Investigation of Genes That May Be Required for the Predictive Ability of the *Phsp-16.2::GFP* Reporter

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Abstract

In *Caenorhabditis elegans* expression of the fluorescent reporter (*Phsp-16.2::GFP*) is driven by transcription of a stress-responsive promoter sequence and has been shown to predict subsequent stress resistance (thermotolerance) and lifespan of individual animals within an isogenic population (Rea et al., 2005). The mechanism behind the predictive nature of this reporter is poorly understood. To better understand how this reporter works, stress-responsive genes were examined to determine if they were required for the ability of the reporter to predict thermotolerance or lifespan. Animals that had low or high expression of the *Phsp-16.2::GFP* reporter and contained loss-of-function mutations in select stress-responsive genes were tested to determine whether the animals with higher expression of the reporter still had improved thermotolerance or lifespan. The results revealed that none of the tested stress-responsive genes were necessary for the predictive power of the reporter. In some mutants, the reporter maintained its predictive ability even though the overall thermotolerance was greatly reduced. This implies that the predictive ability of the reporter is not linked to the stress resistance (thermotolerance) of the animal, as originally expected.

Introduction

Populations of the soil nematode, *Caenorhabditis elegans,* with zero genetic difference between animals (isogenic), have as much natural variation in lifespan as the population of the United States (Finch and Kirkwood, 2000). In both species, this diversity in lifespan is likely due to stochastic variation, countless unquantifiable individual experiences, many of them small, that together make a significant contribution to when one dies (Vaupel et al.,1998; Finch and Kirkwood, 2000; Herndon et al., 2002; Kirkwood and Finch, 2002; Rea et al., 2005). In individual wildtype *C. elegans*, levels of certain biomarkers, such as *sod-3*, *unc-54*, *mir-71, and mir-246*, can reliably predict lifespan, and therefore presumably the effects of stochastic variation (Pincus et al., 2011; Sánchez-Blanco and Kim, 2011). One of these biomarkers is the fluorescent reporter, *Phsp-16.2::GFP* (Rea et al., 2005).

Phsp-16.2::GFP can reliably predict stress resistance and lifespan in an isogenic population of *C. elegans*. The mechanism behind the predictive ability of the *Phsp-16.2::GFP* reporter is not well understood. Levels of gene expression in the worms may be part the stochastic variation that modulates lifespan, which can then be predicted by the reporter. The objective of this project was to provide more insight on how this reporter functions by examining candidate genes that could play a crucial role in the reporter's predictive ability. Examining the thermotolerance (stress resistance) and lifespan of isogenic nematodes selected for low and high expression of *Phsp-16.2::GFP,* in strains also carrying mutations in select candidate genes, showed whether these genes are necessary for the predictive nature of the reporter.

Background

C. elegans as a Model Organism:

C. elegans is a nonparasitic soil nematode found around the world (WormClassroom.org). It was introduced to the research world as a genetic model by Sydney Brenner in 1974 and has successfully been used to study aging, development, pathology, and neurology (Brenner, 1974; WormClassroom.org). Working with *C. elegans* is cost-efficient since they are small (about 1.3 mm as adults) and thousands at a time can live on a single Petri dish containing either agar-based or liquid medium (Kaletta and Hengartner, 2006). The animals are raised on a diet of *E. coli* and are maximally fertile at 20°C. *C. elegans* is also transparent, which permits the detection of *in vivo* reporters, such as the fluorescent *Phsp-16.2::GFP* reporter, by noninvasive methods (Kaletta and Hengartner, 2006).

Despite its being a relatively simple organism, there is strong conservation of many genes between *C. elegans* and mammals. In fact, researchers have identified *C. elegans* homologues for 60-80% of human genes (Kaletta and Hengartner, 2006). The 959 somatic cells that make up the hermaphrodite form of the animals include a wide range of cell types such as muscle, skin, glands, nervous, reproductive, and digestive (Figure 1) (Kaletta and Hengartner, 2006). Additionally, the entire genome of *C. elegans* has been sequenced (WormBase.org).

C. elegans is ideal for aging research as it has a short lifespan (two to three weeks). Further, it develops from an egg to an adult in three days, and each hermaphrodite has around 300 progeny, allowing for rapid amplification of populations (Kaletta and Hengartner, 2006; WormClassroom.org). Although males exist within the

population, the large majority of animals are self-fertilizing hermaphrodites, so isogenic populations are easily obtainable (Kaletta and Hengartner, 2006).

Figure 1: The Basic Anatomy of *C. elegans*

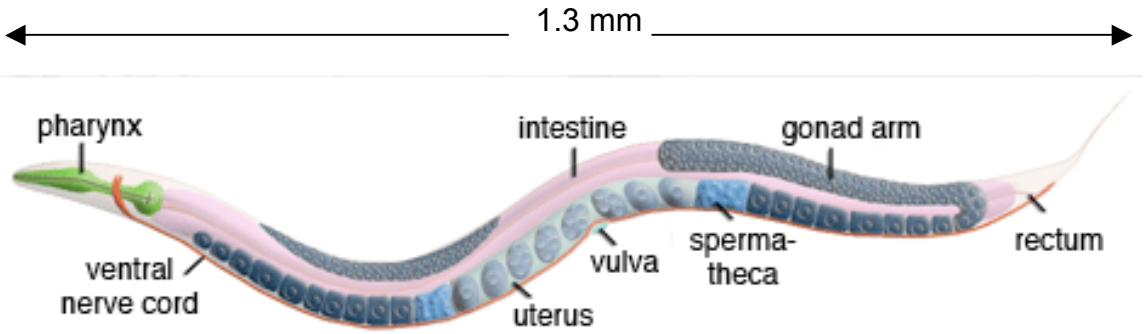


Figure from http://www.sfu.ca/biology/faculty/hutter/hutterlab/research/Celegans.html.

Stochastic Variation Likely Causes Differing Stress Resistance and Survival within an Isogenic Population:

In almost all species studied, including humans, only 10-40% of variation in lifespan is due to genetics (Finch and Tanzi, 1997). In the soil nematode *C. elegans*, isogenic populations exhibit large differences in lifespan, even when environment is held as constant as possible (Kirkwood and Finch, 2002). This variance is likely due to stochastic phenomena, numerous randomly acting and unquantifiable variables that affect the physiological state of the animal and contribute to (or inhibit) its ability to thrive (Vaupel et al., 1998; Finch and Kirkwood, 2000; Herndon et al., 2002; Kirkwood and Finch, 2002, Rea et al., 2005).

As a hypothetical example, whether an animal spends most of its time near the center of the Petri dish or near the edge may, in some small, unquantifiable way, affect the ultimate stress resistance or lifespan of the animal. It is unlikely that we will ever

completely untangle most stochastic contributions to lifespan. However, it is possible to predict the collective effect of stochastic phenomena on lifespan using the *Phsp-16.2::GFP* reporter.

Background on the *Phsp-16.2::GFP* Reporter:

In order to visualize the stress response in *C. elegans*, the stress-responsive transgenic reporter, *Phsp-16.2::GFP*, was developed. This reporter contains the promoter sequence from the *heat shock protein (hsp) 16.2* gene linked to the gene encoding green fluorescent protein (GFP) from the jellyfish (Link et al., 1999). Although this reporter does not encode the HSP-16.2 protein product, Link et al. (1999) showed that expression of GFP from the reporter paralleled the upregulation of HSP-16.2 from the endogenous nematode *hsp-16.2* gene, indicating that *Phsp-16.2::GFP* is a reliable reporter for *hsp-16.2.*

HSP-16.2 is a molecular chaperone involved in a defensive mechanism against stress that is known as the heat shock response (Morimoto et al., 1997; WormBase.org). While reporting the levels of endogenous HSP-16.2, *Phsp-16.2::GFP* is also reporting the ability of the worm to handle stress, such as lethal heat. However, it has not been shown that the variation in stress resistance reported by *Phsp-16.2::GFP* is due to the effects of HSP-16.2.

The transparency of *C. elegans* allows for visualization of the stress response via this reporter transgene. Exposure to heat induces expression of the reporter, causing the animals to glow green when viewed through a fluorescent microscope. Animals

mounting a stronger heat shock response will glow a brighter green than those with a weaker heat shock response.

Rea et al. (2005) showed that levels of production of GFP predicted subsequent survival during exposure to lethal heat or during normal aging. The same authors showed that other reporter constructs, reflecting expression of non-heat-shock genes, including *myo-2*, *mtl-2*, and *gst-4*, did not predict lifespan. For the *Phsp-16.2::GFP* reporter, GFP is not expressed constitutively, but only expressed following a period of stress (Rea et al., 2005). After a one or two hour heat shock at 35°C, GFP can be detected as early as ten hours later, and peaks around 18 hours post heat shock (Rea et al., 2005). Because of this, 18 hours is a good time to select bright and dim worms to use for thermotolerance or lifespan tests (Rea et al., 2005).

