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Characterization of the Responses to Chronic Stress in Caenorhabditis elegans

Amy Laura Knight

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by

Amy Laura Knight

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We the undersigned committee hereby approve the attached thesis,
"Characterization of the Responses to Chronic Stress in *Caenorhabditis elegans"*

by

Amy Laura Knight

_____

Eric A. Guisbert, Ph.D.
Biomedical and Chemical Engineering and Sciences
Major Advisor

_____

Melissa A. Borgen, Ph.D.,
Assistant Professor
Biomedical and Chemical Engineering and Sciences

_____

Brooke E. Wheeler, Ph.D.,
Assistant Professor
College of Aeronautics

_____

Andrew D. Knight, Ph.D.,
Department Head
Biomedical and Chemical Engineering and Sciences

Abstract

Title: Characterization of the Responses to Chronic Stress in *Caenorhabditis elegans*

Author: Amy Laura Knight

Advisor: Eric A. Guisbert, Ph.D.

Exposure to chronic temperature stress influences organismal phenotypes that are important for human health, agriculture, and ecology. In this thesis, the model organism *Caenorhabditis elegans* was used to study the effects of temperature stress on reproduction and lifespan. It was found that worms demonstrated a rapid shut down in egg-laying between 18-24 hours of exposure to 28°C. Despite this reproductive defect, the overall lifespan of worms was unaffected. At the molecular level, heat shock factor 1 (HSF-1), a regulator of the protective molecular pathway known as the heat shock response (HSR), was identified as important for progeny production during heat stress and recovery of fecundity and lifespan after heat stress. The GAL4-UAS system established in *C. elegans* (cGAL) was utilized to generate worms with tissue-specific HSF-1 overexpression in order to determine which tissues were relevant for this protection.

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Chapter 1

Introduction

Organisms are exposed to fluctuating temperatures throughout their lifetime. Organismal survival during temperature stress requires stress response mechanisms that detect the stress and implement protective strategies to counter the negative effects. In addition to temperature, other environmental conditions, chemical exposures, developmental transitions, and pathophysiological states can induce stress responses (Morimoto 1998).

From an ecological perspective, environmental temperature stress can significantly affect population size and biodiversity. Both acute and chronic temperature stress can be lethal if the organism has not developed protective mechanisms (Morimoto 1998, Guisbert *et al.* 2013). An increase in the global mean surface temperature of 3.7 °C by the year 2100 is predicted to lead to an extinction rate of > 8.5% for all species (Urban 2015, Ali *et al*. 2020). If temperature continues to rise, 1 in 6 species face the risk of extinction due to climate change (Urban 2015). Species extinction can impact genetic diversity and may influence evolutionary adaptation (Kristensen *et al*. 2020).

Temperature fluctuations have many direct and indirect effects on physiology. For example, temperature stress can influence livestock reproduction, quantity and quality of meat and milk production, as well as egg yield (Sejian *et al.* 2018, Plagens *et al*. 2021). Increased temperature can also affect metabolic and

immune responses and induce temperature-related diseases (Ali *et al.* 2020). Exploring genetic pathways in response to environmental stress on an organism's overall fitness could provide insight into organismal recovery after cellular or tissue damage from environmental temperature stress.

Exposure to extreme temperature conditions can impact protein homeostasis and lead to an accumulation of misfolded proteins. Protein misfolding and aggregation are key features of both aging and age-related diseases (Jovic *et al.* 2017). The primary cellular stress response to temperature is the highly conserved heat shock response (HSR). The HSR aids in the maintenance of protein folding homeostasis by activating transcription of heat shock genes to produce molecular chaperones and heat shock proteins (hsps) that assist in repairing protein damage after stress (Morimoto 1998, Li *et al*. 2017). Heat shock factor 1 (HSF-1) is the master regulator of the HSR and is essential in activating this protective molecular response (Akerfelt *et al.* 2010, Vihervaara and Sistonen 2014).

The effects and responses to heat stress on reproduction have been studied in the model organism *Drosophila melanogaster*. Several strains of *D. melanogaster* induced male sterility at temperatures above 30 °C; however, if returned to a more permissive temperature, spermatogenesis can be rescued. Interestingly, sterile males were also noted at a lower temperature of 13 °C, suggesting that exposure to a wide range of temperature conditions can affect fertility (David *et al.* 1976, David *et al.* 2005). Similarly, in mammals, an increase in testicular temperature in males can negatively impact spermatogenesis and

impair embryonic development after fertilization. For females, oocyte function and development can be impaired at high temperatures resulting in altered gene expression, imbalanced hormone secretion and longer duration of oocyte and embryonic maturation (Hansen 2009).

C. elegans have also been used as a model to study temperature stress. Their genome is approximately 100 million basepairs with around 20,122 protein coding genes (database version WS274; https://wormbase.org//about/wormbase_release_WS274#0--10). Over two-thirds of the *C. elegans* proteome shares homology with genes associated with human disease, facilitating more research into human gene function and signal transduction pathways (Henricson *et al*. 2004, Hillier *et al.* 2005, Zhang *et al.* 2020). The first published study using *C. elegans* as a model organism to analyze neuronal development and function was performed by Sydney Brenner in 1974. His ability to use forward genetics resulted in characterization and mapping of several genes involved in motility. This research highlighted the value of using *C. elegans* as a genetic model organism for human disease studies (Walsh *et al.* 2020).

C. elegans have two sexual forms, a self-fertilizing hermaphrodite and a less common male form. Sex determination is based on the X chromosome where homozygous (XX) animals are hermaphrodites and hemizygous (XO) animals are males. The self-fertilizing hermaphrodite has a brood size of ~300 offspring. Of the progeny, 0.2-0.5% are male due to chromosomal non-disjunction. The frequency of males in a population can be increased upon exposure to heat stress as well as by

genetic mutations that increase the frequency of chromosomal non-disjunction (Walsh *et al.* 2020, Hodgkin 1983, Hodgkin *et al*. 1979). An outcrossed hermaphrodite that has mated with a male can produce approximately 1,400 offspring; however, this has proved selectively unfavorable during optimal growth despite the increase in progeny (Chasnov 2013).

Adult *C. elegans* have an invariant cell lineage for both hermaphrodites and males (959 and 1,031 somatic cells, respectively). Of these cells, the nervous system is comprised of 302 neurons in hermaphrodites and 381 neurons in males. With a definitive cell number, it is easy to target and select cells and/or tissues for *in vivo* experimentation. Due to their transparent body, visualization using florescence microcopy or differential interference contrast (DIC) of tissues can be advantageous especially during early stages of development and aging (Silverman *et al.* 2010). Construction of transgenic lines or gene fusions with the use of florescent gene reporters can give insight into specific gene function in cells or tissue types (Henricson 2004).

Caenorhabditis elegans is an established eukaryotic multicellular model organism that is widely used to research the effects and responses of temperature stress *in vivo.* The majority of studies have focused specifically on acute temperature stress by exposing the organism to short durations of temperatures between 33-35 °C. Transcriptome analysis reveals that expression of genes involved in reproduction, metabolism and development is altered after a two-hour exposure to 35 °C and a thirty-minute exposure to 33 °C. These studies have

demonstrated initiation of the HSR, which is dependent on the activation of HSF-1, to provide the organism with a protective strategy to reduce damage from heat stress (Jovic 2017, Snoek *et al.* 2017, Brunquell *et al*. 2016, Morimoto, 2008). Few studies have researched *C. elegans* response to prolonged temperature exposure.

A macro-level model was developed to understand how chronic temperature stress can affect egg laying in *C. elegans* (McMullen et *al*. 2012). The number of eggs that were laid by individual worms was recorded after exposure of young adults to different temperatures (20-30 °C). At 28°C, total brood sizes are reduced compared to worms exposed to lower temperatures (20-25 °C). Analysis of single worms revealed that chronic temperature stress has a heterogenous effect on reproduction. It was proposed that some worms anticipate the return of favorable temperature conditions (fast egg laying), whereas others reduce their reproduction rate in order to conserve their egg laying ability during prolonged stress. This could be an adaptive coping mechanism for rapid temperature fluctuations.

A more recent study by our laboratory examined chronic heat stress and its effect on reproduction and reproductive recovery in *C. elegans* (Plagens *et al*. 2021). Worms exposed to 28 ℃ for 24 hours, have a 2-fold decrease in egg-laying compared to WT worms at an unstressed temperature of 20 ℃. This decrease was not observed at the 12-hour timepoint. After 36 hours of heat stress, worms stop laying eggs all together. Recovery of egg laying was examined after 24 hours of temperature stress followed with a 3-day recovery period at an unstressed temperature of 20 ℃. WT worms showed some recovery in egg laying; however,

the total egg count was far less than the normal expected brood size obtained from unstressed worms.

Transcriptome analysis using RNAseq revealed upregulation and downregulation of specific genes in response to chronic stress. Analysis of genes associated with reproduction found that approximately 4% of oogenesis genes were downregulated 4-fold by heat stress (HS) at 28 ℃ for 24 hours. Other phenotypes in the worm were also affected by chronic heat stress such as neuronal signaling, which has a role in reproduction. Titration of chaperone proteins was shown to negatively impact endocytosis, which is needed for loading vitellogenin genes into oocytes. By overexpressing HSF-1 and upregulating chaperone genes, partial rescue of endocytosis as well as egg laying can be observed in *C. elegans* after exposure for 24 hours at 28 ℃ (Plagens *et al*. 2021).

These studies have raised important questions regarding how organisms respond to chronic temperature stress. For example, the boundaries of the phenotypic space of recovery of reproduction have not been determined. Exploration of the capacity for recovery may give important insights into the nature of the cellular damage and the success of organismal defense mechanism. Is there a point at which the damage accumulated in the worm is catastrophic, and the egg laying ability is no longer recoverable? After shorter periods of chronic heat stress, can the total brood size of the worm be fully recovered? Aside from reproduction, the other consequences of chronic temperature stress on organismal health, such as lifespan, have also not been carefully examined. Does the damage from chronic

temperature stress reduce the lifespan of the organism, and can the typical lifespan be recovered once the worms are returned to unstressed conditions?

