Together, this strongly suggests that the presence of glycogen is a major determinant of animal survival in this environment. There are multiple potential explanations for why a significant number of GSG(+) knockdowns did not display increases hyposmotic-anoxia survival. First, it is possible that some of these knockdowns contain glycogen that is less accessible for use as an energetic substrate. In this case, the pool of glycogen usable by the nematode could be similar to or diminished relative to wild type in spite of an increase in total measured glycogen. Second, it is also possible that some of these knockdowns possess increased glycogen consumption rates during hyposmotic anoxia, such that glycogen is depleted in the same amount of time as wild type in spite of a larger preexisting pool of usable glycogen. We have provided data for one pathway (insulin/IGF signaling) regulating glycogen consumption rates; it is possible that many others do the same. A variety of cellular processes are represented among

the gene knockdowns that produce increases in glycogen content and hyposmotic-anoxia survival, including protein translation, transcription, and cell division. While knockdown of tRNA synthetases in *C. elegans* was previously shown to blunt the unfolded protein response and increase animal survival of oxygen deprivation (Anderson *et al.* 2009), this survival was not significantly associated with decreased animal oxygen consumption (Scott *et al.* 2013). Our data suggest that alterations in anaerobic metabolism, as opposed to respiration, may serve as the basis for increased survival.

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Author contributions: J.C.L., H.N.F. and M.B.R. designed experiments. J.C.L. and H.N.F. conducted experiments and analyzed data. J.C.L. and H.N.F. wrote the manuscript.

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GENETICS

Supporting Information

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Glycogen Fuels Survival During Hyposmotic-Anoxic Stress in *Caenorhabditis elegans*

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A.



B.



Figure S1 Nematode glycogen content is decreased upon knockdown of glycogen synthase but not glycogen phosphorylase. Representative micrographs of first-day adult animals following glycogen staining (rust color); **(A)** *control* RNAi (top animal) and *gsy-1* RNAi (bottom animal), **(B)** *control* RNAi (top animal) and *phosphorylase* RNAi (bottom animal).

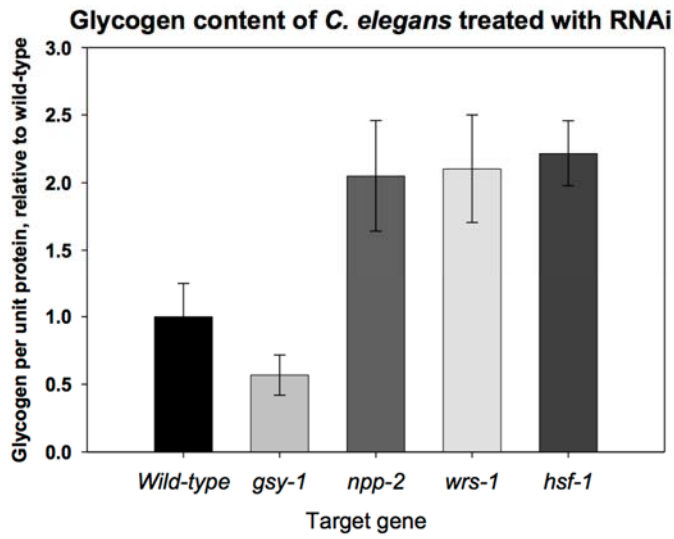


Figure S2 The relative glycogen content of five groups of nematodes treated with RNAi determined by an enzymatic assay. Data represent means of at least three independent experiments, +/- SEM.

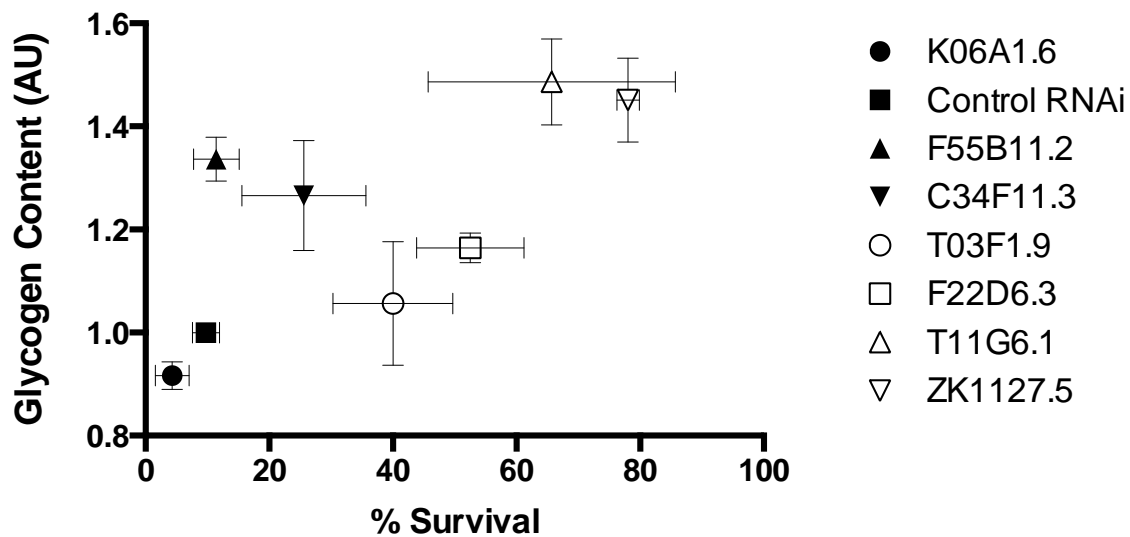


Figure S3 Relationship between nematode glycogen content and hyposmotic-anoxia survival rate in GSG(+) knockdowns. Glycogen content measured in N2 L4 animals raised in normoxia following respective RNAi treatment plotted against 24 h H-A survival of same knockdown. Pearson $R^2 = 0.4776$ ($P = 0.058$). Each data point represents glycogen measurements in at least five RNAi-treated animals (normalized to RNAi controls) and a minimum of four independent H-A trials (each with at least 20 animals). Error bars denote \pm standard error of the mean.

Table S1 Glycogen storage gene knockdowns—glycogen phenotypes (upper panel), survival in hyposmotic-anoxia and functional categorization (lower panel).

Available for download as an Excel file at www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.179416/-/DC1