The Heat Shock Response, Stress Resistance, and Their Correlation to Lifespan:

Heat shock proteins (HSPs), such as HSP-16.2, are stress-responsive proteins which are highly conserved among all living things, and which often act as molecular chaperones to guide protein folding (Li et al., 2004). When organisms are stressed, whether it is by thermal stress or another kind (Figure 2), their proteins tend to unfold, misfold, or aggregate. In order to maintain homeostasis, HSPs are upregulated to deal with the dysfunctional proteins (GuhaThakurta et al., 2002). This phenomenon is known as the heat shock response.

The ability of the worm to respond to stress, such as lethal temperatures, UV radiation, and reactive oxygen species, is predictive of lifespan (Larsen, 1993; Lithgow et al., 1994; Lithgow et al., 1995; Martin et al., 1996; Murakami and Johnson,1996;

Johnson et al., 2002; Walker et al., 2003; Rea et al., 2005). Thermotolerance (the animal's ability to tolerate lethal heat) is the best predictor (Johnson et al., 2002). In an isogenic population of *C. elegans*, the natural variation in stress resistance is correlated to the natural variation in lifespan, implying that stochastic phenomena affect the worms' stress resistance and lifespan in a similar fashion (Rea et al., 2005; Sánchez-Blanco and Kim, 2011). Because of this, observing how an animal in an isogenic population reacts to stress using the *Phsp-16.2::GFP* reporter allows us to predict how long it will live.

Figure 2: Conditions that Activate the Heat Shock Response

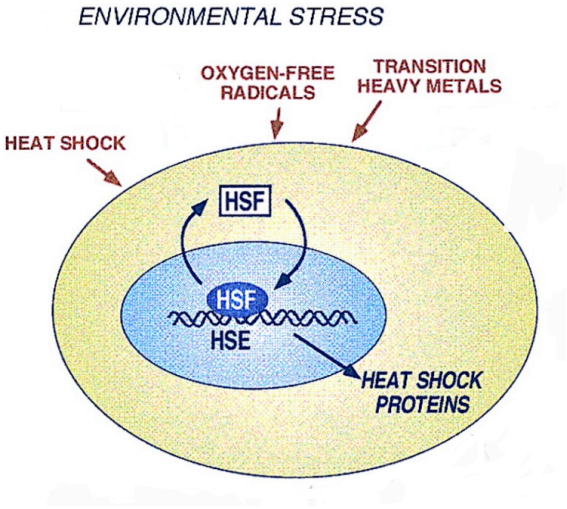


Figure modified from Morimoto et al. (1998).

Mutant Strains That Were Tested for Their Involvement in the Predictive Ability of *Phsp-16.2::GFP*:

Genes involved in the heat shock response, or stress response in general, presented themselves as logical candidates to test for a role in the predictive ability of

the *Phsp-16.2::GFP* reporter for two reasons. First, they are upregulated at the same time as the reporter (GuhaThakurta et al., 2002; Prahlad et al., 2008). Second, they are believed to be involved in combating the effects of stress (Li et al., 2004; Mansisidor et al., 2011; Prahlad et al., 2008); therefore, they may play a role in modulating the variance in stress resistance (and consequently lifespan) within a population. A description of the genes tested is given below:

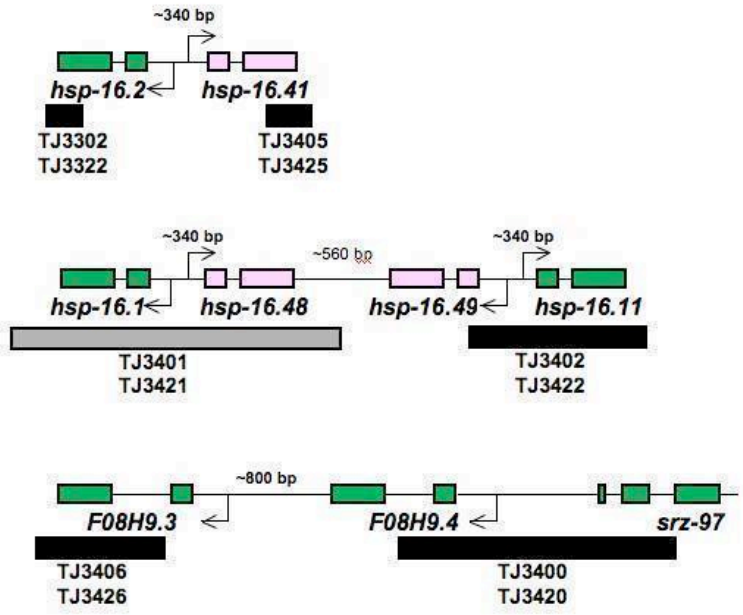
The *hsp-16* gene family:

The *hsp-16* gene family includes eight homologous genes that encode 16 kDa heat shock proteins (Figure 3) (Candido et al., 1989; Shim et al., 2003). Because *hsp-16.1* and *hsp-16.48* are perfect duplicates of *hsp-16.11* and *hsp-16.49*, respectively, these eight genes, in fact, only produce six different proteins (Candido et al., 1989).

The *hsp-16* gene family is not expressed in *unstressed* animals until old age (Rea et al., 2005). One regulatory pathway of this gene family is the insulin-like response pathway. A critical upstream component of this pathway is the insulin-like response receptor protein, DAF-2 (which is homologous to both the human insulin and insulin-like growth factor 1 receptors), and downstream signaling is mediated by the transcription factor DAF-16, homologous to the mammalian FOXO3 transcription factor. Unstressed, nonmutant functioning of the insulin-like response pathway inhibits production of HSPs (Ogg et al., 1997; Lin et al., 2001). When this pathway itself is inhibited by stress or mutation, another transcription factor, Heat Shock Factor 1, is triggered to upregulate HSPs such as the *hsp-16.2* gene family (Morley et al., 2004; Hsu et al., 2003).

In *C.elegans,* mutants of the genes in the insulin-like response pathway tend to be long-lived. For example, a loss-of-function mutation in DAF-2 increases lifespan two-fold (Halaschek-Wiener et al., 2005). In this particular long-lived mutant, six tested genes from the *hsp-16* gene family (*hsp-16.2, hsp-16.48, hsp-16.49, hsp-16.1, hsp-16.11, and hsp-16.41)* were upregulated an average of 60-fold when compared to the wild-type (control) animals (Halaschek-Wiener et al., 2005). This suggests that the genes of the *hsp-16* gene family may be part of the stochastic phenomena modulating aging, or at least may be expressed concordantly with the overall stochastic influences. These genes may therefore affect the ability of the transgene, *Phsp-16.2::GFP,* to predict lifespan.

Figure 3: The *hsp-16* Gene Family



The hsp-16 gene family is shown above with black or grey bars underneath each gene representing the position of the loss-of-function mutation present in the tested strain. Mutations are out-of-frame (black) or in-frame (grey). All strains illustrated (with strain names beginning with "TJ") carry both the mutation and a single copy of the Phsp-16.2::GFP reporter. The strain carrying the larger number within each pair has been backcrossed to the control strain, TJ3001, at least four times. One mutation (TJ3400/TJ3420) affects an unrelated downstream gene, srz-97. Modified from an unpublished figure by James Cypser Ph.D.

hsp-17:

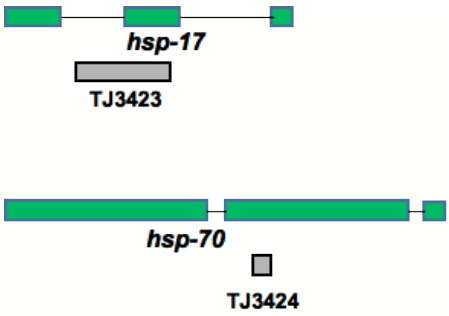
The precise role of *hsp-17* has yet to be determined but it is predicted to act similarly to members of the *hsp-16* gene family and be a part of the heat shock response (WormBase: Gene *hsp-17*, 2004). *hsp-17* is evolutionarily related to the eight genes in the *hsp-16* gene family by paralogy, meaning that the genes were all derived

from the same gene but separated by a duplication event (WormBase: Gene *hsp-17*, 2004).

hsp-70:

hsp-70 is another molecular chaperone upregulated via the heat shock response (GuhaThakurta et al., 2002). Overexpression of *hsp-70* results in an increased lifespan (Tatar et al., 1997; Yokoyama et al., 2002; Singh et al., 2007). Its ability to affect the aging process makes it a candidate gene necessary for the predictive ability of the *Phsp-16.2::GFP* reporter.