The heat shock response has been shown to play a beneficial role in the recovery of egg laying through whole worm overexpression of HSF-1. However, it is not yet known which relevant tissue types are needed for this recovery. Does the location of HSF-1 overexpression have differing effects on the organismal health after chronic heat stress? This thesis explores these and other important questions by measuring the effects of temperature stress on reproduction, lifespan, and transgenic worms with tissue specific HSF-1 overexpression.

Chapter 2

Materials and Methods

2.1 Worm maintenance and strains

Caenorhabditis elegans were maintained at 20 ℃ on petri dishes containing nematode growth media (NGM) seeded with OP50 *E. coli* bacteria according to standard procedures (Brenner, 1974). Bleach synchronization was performed according to standard procedures. The following strains were used: Bristol N2 (wild-type); HSF-1 overexpression, EQ140 (iqIs37[pAH76(hsf-1p::myc-hsf-1)+pRF4(rol-6p::rol-6(su1006)]; and HSF-1 mutant, PS3551 (hsf-1(sy441)I)). Driver strains from the Paul Sternberg lab were: neuronal driver (PS6961); syls334 [rab3p::NLS::GAL4SK::VP64::let-858 3' UTR + unc-122p::RFP + pBlueScript], body wall muscle (BWM) driver (PS6936); syls321 [myo-3p::NLS::GAL4SK::VP64::unc-54 3' UTR + myo-2p::NLS::mCherry + pBlueScript], intestinal driver (PS6916); syls317 [nlp-40::NLS::GAL4SK::VP64::unc-54 3' UTR + myo-2p::NLS::mCherry + pBlueScript]. The following effector strains were generated: effector #1, EAG012; eagIS2[UASp::hsf-1::myc;myo2p::gfp], effector #2, EAG013; eagIS3[UASp::hsf-1::myc;myo2p::gfp], and effector #3, EAG014; eagIS4[UASp::hsf-1::myc;myo2p::gfp]. All effector strains were backcrossed x4 with WT (N2) worms. The extra-chromosomal array strain (EAG010); eagEx1[hsf-1::myc-2p::gfp] spontaneously integrated to generate effector #4, EAG019; eagIS5 [UASp::hsf-1::myc;myo2p::gfp]. Driver-effector crosses used: neuronal-effector

#1, neuronal-effector #2, neuronal-effector #3, BWM-effector #1, BWM-effector #2, BWM-effector #3, neuronal-effector #4, BWM-effector #4. The tau strain CK1046 [pAex-3::Tay 4R1N P301S Tg; eri-1 (mg366); lin-15B (n744)] was obtained from Brian Kraemer. This strain was backcrossed with WT N2 (x4) to remove any RNAi sensitization. Additional crosses include a tau-neuronal HSF-1 overexpression line and tau-BWM HSF-1 overexpression line. These strains were generated from crossing the backcrossed CK1046 strain with the neuronal-effector #4 line and BWM-effector #4 line.

2.2 Microscopy

Individual worms were mounted for fluorescence imaging onto 3% agarose pads in 1 mM levamisole. Fluorescence of worms was observed using a 4-line Solid State Laser Confocal Nikon C2 with fully motorized inverted microscope Nikon Ti2 at 20x objective lens. Additional microscopy was carried out using a stereomicroscope with a NightSea stereomicroscope fluorescence adaptor for visualization of GFP, RFP and mCherry in effector and driver strains.

2.3 Thrashing assay: motility

Thrashing was scored at room temperature (RT) in a droplet of M9 buffer (~10 µL) on an unseeded NGM plate. M9 buffer was prepared using standard procedures. Individual worms were placed on the droplet and allowed to acclimate for 30 seconds, and then body bends (thrashes) were counted for 1 minute. A single

thrash is determined when either the worm's head or tail reaches the midpoint of the body and then returns to its original position.

2.4 Chronic stress progeny-counting experiments

Two methods (A and B) were used for chronic stress progeny-counting experiments:

A) Synchronized worms (WT, N2) were singled out prior to L4 larval stage onto 35 mm x 10 mm nematode growth media (NGM) plates seeded with 150 µL of OP50 *E. coli* bacteria and kept at 20 ℃. Eggs were counted at approximately 60 hours post synchronization when worms were day 1 egg-laying adults (this represents the 0-hour timepoint). Worms were then transferred to a fresh 35 mm plate and shifted to 28 ℃ for HS or maintained at 20 ℃ for control conditions. Worms were transferred to a fresh NGM plate every 12 hours for a total of 36 hours. For the HS worms, fresh pre-warmed plates at 28 ℃ were used. The number of progeny produced was scored at each timepoint. Transferring occurred at room temperature (RT) with minimal exposure (<5 min) to temperature fluctuations.

B) Synchronized worms (WT, N2; HSF-1 overexpression, EQ140 and HSF-1 mutant, PS3551) were singled out prior L4 larval stage onto 35 mm x 10 mm NGM plates seeded with 150 µL of OP50 and kept at 20 ℃. Approximately 60 hours post synchronization, day 1 egg laying adult worms were then shifted to 28 ℃ for HS for a total duration of either 12, 18

or 24 hours. The number of progeny produced was counted at each timepoint.

2.5 Reproduction recovery experiments

Synchronized day 1 WT (N2) adult worms, HSF-1 overexpression (EQ140) worms and loss-of-function mutant HSF-1 *sy441* (PS3551) worms were singled out onto 35 mm x 10 mm NGM plates seeded with OP50 *E. coli* bacteria. The worm plates were then shifted to 28 ℃ for12, 18 or 24 hours and then shifted back down to 20 ℃ for 5 days recovery. The adult worms were quickly transferred onto a fresh 28 ℃ pre-warmed plate and then allowed to recover in a 20 ℃ incubator. After the HS timepoint at 28 ℃, the total number of progeny were counted. Cumulative progeny included both eggs and larva. During the recovery period of 5 days, adults were transferred daily, and the number of progeny were counted.

2.6 Lifespan assays

Synchronized day 1 WT (N2) adult worms, HSF-1 overexpression (EQ140) worms and loss-of-function mutant HSF-1 *sy441* (PS3551) worms were shifted to 28 ℃ for 24 hours or 48 hours. Control worms were exposed to constant temperatures of 20 ℃ or 28 ℃. After HS for 24 hours or 48 hours, the worms were downshifted to 20 ℃ for the remainder of the lifespan. Worms were transferred to a new plate every 2 days for the first 6 days of the experiment to remove any

progeny. Worms were scored as dead in the absence of touch response and then removed. Missing worms were censored from the analysis.

2.7 Statistics, figures, and tables

All bar charts were prepared with statistics from Microsoft Word Excel Version 16.61.1. Student *t*-test was performed to obtain *p*-values. A p-value < 0.05 was considered statistically significant. Lifespan assay plots were generated using the Online Application for the Survival Analysis of Lifespan Assays Performed in Aging Research (OASIS) (Yang *et al.* 2011). Statistical analysis included Kaplan Meier estimator, Mean/Median lifespan, Log-Rank Test and Fisher's Exact Test. Lifespan assay tables were generated from the statistics analysis data in OASIS.

2.8 Integration of extrachromosomal array

On an uncovered, bacteria-free nematode growth media (NGM) plate, day 1 adult worms were UV irradiated at 360 μJ and 720 μJ using a Stratagene Stratalinker® UV 1800 crosslinker. Worms were transferred after UV irradiation to an OP50 *E. coli* bacteria seeded NGM plate and allowed to recover at 20 ℃ incubator overnight. Worms were monitored for survival and egg production. GFP fluorescence was observed in the resulting F1 generation.

2.9 Generation of tissue specific HSF-1 overexpression strains

Hermaphrodites from effector #1, #2, #3 and #4 were crossed with WT (N2) males (3:11 or 4:12). Successfully crossed males showed GFP expression in the pharynx of the worm. Effector males were crossed with either neuronal, body wall muscle (BWM) or intestinal driver strains to generate a tissue specific HSF-1 overexpression (O/E) strain (11:3 or 12:4). Resulting worm strain was confirmed based on expression of GFP, RFP and mCherry in the appropriate tissue types. Tau-neuronal HSF-1 O/E strain and Tau-BWM HSF-1 O/E strain were generated using neuronal-effector #4 males and BWM-effector #4 males crossed with tau (CK1046) hermaphrodites (15: 5). Resulting worm strains was confirmed based on expression of GFP, RFP and mCherry in the appropriate tissue types

2.10 Thermorecovery assay

Synchronized day 1 adult worms (for all strains) were heat shocked in a circulating water bath at 33 ℃ for 6 hours and then allowed to recover for 48 hours in a 20 ℃ incubator. NGM plates seeded with OP50 *E. coli* bacteria were wrapped in parafilm to prevent water contamination. This was removed prior to recovery. Worms were scored based on normal or abnormal motility after recovery. Normal movement included sinusoidal body bends, with the worm having a fast reaction to physical stimulation from a worm pick. Abnormal movement was determined by jerky, uncoordinated movements, and a slow reaction to physical stimulation or paralysis.

Chapter 3

Results

3.1 Effects of chronic temperature stress on reproduction

Exposure of *C. elegans* worms to a chronic temperature stress of 28 ℃ inhibits egg laying. To explore these conditions in greater detail, the reproducibility of the previous research was first determined. Exposure of wild-type, day 1 egg-laying adult worms to 28 ℃ caused no significant difference between heat stressed worms and control worms at 20 ℃ with regard to the number of eggs laid at the 12-hour timepoint (Figure 1). After 24 hours, there was a dramatic decrease in the number of eggs laid by worms exposed to 28 ℃ compared to the control. The heat stressed worms laid 18 eggs and the control worms laid 48 eggs (p-value = 4.40E-07). After 36 hours of heat stress, worms showed almost a complete cessation of egg laying ability at 28 ℃ compared to 56 eggs from control (p-value = 8.31E-07). The control worms laid approximately the same number of progeny between 12 and 24 hours with a slight increase after 36 hours.