Figure 4: *hsp-17* and *hsp-70*



hsp-17 and hsp-70 are shown above with black or grey bars underneath each gene representing the position of the loss-of-function mutation present in the tested strain (grey coloring indicates mutations are in-frame). Both strains carry both the mutation and a single copy of the Phsp-16.2::GFP reporter. Modified from an unpublished figure by James Cypser Ph.D.

RNAi deficient 4 (rde-4):

RDE-4 is a protein required for the RNA interference (RNAi) mechanism. RNAi is a way for an organism to regulate gene expression by degrading sequence-specific

double-stranded RNA (Parker et al., 2006), and is thought to have evolved as a defense against viruses. This process requires small-interfering RNAs (siRNAs), which are produced through double-stranded RNA processing by the enzyme DICER (Parker et al., 2006). The DICER enzyme requires double-stranded RNA-binding proteins, such as RDE-4, to function (Parker et al., 2006).

Mansisidor et al. (2011) found that the endogenous RDE-4 protein affects the insulin-signaling pathway that is involved in stress resistance and lifespan modulation by indirectly repressing a member of this pathway, *pdk-1.* These authors provide evidence that siRNAs produced by RDE-4 bind to and negatively regulate *pdk-1*. In *C. elegans, rde-4* mutants had an increased expression of *pdk-1* and were less resistant to both oxidative and thermal stress (Blanchard et al., 2011; Mansisidor et al., 2011). These *rde-4* mutants also had reduced lifespan (Mansisidor et al., 2011).

guanylyl cyclase 8 (gcy-8):

GCY-8 is a receptor-type guanylyl cyclase that is uniquely expressed in the AFD thermosensory neurons of the worm and required for the function of these neurons (WormBase: Gene *gcy-8,* 2006). The AFD neurons work with their postsynaptic partners, the AIY neurons, to systemically regulate the heat shock response in *C. elegans* (Prahlad et al., 2008). It appears that these latter thermosensory neurons work by activating HSF-1 (Prahlad et al., 2008). *gcy-8* mutants have reduced expression of *hsp-16.2* and *hsp-70* following a heat shock and are less resistant to lethal heat (Prahlad et al., 2008), implying that the mutants lose function of the AFD neurons, leading to a diminished heat shock response.

Materials/Methods

Test Strains:

A control strain of *C. elegans* engineered to carry only a single copy of the *Phsp-16.2::GFP* reporter transgene was already available in the Johnson lab at the University of Colorado, Boulder (having been constructed by A. Mendenhall). Mutant strains carrying the reporter plus mutations in candidate modulatory genes were obtained from the Caenorhabditis Genetics Center at the University of Minnesota. The mutant strains were backcrossed to the control animals one to eleven times ("11X-backcrossed") to reduce interference from unidentified background mutations that may have been present. For this purpose, the strains were considered sufficiently backcrossed after four generations ("4X-backcrossed"), the point at which the mutant animals had a genetic background that was 93.75% identical to the wildtype worms. Strains that were backcrossed more than four times had genetic backgrounds that were greater than 93.75% identical to the wildtype worm.

Some strains were tested before being sufficiently backcrossed. In such cases, the two strains were given different names (in this project, the further-backcrossed strain received a name including a higher strain number). Below is a list of the *hsp-16.2::GFP* reporter strains and the candidate mutations they contain. All the mutant alleles contain large deletions, and so were presumed to be loss-of-function (see for example Figure 3, schematic illustrating the *hsp-16* gene family, with in-frame deletions indicated by gray bars, and out-of-frame deletions indicated by black bars).

Table 1: Test Strains and Loss-of-Function Mutations

Strain (Backcross Number)	Gene(s) Mutated	Allele	References
TJ3001	None – control strain	N/A	N/A
TJ3302 (1X) TJ3322 (4X)	*hsp-16.2*	*gk249*	Rea et al., 2005; Link et al., 1999; Halaschek-Wiener et al., 2005; WormBase.org
TJ3425 (4X)	*hsp-16.41*	*tm1093*	Halaschek-Wiener et al., 2005; WormBase.org
TJ3421 (4X)	*hsp-16.1* and *hsp-16.48*	*ok577*	Halaschek-Wiener et al., 2005; WormBase.org
TJ3402 (1X)	*hsp-16.11* and *hsp-16.49*	*tm1221*	Halaschek-Wiener et al., 2005; WormBase.org
TJ3426 (4X)	*F08H9.3*	*tm5012*	WormBase.org
TJ3400 (1X) TJ3420 (4X)	*F08H9.4*	*ok1976*	WormBase.org
TJ3423 (4X)	*hsp-17*	*tm5013*	WormBase: Gene *hsp-17*, 2004
TJ3424 (4X)	*hsp-70*	*tm2318*	GuhaThakurta et al., 2002; Tatar et al., 1997; Yokoyamma et al., 2002; Singh et al., 2007; WormBase.org
TJ3314 (5X)	*rde-4*	*ne299*	Parker et al., 2006; WormBase.org
TJ3301 (11X)	*gcy-8*	*oy44*	Prahlad et al., 2008; WormBase: Gene *gcy-8*, 2006

Growing up Populations:

The nematode is characteristically an inbreeding hermaphrodite and all strains had been selected to be uniformly homozygous, so the genetic makeup of each strain

was maintained in all the progeny. Each candidate strain was grown up alongside the control strain (TJ3001) at 20^{o}C on agar plates spread with the bacterial food (*E. coli* strain OP50). The animals were age-synchronized using a hypochlorite solution that allows only eggs to survive.

Heat Shock:

Once there were approximately 6,000 worms or more in each strain (split onto 9 cm plates of 3,000 worms each) and the worms were in their first day of adulthood (four days old), they were heat-shocked. Heat shock occurred at 35^{o}C for one hour in a shaker-incubator rotating at 80 RPM. Worms were heat-shocked in a liquid medium (at 300 worm/ml) containing S. Basal, OP50 (at 1x10^9 cells/ml), and cholesterol (at 2 ul/ml). At the end of the heat shock, the worms were transferred to flasks containing equivalent liquid medium at 20°C and were permitted to recover for 18 hours in a shaker-incubator rotating at 80 RPM.

Sorting:

At 18 hours post-heat shock, the mean of GFP production approached maximum (Rea et al., 2005). At this time, a COPAS Biosort (worm sorting apparatus, Union Biometrica, Holliston, MA) sorted the worms by degree of fluorescent GFP expression (green glow) by shining a laser onto the worms and then selecting a specified percentage of “bright” and “dim” worms. Approximately 60 worms from the top 10% of expression (“bright”) and 60 worms from the lowest 10% of expression (“dim”) were selected from each of the strains (control and mutant). The COPAS Biosort

deposited the selected worms onto plates containing standard solid nematode growth medium, or into a small volume of the liquid medium described above, and then the animals were inspected for removal of any dead individuals. The animals were then ready to be tested for either thermotolerance or lifespan.

Assessment of Thermotolerance:

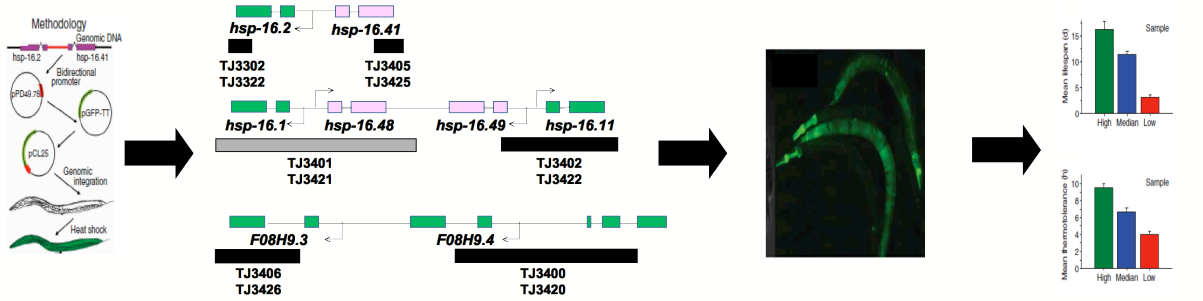
The worms that were tested for thermotolerance were kept at 20°C for an additional 12 hours after sorting and then placed onto solid NGM plates and into a 34°C incubator. Commencing 10 to 12 hours later, the worms were scored as alive or dead every two hours (there is usually very little mortality of worms before 10 hours at 34°C in the incubator). Worms were considered dead if they exhibited no movement and no turgor pressure. Worms that resisted the researcher's attempt at folding them using a small wire were judged as having turgor pressure and scored as alive. The assessment was complete after all animals died, which usually required an additional 12 hours. Statistical comparisons (p-values) of the survival of "bright" and "dim" animals within each strain were calculated using the log-rank test at a level of significance of $p < 0.05$.