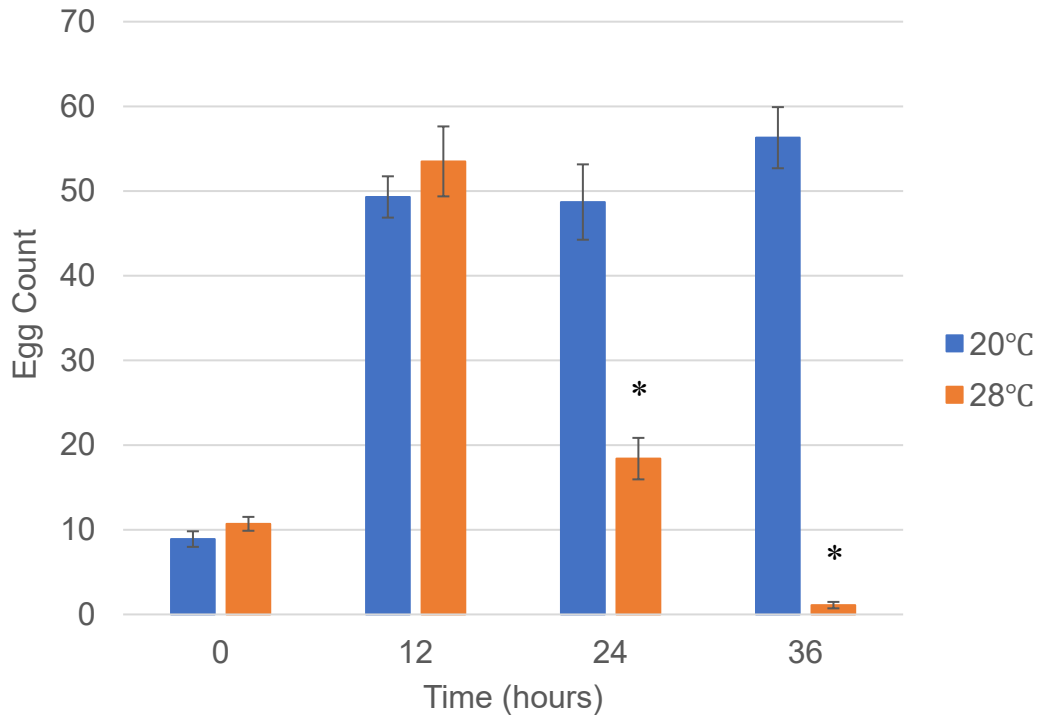

Figure 1. Egg laying during heat stress of adult worms at 28 °C and unstressed conditions at 20 °C. Worms were exposed to 28 °C at day 1 of adulthood and transferred every 12 hours to a fresh, pre-warmed NGM plate. Data represents mean ± SE of n = 10. The difference in the number of eggs laid between 12 and 24 hours for worms exposed to 28 °C was significantly reduced (*p*-value = 4.40E-07), whereas the difference in the number of eggs laid from worms exposed to 20 °C was insignificant. The difference in eggs laid at 24 hours compared to 36 hours was significantly reduced for worms exposed to 28 °C (*p*-value = 8.31E-07).

The dramatic reduction in egg laying observed between 12 and 24 hours of heat stress, prompted the inclusion of an additional timepoint of 18 hours to determine whether this reduction in egg laying resembled more of a gradual decline or a rapid sharp drop off in progeny production. Additionally, to ensure that damage to the worms or temperature fluctuations during transfer did not impact the data, a second methodology was used. In this experiment the worms were exposed to a

specific duration of heat stress (12, 18 or 24 hours) at 28 ℃ and analyzed at a single timepoint. The average cumulative progeny was recorded for worms exposed to 0-12, 0-18, and 0-24 hours of heat stress at 28 ℃. This data was then used to calculate the average number of progeny produced per hour. During the initial 12 hours of heat stress at 28 ℃ worms on average produced approximately 4 progeny per hour. Between 12-18 hours of heat stress, worms produced approximately 3 progeny per hour. However, between 18 and 24 hours of heat stress, worms significantly reduced egg laying and only produced approximately 1 progeny per hour (Figure 2). This dramatic reduction in progeny count per hour suggests that shut down of egg laying ability occurs at a time point between 18 and 24 hours.

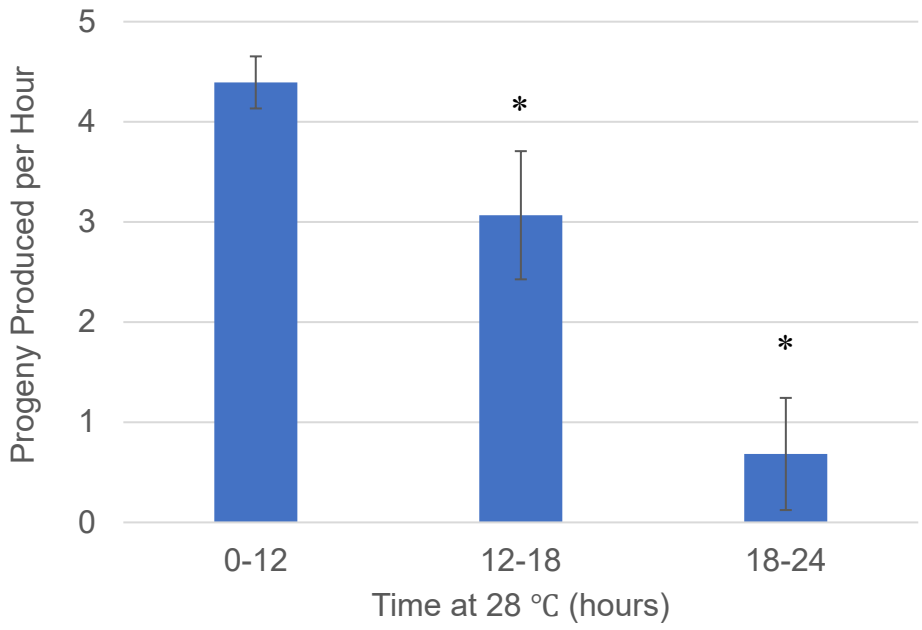

Figure 2. Progeny produced by WT worms in the first 12 hours of heat stress, between 12 and 18 hours of heat stress (HS) and between 18 and 24 hours of HS at 28 ℃. There is a significant reduction in progeny produced per hour between 0-12 and 12-18 hours (p-value = 0.03); however, there is an even greater reduction in progeny produced between 12-18 and 18-24 hours (p-value = 3.00E-03). Progeny represents both eggs and larva collected. Worms were exposed to 28 ℃ at day 1 of adulthood. Data represents a mean ± SE of n =30 worms, collected across 3 independent trials.

3.2 Effects of chronic temperature stress on lifespan

Having more clearly defined the reproductive scope during chronic temperature stress, the overall health of the worms during chronic temperature stress was assayed by examining their lifespan. A lifespan assay was performed on WT worms at various durations of chronic temperature stress (28 ℃). Conditions included 24 hours and 48 hours at 28 ℃ with a return to a normal unstressed temperature (20 ℃) for the remainder of the lifespan, constant exposure to 28 ℃, and a control condition where worms were maintained at 20 ℃ (Figure 3 and Table

1). The average lifespan for worms at 20 ℃ and the average lifespan after 24 hours of heat stress with recovery were both approximately 15 days. This indicates that after 24 hours of heat stress with recovery, the lifespan of the worm is not affected. It also suggests that, if there is any damage from the stress, it does not impact the lifespan of the worm. After 48 hours at 28 ℃ with recovery, the average lifespan is reduced to 13 days. This increase in the duration of heat stress past 24 hours, could indicate an accumulation of damage that can no longer be reversed by returning to normal temperature conditions. After constant exposure to 28 ℃, a dramatic reduction in lifespan was observed to approximately 6 days (40% decrease in lifespan compared to that of unstressed worms). This indicates that at some point after 24 hours constant exposure to heat does affect overall lifespan.

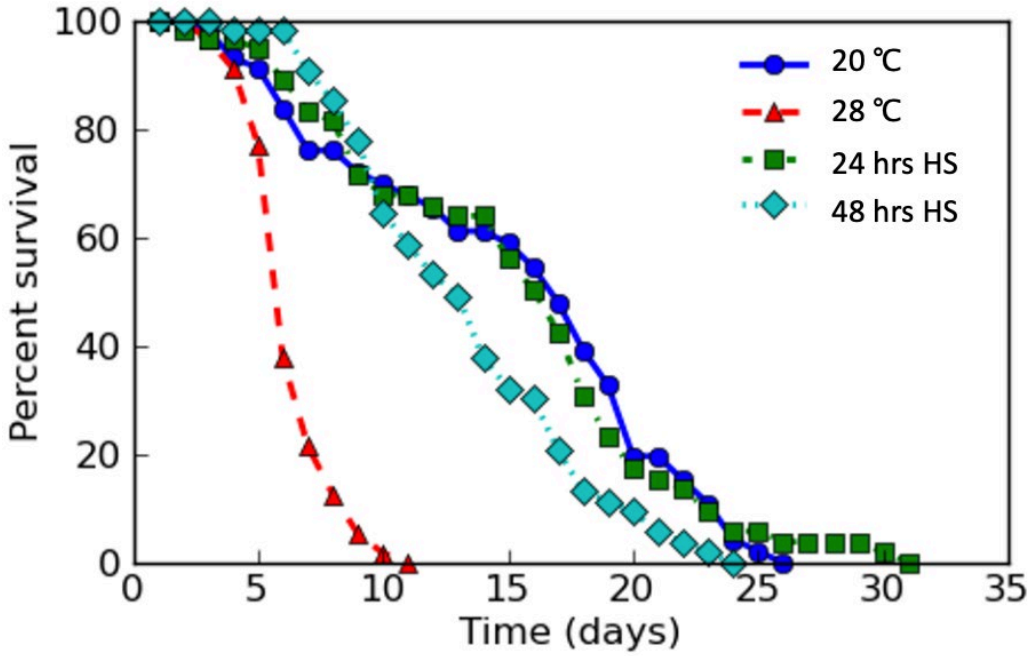

Figure 3. Lifespans of adult WT N2 worms at four different temperature conditions (20 ℃ constant (blue); 28 ℃ constant (red); 24 hrs heat stress (HS) at 28 ℃ (green); 48 hrs HS at 28 ℃ (light blue)). Significant reduction in average lifespan was recorded for worms exposed to constant 28 ℃ compared to that of unstressed worms at 20 ℃, 24 hrs HS, and 48 hrs HS (p-value < 0.05). No significant difference was observed for the worms exposed to 20 ℃ and 24 hrs of HS. Significant decreased in lifespan was observed between the 24 hrs HS condition and the 48 hrs HS (p-value = 0.02). Representative data is from 3 biological replicates. Worms were exposed to temperature conditions at day 1 of adulthood. For additional statistical data see Table 1.

Table 1. Additional data from the lifespans of WT (N2) *C. elegans* exposed to four temperature conditions. Heat stress (HS) was performed at 28 °C, and worms were shifted to 20 °C for the remainder of their lifespan. All other temperature conditions were kept constant. Confidence interval is represented as C.I.

Condition	**No. of Subjects**	**Restricted Mean**		
		Days	*Standard Error*	*95% C.I.*
20 °C	60	15.23	± 0.93	13.40 ~ 17.05
28 °C	60	6.44	± 0.22	6.02 ~ 6.87
24 hrs HS	60	15.31	± 0.91	13.53 ~ 17.10
48 hrs HS	60	13.41	± 0.64	12.16 ~ 14.66

3.3 Recovery of fecundity

Since there was no effect on lifespan was observed after 24 hours of heat stress at 28 ℃ for WT worms; we next determined whether fecundity could be recovered at this timepoint. The average number of progeny produced during a 5-day recovery period at 20 ℃ was recorded after exposure to different durations of heat stress at 28 ℃ (Figure 4). After 12 hours of heat stress with recovery, worms produced approximately 185 progeny. This number, when added to the total number of progeny produced during the 12 hours of heat stress, suggests an estimated brood size of approximately 241 worms (80 % of the brood size from an unstressed worm, approximately 300). The resulting percent value indicated that after short periods of chronic stress (12 hours), fecundity can be recovered with little impact on overall brood size. After 18 hours of heat stress with recovery, worms produced approximately 222 progeny (148 progeny during the recovery

period and 74 during the heat stress). This is approximately 74% of the brood size from an unstressed worm. A reduction in progeny production was observed at this 18-hour timepoint when compared to the 12-hour timepoint; however, it still indicated that recovery of egg laying can be achieved after longer durations of heat stress. Progeny produced after 24 hours of heat stress with recovery was approximately 147 (69 progeny during the recovery period and 78 progeny during the heat stress). The data indicated about 49% of the brood size from an unstressed worm can be rescued after this duration of chronic stress. Despite a significant decrease in percent recovery of brood size between 18 hours and 24 of heat stress with recovery, substantial recovery of fecundity was achieved. This suggests that during the 18-24 hours of heat stress, worms shut off reproduction before irreversible damage occurs.

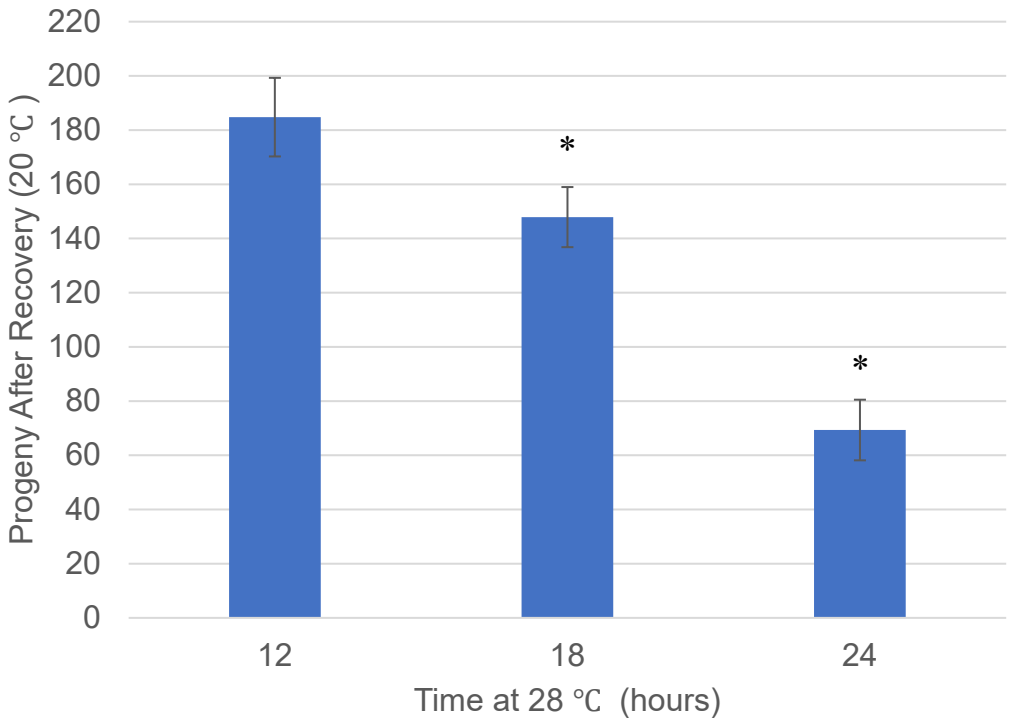

Figure 4. Recovery of fecundity after heat stress at 28 ℃ followed by a 5-day period at 20 ℃ for WT (N2) worms. Progeny production during the recovery period was reduced between 12 and 18 hours of heat stress and 18 and 24 hours of heat stress (*p*-value = 0.02 and *p*-value = 3.62E-06, respectively). Worms were exposed to 28 ℃ at day 1 of adulthood. Data represents mean ± SE of n=30 worms, collected across 3 independent trials.

3.4 Molecular role of HSF-1 in chronic stress

Having established that recovery of both lifespan and fecundity can occur after 24 hours of heat stress, we next determined the basis for these effects. The molecular pathway known as the heat shock response (HSR) is an established response to acute temperature stress; however, few studies have analyzed whether it is important for responses to chronic temperature stress. The hourly rate of progeny was recorded for a loss-of-function HSF-1 (*sy441*) mutant strain and compared to

WT for 0-12 hours, 12-18 hours and 18-24 hours of heat stress at 28 ℃ (Figure 5). There was a significant reduction in progeny produced by the mutant strain for both the 0-12 hour and 12-18 hour timepoints in comparison to the WT (*p*-value = 1.50E-23 and *p*-value = 4.80E-05, respectively). There was no significant difference between the progeny produced per hour at the 18-24 hour timepoint for mutant and WT. At all three timepoints, the HSF-1 mutant strain recorded below 1 progeny per hour, showing that the strain is very sensitive to chronic temperature stress. These results indicate that HSF-1 is necessary for progeny production during chronic heat stress conditions.

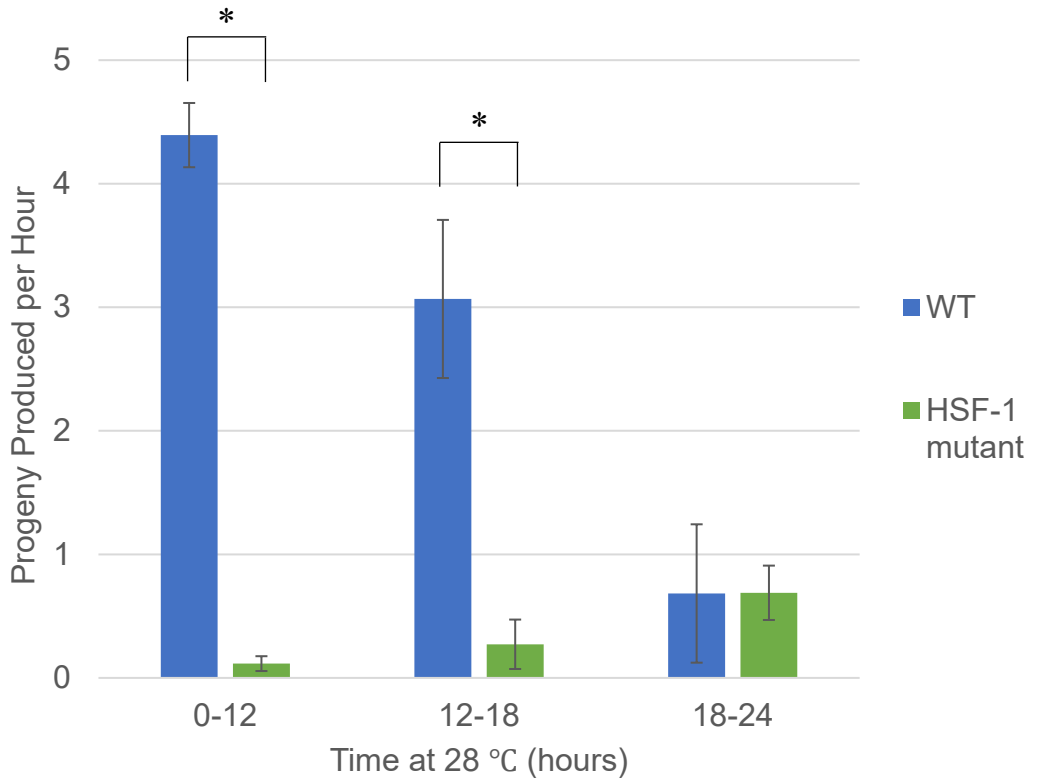

Figure 5. HSF-1 is necessary for egg laying during chronic temperature stress at 28 ℃. A significant reduction in progeny produced per hour for the HSF-1 mutant, PS3551 (green) compared to WT, N2 (blue) for 0-12 hours and 12-18 hours (*p*-value = 1.50E-23 and *p*-value = 4.80E-05, respectively). Difference in progeny production was not significant at the 18-24 hour timepoint for both strains. Worms were exposed to 28 ℃ at day 1 of adulthood. Data represents mean ± SE of n=30 worms, collected across 3 independent trials.