Assessment of Lifespan:

The worms that were tested for lifespan were scored as dead or alive every two or three days. Any worms that were lost, or that died from non-aging causes (internal hatching of eggs or accidents) were not included in final calculations. Worms were transferred to fresh liquid medium every day during the first week while they were still laying eggs, to avoid confounding of test animals with their own progeny. Once

reproduction had ceased, worms were transferred to new liquid medium once a week. A typical lifespan experiment required three weeks to complete. Statistical comparisons between the longevities of “bright” and “dim” animals were made within each strain using the log-rank test at a level of significance of $p < 0.05$. Age at death was reported as days since egg hatching (rather than days since heat shock or days of adulthood).

Figure 5: Flowchart of Methodology



Engineer worms with heat-inducible reporter, *Phsp-16::GFP,* to create strain TJ3001

Obtain mutant strains of *C.elegans* with deletions to one or two stress-responsive genes and mate these mutants with TJ3001 to obtain the doubly-mutant strains shown above

Heat shock strains to make the worms glow differentially, and select the “bright” and “dim” worms

Test mutant strains for survival during aging and thermotolerance

Modified from a published figure by Rea et al. (2005) and an unpublished figure by James Cypser Ph.D.

Results

To determine whether select candidate genes are required for the predictive ability of the *Phsp-16.2::GFP* reporter, isogenic animals carrying mutations in the candidate genes with low ("dim") or high ("bright") expression of the reporter were examined for their thermotolerance and lifespan. Differential survival between the "bright" and "dim" worms indicated that the reporter was still predictive of thermotolerance or lifespan. All strains were sufficiently backcrossed (at least four times) to make genetic backgrounds uniform unless otherwise indicated.

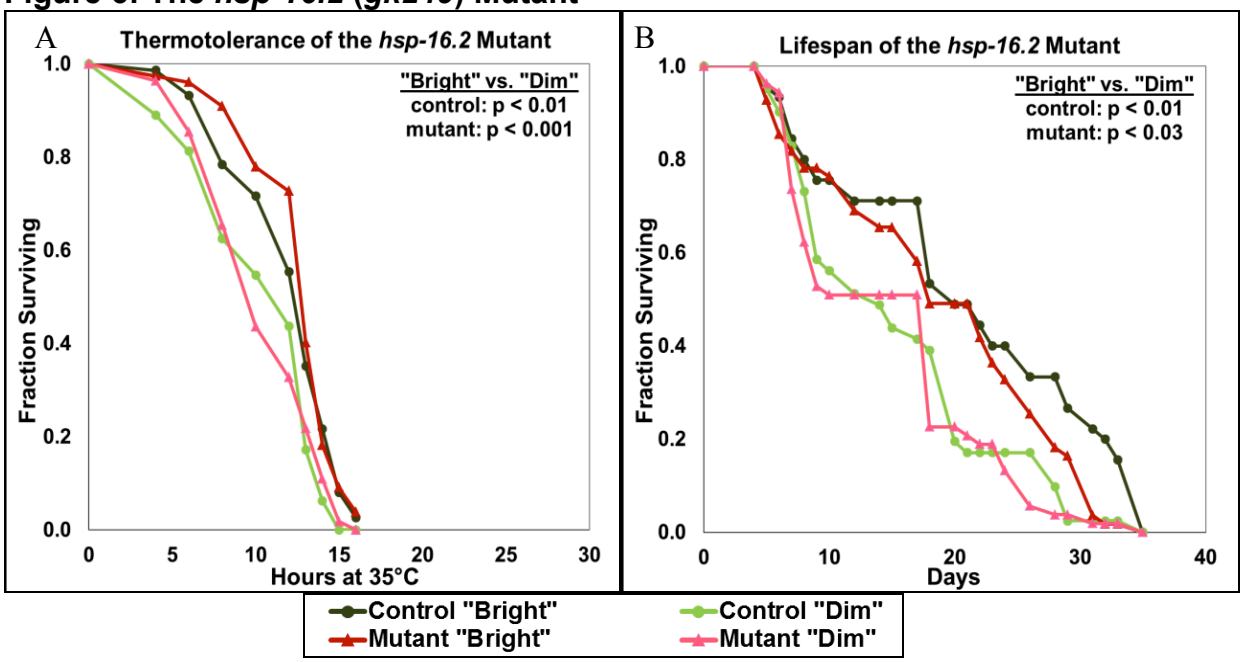
The *hsp-16.2* (*gk249*) mutant:

Strain TJ3322, carrying a mutation in *hsp-16.2* (*gk249*) was tested to see if the *Phsp-16.2::GFP* reporter could predict thermotolerance and lifespan in the mutant. Since its promoter sequence is identical to the sequence that drives transcription of the reporter, it was arguably the gene most likely to be necessary for *Phsp-16.2::GFP*'s predictive ability. In the thermotolerance test, the "bright" and "dim" animals of the control strain (TJ3001) were significantly different ($p < 0.01$; Figure 6A), indicating that the *Phsp-16.2::GFP* reporter was predicting thermotolerance as expected. In the *hsp-16.2* (*gk249*) mutant strain, the thermotolerance of "bright" and "dim" animals was also significantly different ($p < 0.001$); the mean hour of death while at lethal heat was 12.7 ± 0.3 hours for the "bright" animals and 10.5 ± 0.4 hours for the "dim" animals (Figure 6A; Table 2). The difference between the "bright" and "dim" animals of the mutant strain revealed that *hsp-16.2* (*gk249*) was not required for the reporter to be predictive of thermotolerance (Figure 6A; Table 2).

The results for the *hsp-16.2* (*gk249*) mutants that were tested for lifespan reveled that the lifespans of the control “bright” and “dim” animals were significantly different as expected ($p < 0.01$) (Figure 6B). Additionally, the lifespans of the *hsp-16.2* (*gk249*) mutant “bright” and “dim” animals were significantly different ($p < 0.03$); the mean lifespan was 19.1 ± 1.2 days for the “bright” animals and 14.7 ± 1.1 days for the “dim” animals. This indicated that *hsp-16.2* (*gk249*) was not vital for the reporter’s ability to predict lifespan.

The loss of *hsp-16.2* (*gk249*) did not appear to affect the overall thermotolerance of the animals when compared to the control strain (Figure 6A). Although accurate statistical comparisons of overall thermotolerance between the mutant and control strains would be best done with comparisons between the means of each strain, only data from the extremes (i.e., the data from the “bright” and “dim” worms) was available. Because of this, comparisons of overall thermotolerance were done by comparing the “bright” mutant animals to the “bright” control animals and the “dim” mutant animals to the “dim” control animals.

Figure 6: The *hsp-16.2* (*gk249*) Mutant



The thermotolerance test for the hsp-16.2 (gk249) mutant was done at 35 °C. Thermotolerance tests for all other mutants were done at 34 °C.