Next, similar experiments were conducted for an HSF-1 overexpression strain (HSF-1 O/E) to determine whether it would reduce sensitivity to temperature stress. The HSF-1 O/E strain produced fewer progeny per hour compared to the WT across all three stress durations (Figure 6). This suggests that HSF-1 overexpression is not sufficient to reverse the negative effects during heat stress on progeny production. The difference in progeny between the two strains was only statistically

significant between 0-12 hours (*p*-value = 5.00E-03). Comparison of the HSF-1 O/E strain between timepoints showed a significant reduction in progeny produced per hour between the 12–18 hour timepoint and the 18-24 hour timepoint (*p*-value = 3.00E-04). This dramatic decrease in progeny production observed during the 18-24 hours timepoint is very similar to the WT control suggesting that this strain also shuts off reproduction around 18 hours in anticipation of further damage from longer durations of heat stress. The negative value obtained from the HSF-1 O/E strain during the 18-24 hour timepoint is a outcome of the procedure used (see 2.4 Chronic stress progeny-counting experiments, method B).

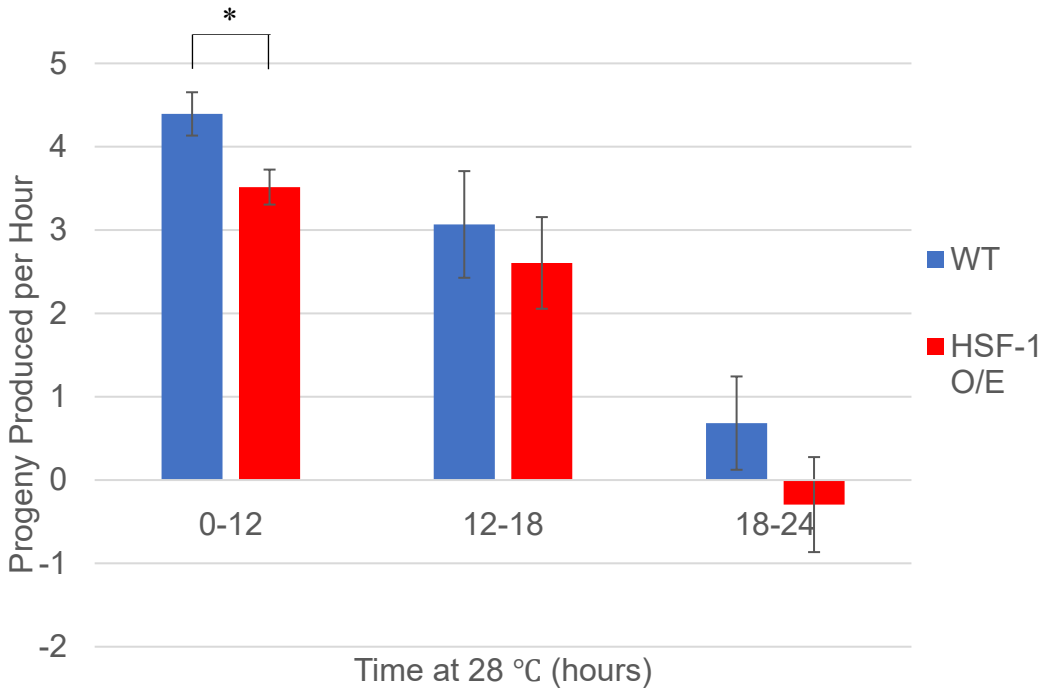

Figure 6. HSF-1 is not sufficient to maintain progeny production during chronic heat stress at 28 ℃. There is a reduction in progeny produced per hour for the HSF-1 O/E, EQ140 (red) compared to the WT, N2 (blue) across all three durations of chronic stress. Only the 0-12 hour timepoint was significant (*p*-value = 5.00E-03). The HSF-1 O/E strain showed a significant decrease between 12-18 hours and 18-24 hours (*p*-value = 3.00E-04). Worms were exposed to 28 ℃ at day 1 of adulthood. Data represents mean ± SE of n=30 worms, collected across 3 independent trials.

Next, the ability of HSF-1 to affect recovery of fecundity after chronic heat stress was examined. Progeny production was recorded during a 5-day recovery period at 20 ℃ after worms were exposed to 12, 18 and 24 hours of heat stress at 28 ℃ (Figure 7). It was found that HSF-1 O/E worms showed a significant increase in progeny production during the recovery period at 20 ℃ after three different chronic stress conditions compared to WT (*p*-value = 0.04, *p*-value =1.97E-05, *p*-value = 1.80E-06, respectively). The number of progeny produced during recovery

was then added to the total progeny produced after chronic heat stress to calculate the overall brood size. This value was compared to the typical wildtype unstressed brood size of 300 progeny to give percent recovery of brood size for the strain. After 12 hours of heat stress with recovery, HSF-1 O/E worms showed an 86% recovery of brood size, and after 18 hours of heat stress with recovery, the strain showed 90% recovery of brood size. The percent recovery after 18 hours of heat stress with recovery was the maximum percent recovery recorded for the HSF-1 O/E. After 24 hours of heat stress with recovery, HSF-1 O/E worms had 70% recovery of brood size. This suggests that HSF-1 is important for the recovery of fecundity after chronic stress.

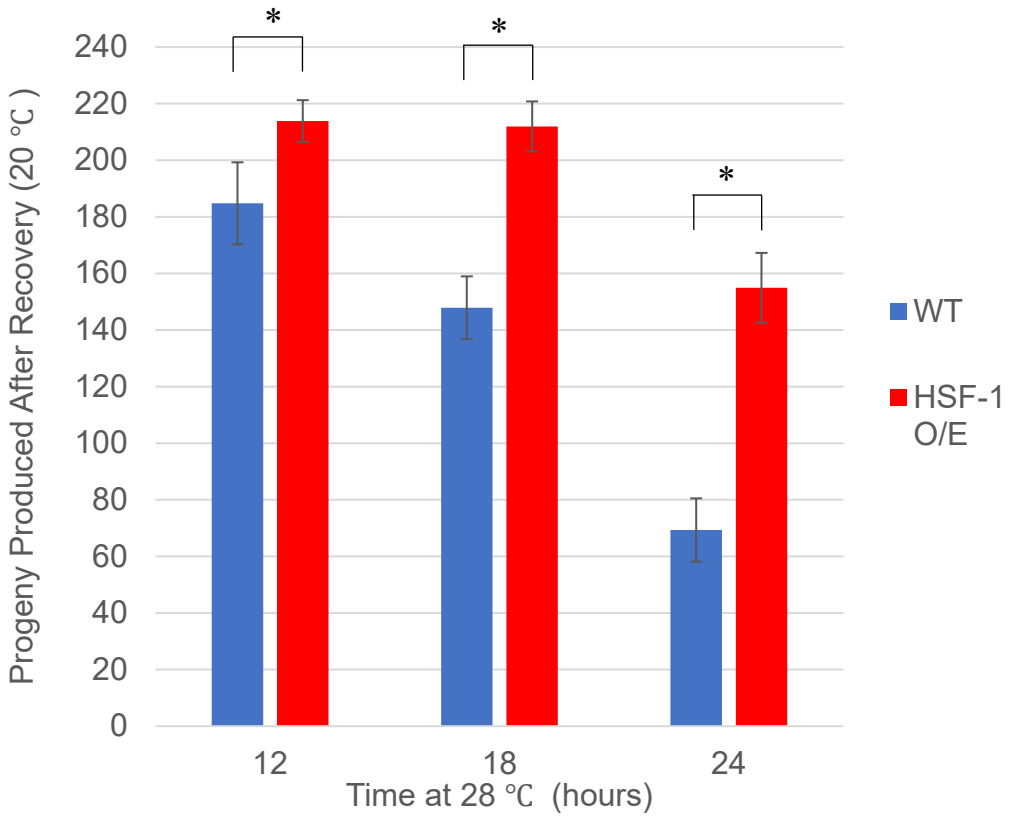

Figure 7. Whole worm HSF-1 O/E is sufficient to recover fecundity after chronic heat stress at 28 ℃ followed by a 5-day recovery period at 20 ℃. The HSF-1 O/E strain, EQ140 (red) showed a significant increase in progeny production during the recovery period compared to WT, N2 (blue) across all 3 timepoints (*p*-value = 0.04, *p*-value =1.97E-05, *p*-value = 1.80E-06, respectively). Worms were exposed to 28 ℃ at day 1 of adulthood for varying timepoints (12, 18 and 24 hours). Data represents mean ± SE of n=30 worms, collected across 3 independent trials.

Unexpectedly, the HSF-1 mutant strain also showed recovery of fecundity after heat stress (Figure 8). The HSF-1 mutant strain produced significantly fewer progeny when compared to the WT for all three chronic stress timepoints (*p*-value = 2.2E-04, *p*-value = 1.2E-01, and *p*-value = 0.02, respectively); however, these values were still greater than 0. This could suggest that perhaps another molecular

response is responsible for some of the reproduction recovery seen after chronic heat stress in the HSF-1 mutant.