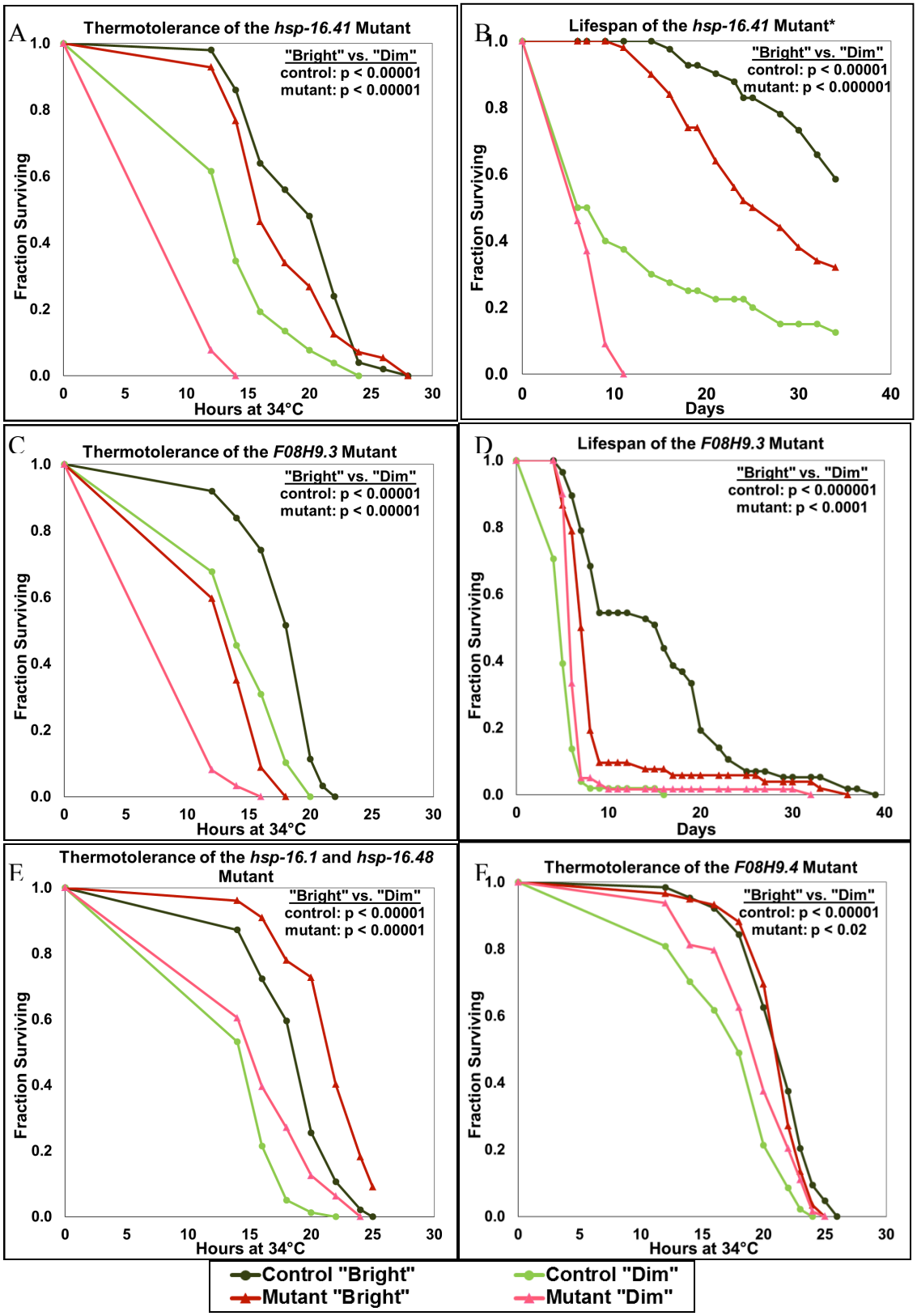
Other *hsp-16* gene family mutants:

The *Phsp-16.2::GFP* reporter was predictive of thermotolerance for all other *hsp-16* gene family mutants tested, including strain TJ3425 carrying an *hsp-16.41* (*tm1093*) mutation, strain TJ3421 carrying an *hsp-16.1* and *hsp-16.48* (*ok577*) mutation, strain TJ3426 carrying an *F08H9.3* (*tm5012*) mutation, and strain TJ3420 carrying an *F08H9.4* (*ok1976*) mutation (Figure 7A, 7C, 7E, 7F; Table 2). One thermotolerance test was done for each mutant strain. In each thermotolerance test, the “bright” mutant animals were significantly more thermotolerant than the “dim” mutant animals (Figure 7A, 7C, 7E, 7F; see Table 2 for the p values and mean survival during thermotolerance of the “bright” and “dim” mutant animals). A lifespan test was done on both the *hsp-16.41* (*tm1093*) and the *F08H9.3* (*tm5012*) mutants. In both tests, the “bright” mutant animals were significantly longer lived than the “dim” mutant animals (Figure 7B, 7D; see Table 2 for

the p values and mean lifespan of the “bright” and “dim” mutant animals), signifying that the reporter maintained its predictive ability.

The loss of *F08H9.3* (*tm5012*) appeared to cause a large decrease in overall thermotolerance of the animals (Figure 7C). A log-rank test comparing the mutant “bright” and animals to the control “bright” animals revealed that the mutant “bright” animals were significantly less thermotolerant ($p < 0.000001$) (Figure 7C). Additionally, the mutant “dim” animals were significantly less thermotolerant than the control “dim” animals ($p < 0.000001$) (Figure 7C). The *hsp-16.41* (*tm1093*) mutant also appeared to have a decreased thermotolerance, but the reduction was not as severe as seen with the *F08H9.3* (*tm5012*) mutant (Figure 7A); the mutant “bright” animals were not significantly less thermotolerant than the control “bright” animals ($p > 0.1$), but the mutant “dim” animals were significantly less thermotolerant than the control “dim” animals ($p < 0.00001$). Surprisingly, the loss of both *hsp-16.1* and *hsp-16.48* (*ok577*) appeared to increase the thermotolerance of the animals (Figure 7E); the mutant “bright” and “dim” animals were significantly more thermotolerant than the control “bright” and “dim” animals, respectively ($p < 0.01$ for both comparisons). None of the other mutants in this family caused evident changes in the thermotolerance of the worms.

Figure 7: The *hsp-16* Gene Family Mutants



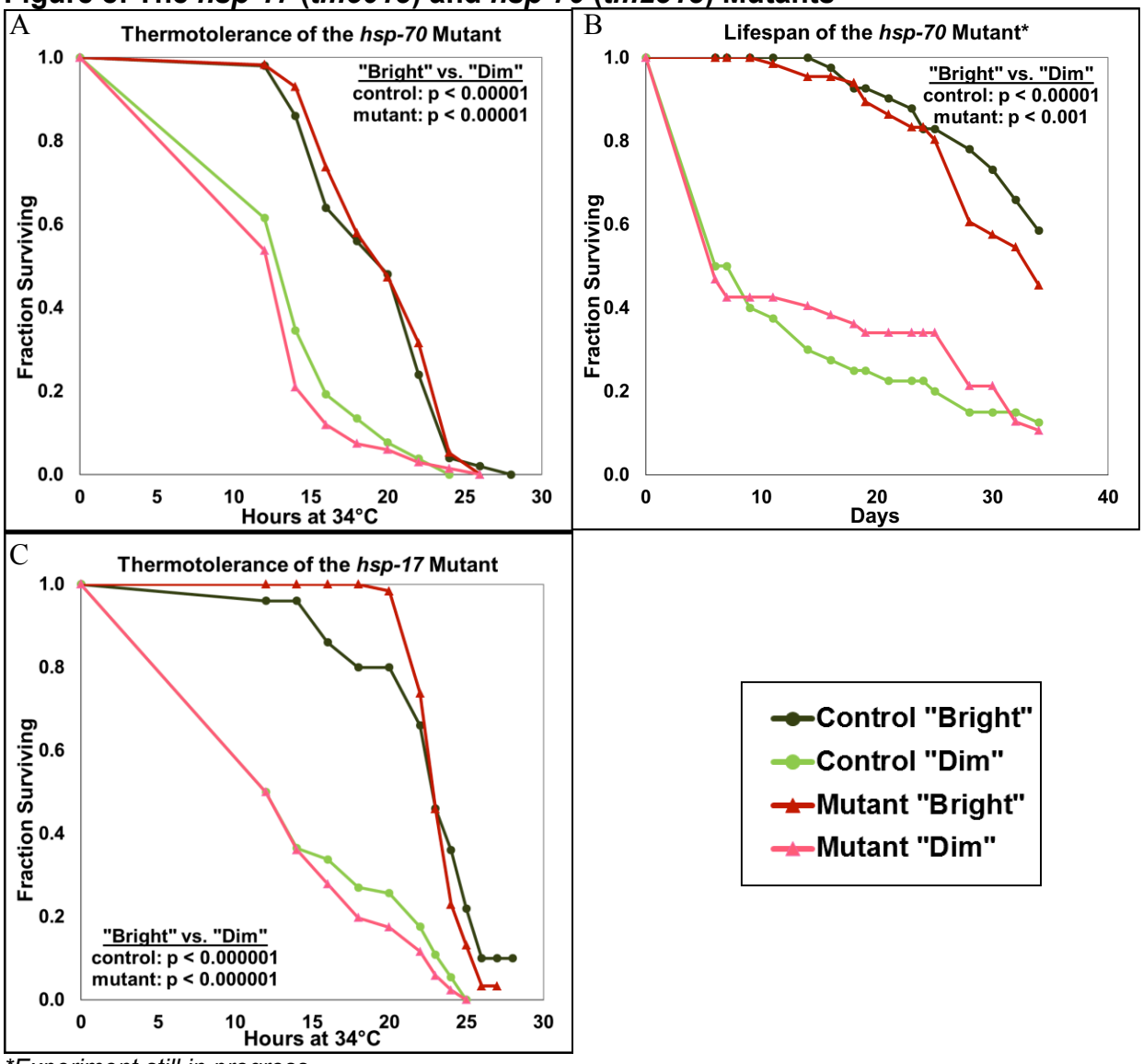
**Experiment still in progress.*

The *hsp-17* (*tm5013*) and *hsp-70* (*tm2318*) mutants:

In the single thermotolerance test done on strain TJ3423, carrying an *hsp-17* (*tm5013*) mutation, there was a significant difference between the thermotolerance of the “bright” and “dim” mutant animals ($p < 0.000001$) (Figure 8C; Table 2), indicating that the reporter preserved its predictive power. The “bright” mutant animals lived an average of 23.5 ± 0.2 hours during the thermotolerance test, while the “dim” mutant animals lived an average of 15.2 ± 0.5 hours (a few animals were still alive in the “bright” group after scoring of worms ceased so the mean value for this group was taken from slightly incomplete data) (Figure 8C; Table 2). The loss of *hsp-17* (*tm5013*) did not appear to affect the overall thermotolerance of the worm (Figure 8A).