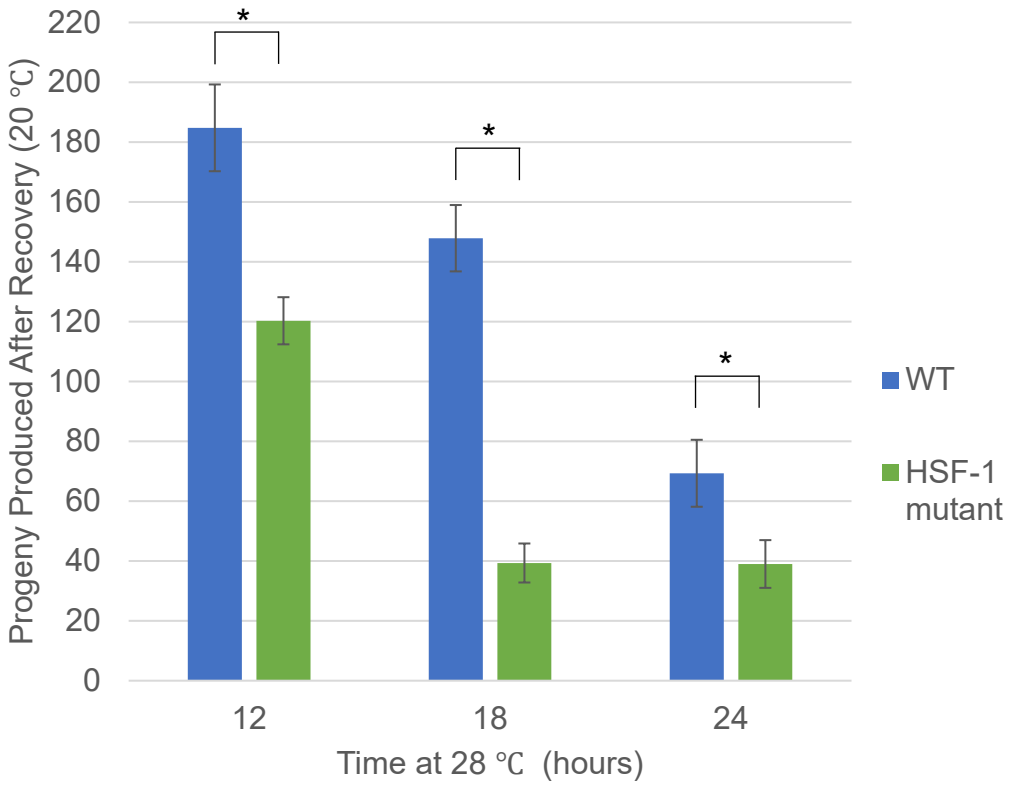

Figure 8. HSF-1 mutant (*sy441*) recovery of fecundity after heat stress at 28 ℃ followed by a 5-day recovery period at 20 ℃. HSF-1 mutant (green) showed significant reduction in progeny produced during recovery for all three timepoints (*p*-value = 2.2E-04, *p*-value = 1.26E-10, *p*-value = 0.02, respectively) compared to WT (N2) worms (blue). Worms were exposed to 28 ℃ at day 1 of adulthood for varying timepoints (12, 18 and 24 hours). Data represents mean ± SE of n=30 worms, collected across 3 independent trials.

Having established the effects of HSF1 on reproduction, the effects of HSF1 on lifespan were measured. A lifespan experiment was performed with the HSF-1 mutant strain at four different temperature conditions (20 ℃, 28 ℃, 24 hours of HS

at 28 ℃ with recovery at 20 ℃ and 48 hours of HS at 28 ℃ with recovery at 20 ℃) (Figure 9 and Table 2). At a control temperature of 20 ℃, the HSF-1 mutant worms live an average of approximately 11 days. This is less than the average WT lifespan of approximately 15 days (Figure 3 and Table 1). After 24 hours of HS with recovery, the HSF-1 mutant worms showed a significant decrease in lifespan compared to the 20 ℃ control (p-value < 0.05) with an average lifespan of approximately 9 days. This reduction in lifespan for the HSF-1 mutant between the 24 hour HS with recovery and 20 ℃ was not observed for the WT worm as both conditions resulted in the same lifespan of 15 days (Figure 3 and Table 1). This suggests that perhaps there is too much accumulated damage after 24 hours of heat stress to the HSF-1 mutant and without activation of the HSR through HSF-1, there is no rescue of lifespan. As the duration increased to 48 hours of heat stress with recovery, the lifespan was further reduced to 6 days. This is approximately 50 % of the WT lifespan recorded after 48 hours of heat stress with recovery. With constant exposure to 28 ℃, the lifespan was reduced to 4 days which is 2 days shorter than the average lifespan of WT worms at 28 ℃. This data showed that without functional HSF-1 there was no recovery of lifespan at 24 and 48 hours of chronic heat stress, and constant exposure to heat stress severely affected overall lifespan.

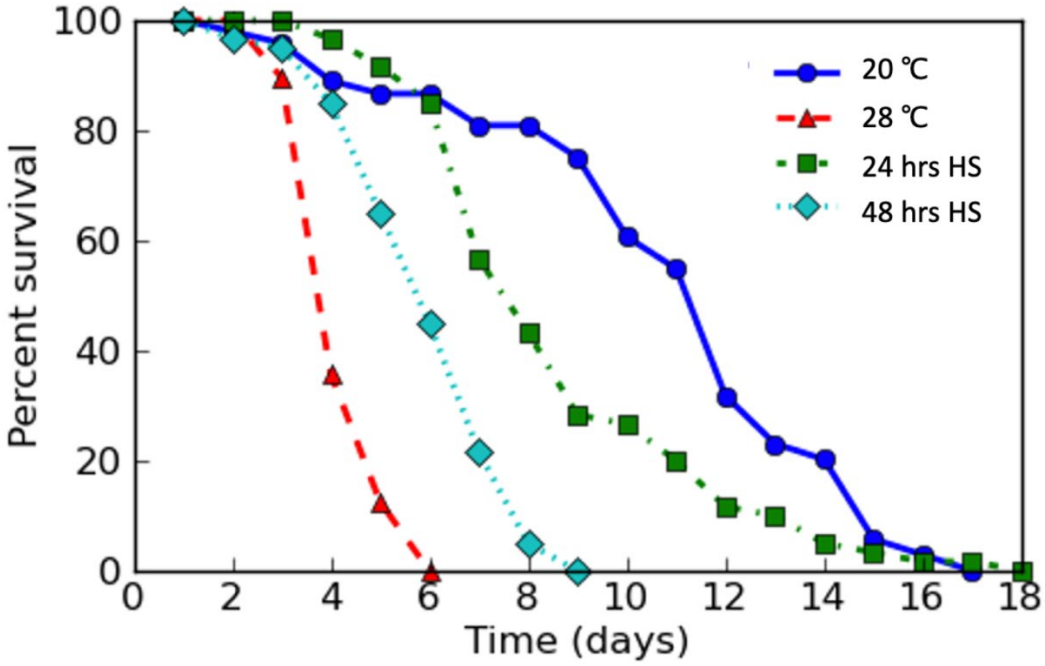

Figure 9. Lifespans of adult HSF-1 mutant (*sy441*) worms (PS3551) at four different temperature conditions (20 ℃ constant (blue); 28 ℃ constant (red); 24 hrs heat shock (HS) at 28 ℃ (green); 48 hrs heat shock (HS) at 28 ℃ (light blue)). Compared to HSF-1 mutant worms at unstressed condition (20 ℃), worms exposed to a constant temperature of 28 ℃ (p-value < 0.05) or heat shocked for both 48 hours and 24 hours at 28 ℃ before shifting to recovery at 20 ℃ (p-value < 0.05 and p-value = 5.5E-03, respectively) had a significantly reduced lifespan. Between heat shock conditions of 24 hours and 48 hours at 28℃, the worm lifespan was also significantly reduced (p-value = 3.0E-09). Worms were exposed to temperature conditions at day 1 adulthood. Representative data is from 3 biological replicates. For additional statistical data see Table 2.

Table 2. Additional data from the lifespans of HSF-1 mutant (*sy441*) *C. elegans* exposed to four temperature conditions. Heat shock (HS) was performed at 28 °C on day 1 of adulthood. Worms were then shifted to 20 °C after HS for the remainder of their lifespan. All other temperature conditions were kept constant. Confidence interval is represented as C.I.

Condition	**No. of Subjects**	**Restricted Mean**		
		Days	*Standard Error*	*95% C.I.*
20 °C	50	10.93	± 0.58	9.80 ~ 12.05
28 °C	60	4.38	± 0.11	4.16 ~ 4.60
24 hrs HS	60	8.82	± 0.38	8.07 ~ 9.57
48 hrs HS	60	6.13	± 0.21	5.72 ~ 6.54

Next, lifespan assays were performed with HSF-1 O/E worms at four different temperature conditions (20 ℃, 28 ℃, 24 hours of HS at 28 ℃ with recovery at 20 ℃ and 48 hours of HS at 28 ℃ with recovery at 20 ℃) (Figure 10 and Table 3). HSF-1 O/E worms at an unstressed temperature of 20 ℃ were previously reported in the literature to have a significantly extended lifespan compared to WT worms. This lifespan extension was observed in one of the three replicate experiments performed (Figure 11 and Table 4). The HSF-1 O/E worms showed an average lifespan of 19 days which was 6 days longer than the average from WT worms. The two other experimental replicates showed HSF-1 O/E worms having a similar lifespan to that of unstressed WT worms. These two experiments had bacterial contamination on the worm plates that may have prevented the typical lifespan extension observed in this strain. In Figure 10, the 24-hour HS with recovery had a similar lifespan to that of the 20 ℃ control (16 days). Therefore

HSF-1 O/E was not sufficient to enhance the recovery of lifespan. After 48 hours of HS with recovery, the lifespan had decreased compared to the 20 ℃ control suggesting that, even after longer durations of heat stress, HSF-1 O/E was not sufficient to recover lifespan back to normal unstressed conditions. If the heat stress is continuous, then HSF-1 O/E worms showed an average lifespan of 6 days which matched that of WT worms exposed to 28 ℃. Therefore, there was no benefit in lifespan from the overexpression of HSF-1 at this constant temperature stress.