Both a thermotolerance and a lifespan test were done on strain TJ3424, carrying an *hsp-70* (*tm2318*) mutation. In both tests, the p values between the “bright” and “dim” mutant animals were less than 0.001 (although the lifespan test is still in progress, the p value was calculated based on the data collected thus far) (Figure 8A, 8B; Table 2). The mean survival during the thermotolerance test was 20.1 ± 0.5 hours for the “bright” mutant animals and 14.1 ± 0.4 hours for the “dim” animals (Figure 8A; Table 2). The mean lifespan was 26.2 ± 1.0 days for the “bright” mutant animals and 13.4 ± 1.6 days for the “dim” mutant animals; however, these mean values were taken from incomplete data as the experiment is still in progress (Figure 8B; Table 2). This revealed that *hsp-70* (*tm2318*) is not needed for the reporter to be predictive of thermotolerance or lifespan. Additionally, the loss of *hsp-70* (*tm2318*) did not appear to affect the overall thermotolerance of the worm (Figure 8C).

Figure 8: The *hsp-17* (*tm5013*) and *hsp-70* (*tm2318*) Mutants



**Experiment still in progress.*

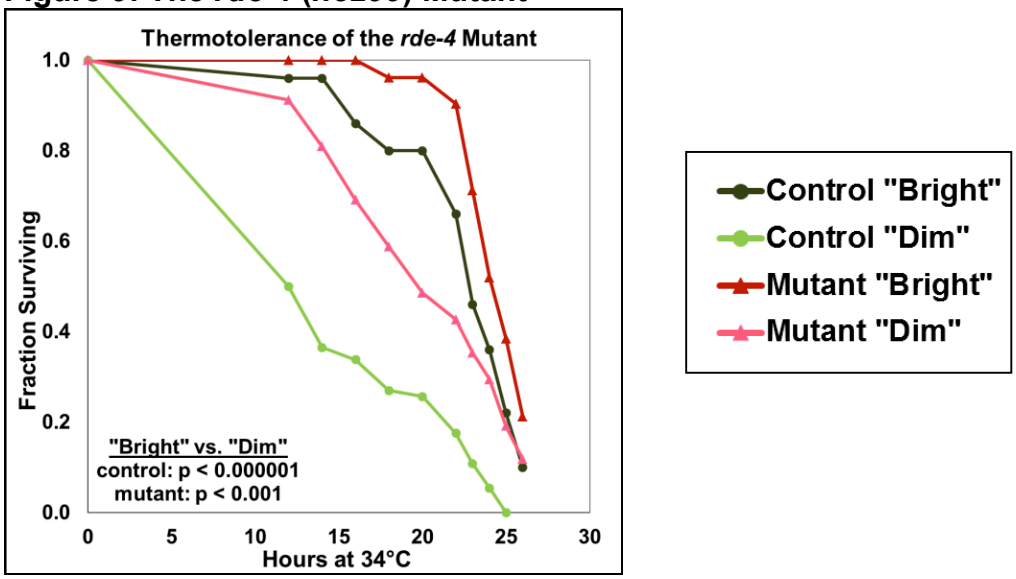
The *rde-4* (*ne299*) mutant:

A single thermotolerance test was conducted on strain TJ3314 carrying an *rde-4* (*ne299*) mutation. In this test, the "bright" mutant animals were significantly more thermotolerant than the "dim" mutant animals ($p < 0.001$) (Figure 9; Table 2). When placed at lethal temperature, the "bright" mutant animals lived an average of 23.9 ± 0.3 hours while the "dim" mutant animals lived an average of 20.3 ± 0.6 hours (a few

animals were still alive in each group after scoring of worms ceased so means were taken from slightly incomplete data) (Figure 9; Table 2). This revealed that *rde-4* (*ne299*) is not necessary for the predictive nature of the reporter (Figure 9; Table 2).

The loss of *rde-4* (*ne299*) also appeared to increase the overall thermotolerance of the animals (Figure 9). The mutant "bright" and "dim" animals were significantly more thermotolerant than the control "bright" and "dim" animals, respectively ($p < 0.02$ for the "bright" vs. "bright" animals and $p < 0.00001$ for the "dim" vs. "dim" animals) (Figure 9).

Figure 9: The *rde-4* (*ne299*) Mutant



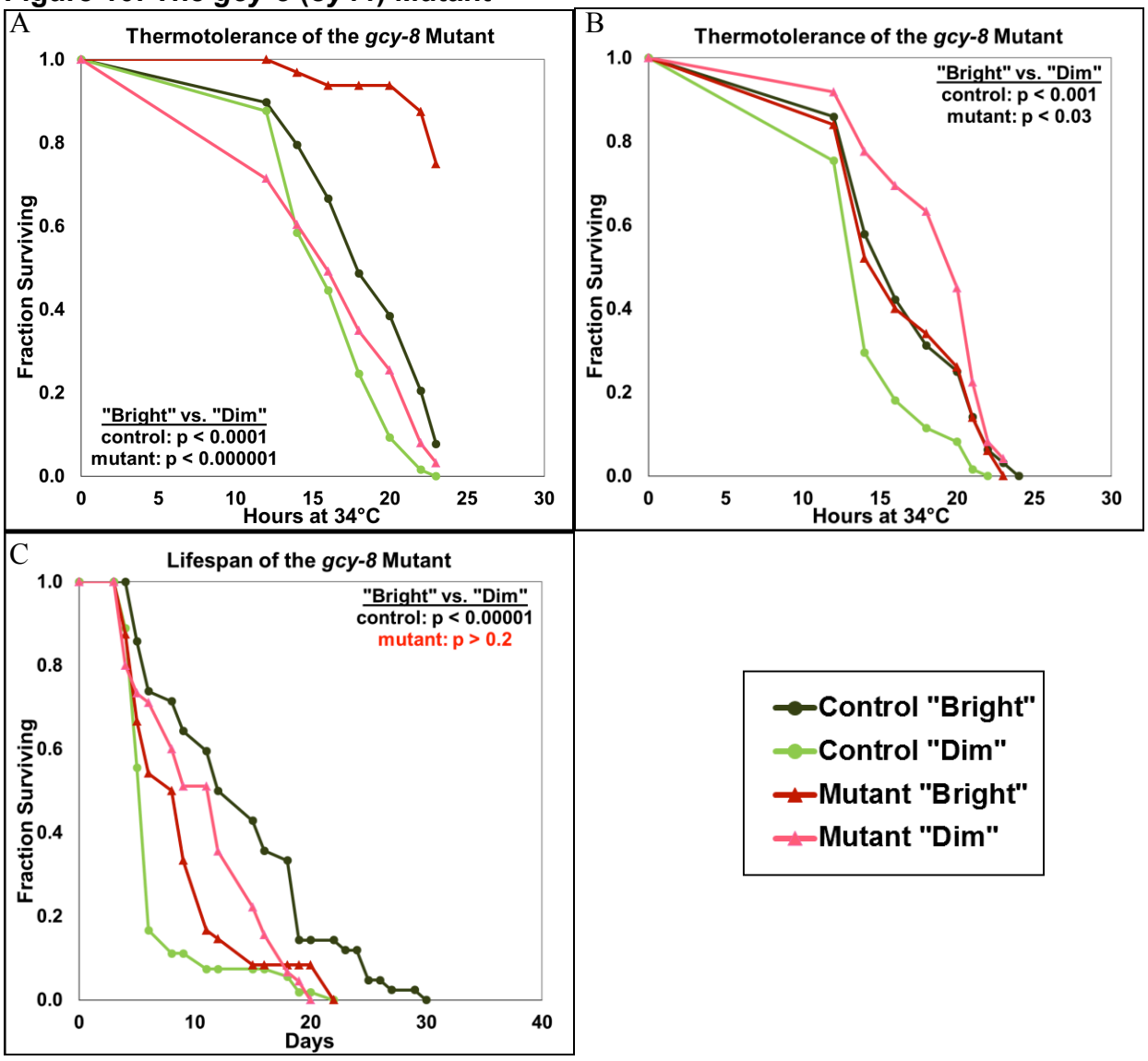
The *gcy-8* (*oy44*) mutant:

In one of the thermotolerance tests done on strain TJ3301 carrying a mutation to *gcy-8* (*oy44*), the normal effect seen with the "bright" and "dim" animals was reversed; the thermotolerance of the "dim" animals was significantly greater than that of the "bright" animals ($p < 0.03$) (Figure 10B; Table 2). The average time of death at lethal heat was 16.7 ± 0.5 hours for the "bright" mutant animals and 18.8 ± 0.5 hours for the "dim" mutant animals (Figure 10B; Table 2). In the other thermotolerance test done on

the *gcy-8* (*oy44*) mutant, the reporter maintained its normal predictive ability but the "bright" animals were extremely more thermotolerant than the "dim" animals ($p < 0.000001$) (Figure 10A; Table 2). The mean survival during thermotolerance was 23.2 ± 0.4 hours for the "bright" mutant animals and 16.9 ± 0.5 hours for the "dim" mutant animals (a few animals were still alive in each group after scoring of worms ceased so means were taken from slightly incomplete data) (Figure 10A; Table 2). In a lifespan test done on the *gcy-8* (*oy44*) mutant, the reporter lost its ability to predict lifespan; the "bright" mutant animals were not significantly longer lived than the "dim" mutant animals ($p > 0.2$) (Figure 10C; Table 2).

Additionally, in both of the thermotolerance tests done on the *gcy-8* (*oy44*) mutant, it appeared that the mutant had an overall increased thermotolerance (Figure 10A, 10B). In the thermotolerance test shown in figure 10A, the mutant "bright" animals were significantly more thermotolerant than the control "bright" animals ($p < 0.000001$) but the mutant "dim" animals were not more thermotolerant than the control "dim" animals ($p > 0.1$). In the thermotolerance test shown in figure 10B, the mutant "bright" animals were compared to the control "dim" animals since there was a reversal of thermotolerant ability in the mutant "bright" and "dim" animals. The mutant "bright" and "dim" animals were significantly more thermotolerant than the control "dim" and "bright" animals, respectively ($p < 0.01$ for the mutant "bright" vs. the control "dim" and $p < 0.03$ for the mutant "dim" vs. the control "bright").

Figure 10: The *gcy-8* (*oy44*) Mutant



1X-backcrossed mutants:

1X-backcrossed mutants, including strain TJ3302 carrying an *hsp-16.2* (*gk249*) mutation, strain TJ3402 carrying an *hsp-16.11* and *hsp-16.49* (*tm1221*) mutation, and strain TJ3400 carrying an *F08H9.4* (*ok1976*) mutation, were tested. Two thermotolerance tests were done on the 1X-backcrossed mutant of *hsp-16.2* (*gk249*). In both tests, the reporter was not predictive of stress resistance (there was no significant difference between the “bright” and “dim” mutant animals during the thermotolerance

tests). In both a thermotolerance and a lifespan test done on the 1X-backcrossed mutant of *hsp-16.11* together with *hsp-16.49* (*tm1221*), the reporter lost its predictive power. The reporter also lost its predictive ability in the thermotolerance test done on the 1X-backcrossed mutant of *F08H9.4* (*ok1976*). Backcrossing mutant strains one time is not sufficient to remove all genetic background differences that may have been present in the strain, so these results may have been driven by mutations other than those being tested.

Table 2: Summary of Results

Strain (Backcross Number)	Gene(s) Tested	Type of Test	Mean ± SEM "Bright" (Days or Hours)	Mean ± SEM "Dim" (Days or Hours)	p-value of "Bright" vs. "Dim"
TJ3302 (1X)	*hsp-16.2*	TT	19.6 ± 0.05	20.1 ± 0.04	p > 0.4
TJ3302 (1X)	*hsp-16.2*	TT	16.1 ± 0.4	15.4 ± 0.5	p > 0.4
TJ3322 (4X)	*hsp-16.2*	TT	12.7 ± 0.3	10.5 ± 0.4	p < 0.001
TJ3322 (4X)	*hsp-16.2*	LS	19.1 ± 1.2	14.7 ± 1.1	P < 0.03
TJ3425 (4X)	*hsp-16.41*	TT	18.0 ± 0.6	12.2 ± 0.5	p < 0.00001
TJ3425 (4X)	*hsp-16.41*	LS	22.0 ± 1.0*	3.5 ± 0.5	P < 0.000001
TJ3421 (4X)	*hsp-16.1* & *hsp-16.48*	TT	21.2 ± 0.3*	16.9 ± 0.5	p < 0.00001
TJ3402 (1X)	*hsp-16.11 & hsp-16.49*	TT	18.0 ± 0.4	19.5 ± 0.5	p > 0.06
TJ3402 (1X)	*hsp-16.11 & hsp-16.49*	LS	23.0 ± 1.2	21.4 ± 1.8	p > 0.8
TJ3426 (4X)	*F08H9.3*	TT	14.1 ± 0.3	12.2 ± 0.1	p < 0.00001
TJ3426 (4X)	*F08H9.3*	LS	9.0 ± 0.9	6.7 ± 0.4	P < 0.0001
TJ3400 (1X)	*F08H9.4*	TT	21.6 ± 0.5	22.4 ± 0.6	p > 0.05
TJ3420 (4X)	*F08H9.4*	TT	21.3 ± 0.3	19.4 ± 0.4	p < .02
TJ3423 (4X)	*hsp-17*	TT	23.5 ± 0.2*	15.2 ± 0.5	p < 0.000001
TJ3424 (4X)	*hsp-70*	TT	20.1 ± 0.5	14.1 ± 0.4	p < 0.00001
TJ3424 (4X)	*hsp-70*	LS	26.2 ± 1.0*	13.4 ± 1.6*	P < 0.001
TJ3314 (5X)	*rde-4*	TT	23.9 ± 0.3*	20.3 ± 0.6*	p < 0.001
TJ3301 (11X)	*gcy-8*	TT	16.7 ± 0.5	18.8 ± 0.5	P < 0.03**
TJ3301 (11X)	*gcy-8*	TT	23.2 ± 0.4*	16.9 ± 0.5*	P < 0.000001
TJ3301 (11X)	*gcy-8*	LS	10.8 ± 0.8	9.0 ± 0.7	p > 0.2

*Means ± SEMs are reported in hours for thermotolerance (TT) tests and in days for lifespan (LS) tests. If the p-value of "Bright" animals vs. "Dim" animals is significant, then the reporter is predictive of lifespan. Non-significant p-values (indicating that the reporter lost its predictive ability) are shown in red. *These values collected from incomplete data; either a few animals were still alive after scoring of worms ceased or the experiment is still in progress. **The reporter predicted the opposite effect in one thermotolerance test for the gcy-8 (oy44) mutant.*

Discussion

None of the results obtained for this thesis provided evidence that any of the tested genes were required for *Phsp-16.2::GFP* to be predictive of stress resistance (thermotolerance) or lifespan in an isogenic population of *C. elegans* (a summary of these results is shown in Table 2). The four mutants for which the reporter lost its ability to predict thermotolerance or lifespan [*hsp-16.2* (*gk249*), *hsp-16.11 and 16.49* (*tm1221*), *F08H9.4* (*ok1976*), and *gcy-8* (*oy44*)] either had contradicting results associated with them [*gcy-8* (*oy44*)], or were obtained from mutants that were not sufficiently backcrossed with the wildtype strain [*hsp-16.2* (*gk249*), *hsp-16.11 and 16.49* (*tm1221*), and *F08H9.4* (*ok1976*)] (Figure 10A-C; Table 2).

The *hsp* mutants:

Because the *Phsp-16.2::GFP* reporter contains the promoter sequence from *hsp-16.2* and is expressed concordantly with endogenous *hsp-16.2* it was reasonable to expect that *hsp-16.2* (*gk249*) was required for the predictive capability of the reporter. However, the loss of *hsp-16.2* (*gk249*), or any other *hsp* gene tested, did not alter the predictive power of the reporter (Figure 6A, 6B, 7A-F, 8A-C; Table 2). This reveals that none of the tested *hsp* genes is individually responsible for the variance in thermotolerance and lifespan seen in isogenic populations of *C. elegans.*

Only three out of the seven mutants tested [representing four *hsp* genes, *hsp-16.41* (*tm1093*), *hsp-16.1* (*ok577*), *hsp-16.48* (*ok577*), and *F08H9.3* (*tm5012*)] appeared to affect the thermotolerance of the animals (Figure 7A-E). This is surprising since all tested genes were predicted to have a role in the stress response. However, given the

extreme homology between members of the *hsp-16* gene family and even *hsp-17* (WormBase.org), it is possible that the proteins encoded by many of the *hsp* genes are functionally redundant. This would explain why some of the losses of individual genes did not have a noticeable effect on the stress resistance of the worms.

Two of the most interesting results were those from the *F08H9.3* (*tm5012*) and the *hsp-16.41* (*tm1093*) mutants. Both the *F08H9.3* (*tm5012*) gene and the *hsp-16.41* (*tm1093*) gene appeared to be required for full thermotolerance of the animals, yet neither of these genes was vital for the predictive nature of the reporter (Figure 7A-D; Table 2). This implies that the reporter's predictive ability is not linked to heat resistance, even though it is driven by a heat-responsive reporter.

For one mutant of the *hsp-16* gene family, the *hsp-16.49* and *hsp-16.11* (*tm1221*) mutant, a sufficiently backcrossed strain was not tested. Until such a strain can be tested, it is unclear whether *hsp-16.49* and *hsp-16.11* (*tm1221*) are required for the predictive ability of the reporter. However, since *hsp-16.11* and *hsp-16.49* (*tm1221*) are perfect duplicates of *hsp-16.1* and *hsp-16.48* (*ok577*), respectively, it is unlikely that they are involved in the capability of the reporter to be predictive, since *hsp-16.1* and *hsp-16.48* (*ok577*) were not required for the predictive ability of the reporter (Figure 7E; Table 2).

The *rde-4* (*ne299*) mutant:

The *Phsp-16.2::GFP* reporter was predictive of thermotolerance in the *rde-4* (*ne299*) mutant (Figure 9; Table 2). This suggests that RNAi may not be involved in the predictive nature of this reporter. The result for the *rde-4* (*ne299*) mutant also showed

that the loss of *rde-4* (*ne299*) increased the thermotolerance of the animals (Figure 9). This is inconsistent with the findings of Mansisidor et al. (2011) and Blanchard et al. (2011), who showed that *rde-4* (*ne299*) mutants have reduced lifespan and resistance to oxidative and thermal stress. The discrepancy between these results could be due to procedural difference. In this project, I tested "induced" thermotolerance, meaning that the animals were subjected to a one-hour heat shock (see methods) before they were permitted to recover, and subsequently tested for thermotolerance. It is therefore possible that differential hormetic induction of stress resistance could account for the increased thermotolerance of the *rde-4* (*ne299*) mutants in this project. Neither Blanchard et al. (2011) nor Mansisidor et al. (2011) subjected their animals to stress pretreatment prior to testing for stress resistance.

The *gcy-8* (*oy44*) mutant:

There was inconsistency among the thermotolerance and lifespan tests of whether *gcy-8* (*oy44*) is required for the reporter to be predictive (Figure 10A-C; Table 2). Additionally, none of the tests on this mutant resulted in a reduced thermotolerance (Figure 10A-C). This was unexpected since Prahlad and Morimoto (2008) showed that *gcy-8* (*oy44*) mutants were less thermotolerant than wildtype animals.

A comparison between the methods used by Prahlad and Morimoto (2008) and the methods used for this project reveals that the *gcy-8* (*oy44*) mutants were grown at different densities and heat shocked differently in the two studies. Prahlad and Morimoto found that the density at which the animals were grown had a significant effect on their results. This finding was replicated in unpublished results from Alex

Mendenhall working in the Johnson Lab at the University of Colorado, Boulder. Prahlad and Morimoto (2008) grew up their animals at the low density of 10 adults per 6 cm plate. I raised my animals at a density of about 3,000 adults per 9 cm plate for this project. Further, Prahlad and Morimoto (2008) heat shocked their animals on solid agar plates and I heat shocked my animals in a liquid medium.

The differences between the two procedures could be the cause of the discrepancy between the results of Prahlad and Morimoto (2008) and those presented here. It is possible that the loss of *gcy-8* (*oy44*) makes the animals more sensitive to slight environmental changes. This sensitivity may also explain why the results of the two thermotolerance and one lifespan test on the *gcy-8* (*oy44*) mutant shown here are inconsistent.

1X-backcrossed mutants:

In all of the tests done on 1X-backcrossed mutants, the *Phsp-16.2::GFP* reporter lost its ability to predict thermotolerance or lifespan (Table 2). In two cases [the *hsp-16.2* (*gk249*) mutant and the *F08H9.4* (*ok1976*) mutant] this result differs from the results of the associated 3X-backcrossed mutant (Figure 6A, 7F; Table 2). It is possible that unknown background mutations in the 1X-backcrossed animals were interfering with the reporter's predictive ability. This reveals the importance of backcrossing strains and indicates that results from 1X-backcrossed mutants should not be given much weight, as they are only preliminary. The only other 1X-backcrossed mutant tested was the *hsp-16.49* and *hsp-16.11* (*tm1221*) mutant. No tests were done on a sufficiently backcrossed mutant of these genes.

Thermotolerance vs. lifespan experiments:

Six mutants were tested for both thermotolerance and lifespan: the *hsp-16.2* (*gk249*), *hsp-16.41* (*tm1093*), *hsp-16.11 and hsp-16.49* (*tm1221*), *F08H9.3* (*tm5012*), *hsp-70* (*tm2318*), and *gcy-8* (*oy44*) mutants. In five of these mutants, the results of the two tests were consistent with each other (Figure 6A, 6B, 7A-D, 8A, 8B; Table 2). Only the thermotolerance and lifespan results of the *gcy-8* (*oy44*) mutant were not consistent (Figure 10A-C; Table 2). However, the two thermotolerance tests done on this mutant also disagreed (Figure 10A, 10B; Table 2), implying that procedural differences may have caused inaccurate or inconsistent results for this mutant. The general consistency between the thermotolerance and lifespan data is further evidence that stress resistance (thermotolerance in particular) confers increased lifespan in *C. elegans.*

Limitations:

None of the results presented in this thesis were replicated enough to give a sufficient amount of confidence in them. Additionally, some of the mutants tested (the 1X-backcrossed mutants) were not sufficiently backcrossed with the wildtype strain so that, apart from the desired mutation, uniformity of the genetic backgrounds of the experimental and control strain cannot be assumed.

In general, the procedure for this project was based on the methods published by Rea et al. (2005). In both the procedure followed by Rea et al. (2005) and the procedure followed in this experiment, slight environmental differences were possible, and may have caused inaccurate or inconsistent results. For example, the density at which the

animals were raised was not tightly controlled and probably varied to some extent within and between experiments. Further, the concentration and amount of food distributed to each strain was controlled within experiments but not between them. Sánchez-Blanco and Kim (2011) found that an *E. coli* diet increased the variation of lifespan within populations of *C. elegans* when compared to a diet of *Bacillus subtilis* (*B. subtilis*). If the animals are sensitive to *E. coli* in general, then varying the *amount* of *E. coli* given to the animals in each experiment could cause more or less variation in thermotolerance or lifespan in the animals in each strain.

Another limitation of the data presented here is that, due to a lack of time, lifespan tests were not done on all mutants. Thermotolerance tests were completed for all strains. Since there is a strong, direct correlation between stress resistance (in particular, thermotolerance) and lifespan (Johnson et al., 2002; Link et al., 1999; Morley et al., 2004; Rea et al., 2005; Walker et al., 2003), the results of the two tests should be broadly similar. However, it is possible that some of the mutations present in tested strains could affect the reporter's ability to predict survival during normal aging but not thermotolerance.

Future Research:

In order to strengthen the claims made here, replicates of all experiments should be conducted. Additionally, lifespan tests should be done on all mutant strains. Results that were inconsistent with previously published data, such as the results for the *rde-4* (*ne299*) and *gcy-8* (*oy44*) mutants, should be replicated or dismissed. For the *gcy-8* (*oy44*) mutants, experiments on how the population density in which the animals are

grown affects the phenotype should be conducted. Tests should also be done to determine whether subjecting animals to small periods of stress prior to a stress resistance test greatly affects the results of a stress resistance test.

Further tests should be done to strengthen the claim that the predictive power of the reporter is not linked to thermotolerance as originally believed. Mutants that have very little thermotolerant ability should be tested to see if the *Phsp-16.2::GFP* reporter can still predict thermotolerance and lifespan of the animals. Finally, the 1X-backcrossed strains should be sequenced and examined for matching mutations, as these mutations may indicate genes required for the predictive nature of the *Phsp-16.2::GFP* reporter.

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