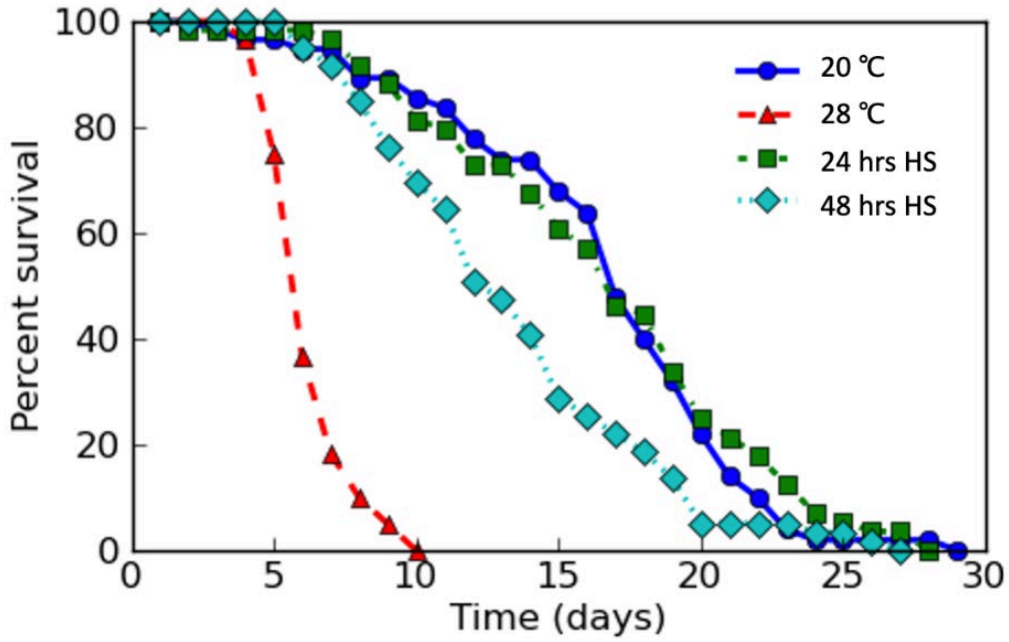

Figure 10. Lifespans of adult HSF-1 overexpression, HSF-1 O/E (EQ140) worms at four different temperature conditions (20 ℃ constant (blue); 28 ℃ constant (red); 24 hrs heat shock (HS) at 28 ℃ (green); 48 hrs heat shock (HS) at 28 ℃ (light blue)). Compared to HSF-1 O/E worms at unstressed condition (20 ℃), worms had a significantly reduced lifespan when exposed to a constant temperature of 28 ℃ (p-value < 0.05) or when heat shocked for 48 hours at 28 ℃ before shifting to recovery at 20 ℃ (p-value < 0.05). Between heat shock conditions of 24 hours and 48 hours at 28 ℃, the worm lifespan was also significantly reduced (p-value < 0.05). No statistical significance was determined between the 20 ℃ condition and the worms heat shocked for 24 hours at 28 ℃. Representative data is from 3 biological replicates. For additional statistical data see Table 3.

Table 3. Additional data from the lifespans of HSF-1 overexpression (*EQ140*) *C. elegans* exposed to four temperature conditions. Heat shock (HS) was performed at 28 °C on day 1 of adulthood. Worms were then shifted to 20 °C after HS for the remainder of their lifespan. All other temperature conditions were kept constant. Confidence interval is represented as C.I.

Condition	**No. of Subjects**	**Restricted Mean**		
		Days	*Standard Error*	*95% C.I.*
20 °C	60	16.66	± 0.71	15.26 ~ 18.05
28 °C	60	6.42	± 0.18	6.06 ~ 6.77
24 hrs HS	60	16.82	± 0.74	15.36 ~ 18.28
48 hrs HS	60	13.59	± 0.63	12.34 ~ 14.83

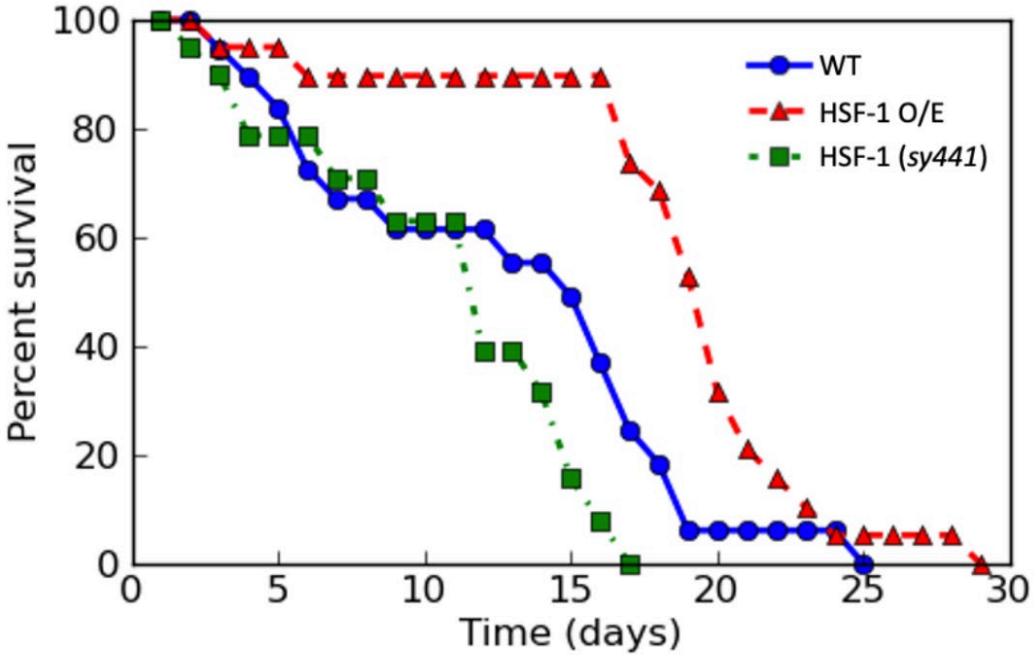

Figure 11. HSF-1 O/E increases longevity in *C. elegans* at unstressed conditions (20 ℃) compared to WT. Adult lifespans for WT, N2 (blue); HSF-1 whole worm overexpression, HSF-1 O/E (EQ140) (red); HSF-1 mutant (*sy441*) (green) worms at 20 ℃. Representative data is from one independent trial, n = 20 worms per strain. For additional statistical data see Table 4.

Table 4. Data from adult lifespans of *C. elegans* strains exposed to 20 °C. Confidence interval is represented as C.I.

Strain	**No. of Subjects**	**Restricted Mean**		
		Days	*Standard Error*	*95% C.I.*
WT (N2)	20	12.98	± 1.49	10.06 ~ 15.89
HSF-1 O/E (*EQ140*)	20	18.73	± 1.26	16.25 ~ 21.20
HSF-1 (*sy441*)	20	10.86	± 1.17	8.56 ~ 13.16

3.5 The cGAL system of gene expression

As overexpression of HSF-1 enhanced the recovery of fecundity after chronic heat stress, the tissue-specificity of this effect was examined. A GAL4-UAS system previously established in *Drosophila* was adapted for targeted gene expression in *C. elegans* (cGAL) (Wang *et al.* 2017). This system uses the yeast *Saccharomyces kudriavzevii* Gal4 protein to activate the transcription of downstream genes. A cryophilic strain of *Saccharomyces* was used due to its ability to function at lower temperatures (23-24 °C) similar to the optimal growth temperature of *C. elegans* (20-25 °C). The Gal4 protein contains both a DNA binding domain (DBD) and a transactivation domain (AD). A cell-specific promoter can be placed upstream of the Gal4 protein therefore driving tissue specific gene expression. The Gal4 protein then binds to an upstream activating sequence (UAS) initiating transcription of the desired downstream gene. This bipartite system of crossing an effector strain, containing the UAS and gene of interest, with a driver strain containing the Gal4 protein and tissue specific

promoter, generates tissue specific overexpression strains of the desired gene. This cGAL system has been validated and used in functional studies such as tissue specific rescue experiments as well as gain-of-function experiments in *C. elegans* (Wang et *al.* 2017).

To generate an effector strain necessary for implementation of the cGAL system, a gene construct containing the desired gene, HSF-1, was produced, cloned, and successfully microinjected into a WT (N2) worm strain (construct design and development was performed by Karen Kim Guisbert). This generated a line carrying an extrachromosomal array (EAG010); eagEx1[hsf-1::myc-2p::gfp], which was then integrated into the worm genome using UV irradiation. Resulting integrated lines (EAG012, referred to as effector #1 and EAG013, referred to as effector #2) showed exact or close to 100% GFP transmission in the F3 generation and were chosen for experimentation. A third line (EAG014, referred to as effector #3) was also selected based on qualitative data suggesting healthier worms in comparison to other effector lines. These lines were then backcrossed (x4) with WT males. The three transgenic lines did not display 100% GFP transmission efficiency after the backcross. From the F2 generation, only 1 out of 10 worm populations showed an efficiency of ~95% for effector #1 (other effector strains recorded data below this percentage) (Strenko & Guisbert, unpublished results). This suggests that the construct may not have been successfully incorporated into the worm genome and therefore was not inherited in subsequent generations.

An additional integrated effector line (EAG019, referred to as effector #4) was produced using the same line carrying the extrachromosomal array (EAG010). UV irradiation was performed however, results indicated unsuccessful incorporation of the HSF-1 gene due to low GFP fluorescence in the F3 generation. After continued maintenance of the extrachromosomal line over several generations, GFP transmission efficiency reached ~95% indicating that the HSF-1 extrachromosomal array was randomly integrated and selectively maintained within the worm genome.

Several experiments were conducted with these four effector strains to obtain a homozygous line with 100% transmission efficiency. Using effector #1, nine worms (at differing developmental stages) were singled out onto OP50 plates and checked for 100% GFP transmission efficiency in the F2 generation. This effector strain was selected over the other strains due to the high GFP transmission efficiency observed in the F3 generation after UV integration. Three plates out of nine contained larva with GFP percent transmission of 90%, 85-90% and 75%. This result indicated a heterozygous effector line. The experiment was repeated in an attempt to obtain a homozygous line; however, all subsequent generations were heterozygous. The other three effector lines showed similar results with all strains determined as heterozygous.

Driver strains were confirmed homozygous based on 100% expression of the fluorescence driver marker (RFP or mCherry). Worms from the effector strains were selected for mating based on GFP expression in the pharynx. The primary cross involved mating the effector line with N2 males to produce “effector males.”

Effector males were then crossed with hermaphrodites from a driver strain. The F1 progeny from this cross was analyzed for the expression of the two fluorescent markers, GFP and RFP or mCherry. If both markers were present in the hermaphrodite, then the worms were determined as having HSF-1 O/E in the specific tissue type. Larva from the F2 generation were then singled out based on visualization of GFP, and the resulting F3 progeny were observed for the presence of fluorescent markers.

The first transgenic worm strain generated involved crossing the neuronal driver with effector #1 to produce a neuronal HSF-1 O/E strain. Hermaphrodites were singled out onto three OP50 plates (A, B and C), and percent progeny with GFP fluorescence was recorded for A and C plates in Table 5. B plates were not recorded due to the presence of starved worms. Adult worms were chosen from the A11, A8 and A7 plates for visualization under confocal fluorescence due to the highest percentage of GFP fluorescence observed in the population. Expression of GFP in the pharynx and RFP in the coelomycetes was observed in 60% of the worms. Adult worms from the C6, C10 and C12 plates were selected for visualization under confocal fluorescence; however, no expression of RFP was observed from these worms.

Table 5. GFP fluorescence (%) of the progeny from the F3 generation of the neuronal-effector #1 cross

Neuronal-effector #1 (Singled-out plate A)		Neuronal-effector #1 (Singled-out plate C)	
Singled-out Plates	GFP Fluorescence (%)	Singled-out Plates	GFP Fluorescence (%)
A1	90	C1	80
A2	50	C2	N/A; no progeny
A3	50	C3	50
A4	50	C4	75
A5	90	C5	50
A6	80	C6 *	90
A7 *	90	C7	75
A8 *	90	C8	90
A9	N/A; low progeny	C9	75
A10	N/A; low progeny	C10 *	95
A11 *	95	C11	65
A12	80	C12 *	95

* Singled out plates chosen for RFP fluorescence analysis.

From the A7, A8 and A11 plates, eight worms were transferred onto a single OP50 plate. From the following generation, 16 worms were singled out onto OP50 plates. This reduced the percentage of successfully crossed worms from F3

generation. The neuronal cross was therefore determined heterozygous. Worms were selected by visualization of both markers prior to experimentation.

Additional HSF-1 O/E strains were generated using effector #1 crossed with the body wall muscle (BWM) driver or crossed with the intestinal driver. Three singled out GFP fluorescent worms were transferred from the original parent cross plates (A, B, C), and twelve singled out worms from the subsequent F2 generation were transferred onto new OP50 plates. The percent of GFP fluorescence was recorded for the F3 generation for the C plates (Table 6). Populations from plates C5, C6, and C9 showed the highest GFP fluorescence, and therefore adult worms were viewed under confocal fluorescence to determine the presence of mCherry in the pharynx. From the C5 plate, all six adult worms expressed GFP and mCherry, whereas only 40% of worms from the C6 plate expressed both fluorescent markers. For the intestinal cross plates, A9, A11 and A12 showed the highest percentage of worms expressing GFP. Worms were selected and analyzed for mCherry expression. From the A9 plate, 30% of the worms had both GFP and mCherry expressed; plate A11 had 30% and plate A12 had 20% expression. The intestinal cross plates had a lower percentage of successfully crossed worms in the F3 generation determining a heterozygous strain. Due to the low percentage of worms expressing both markers, maintenance of this strain was difficult, and the intestinal cross was lost.

Both neuronal and BWM crosses were achieved with effector #2 and #3. These HSF-1 O/E lines were determined as homozygous due to low percentage of GFP fluorescence in the population.

Table 6. GFP fluorescence (%) of progeny from the F3 generation of the body wall muscle (BWM)-effector #1 cross and the intestinal-effector #1 cross.

BWM-effector #1 (Singled-out plate C)		Intestinal-effector #1 (Singled-out plate A)	
Singled-out Plates	GFP Fluorescence (%)	Singled-out Plates	GFP Fluorescence (%)
C1	N/A; No progeny	A1	80
C2	85	A2	80
C3	60	A3 *	80-75
C4	80	A4	70
C5*	95	A5	50
C6 *	95	A6	75
C7	85	A7	75-80
C8	85	A8 *	85
C9 *	90-95	A9 *	85
C10	80-85	A10	70-75
C11	80	A11	80
C12	70	A12 *	85

* Singled out plates chosen for RFP fluorescence analysis.

To verify the successful generation of tissue specific HSF-1 O/E strains, thermorecovery experiments were performed. This assay is used to analyze worm motility after a high magnitude heat stress followed by a recovery period at normal unstressed temperature. Previous literature states that worms overexpressing HSF-1 have improved recovery after temperature stress, and therefore, the results from the thermorecovery assay should indicate a large percentage of the population with normal movement. Worm motility after recovery was characterized as normal or abnormal movement. Normal worm movement included sinusoidal body bends, with the worm having a fast reaction to physical stimulation from a worm pick. Abnormal movement was determined by jerky, uncoordinated movements, and a slow reaction to physical stimulation or paralysis. This experiment tested four transgenic tissue specific HSF-1 O/E strains: two neuronal HSF-1 O/E lines (neuronal-effector #2 and neuronal-effector #3) and two BWM HSF-1 O/E lines (BWM-effector #1 and BWM-effector #2) (Figure 12). Both BWM HSF-1 O/E strains had the highest percentage of worms displaying normal movement after heat stress and recovery (BWM-effector #1; 57% and BWM-effector #2; 67%). The neuronal-effector #3 showed 53% normal movement and the neuronal-effector #2 resulted in the lowest percentage of normal movement (21%). The neuronal-effector #3 worm had a lower percentage of normal movement compared to both the WT and whole worm HSF-1 O/E controls, suggesting unsuccessful overexpression of HSF-1 in the neuronal tissue. All other strains showed better

recovery with percentages of normal movement greater than the WT control (33%) and HSF-1 O/E (46%) (Figure 12).

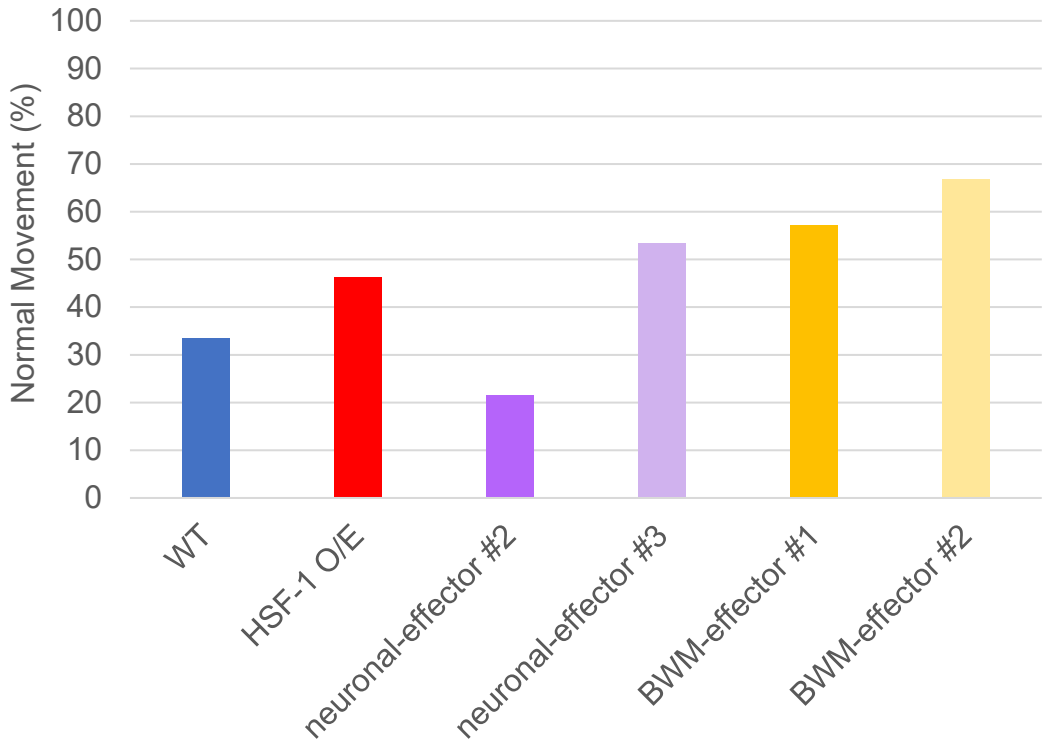

Figure 12. Thermorecovery of four *C. elegans* transgenic tissue-specific HSF-1 O/E strains. Integrated effector lines (effector #1, #2 and #3) were crossed with neuronal and body wall muscle (BWM) driver lines to generate neuronal and BWM tissue specific HSF-1 O/E strains. Percent of normal movement is determined for neuronal HSF-1 O/E strains (purple), BWM HSF-1 O/E strains (yellow), WT; N2 (blue) and whole worm HSF-1 O/E; EQ140 (red), n = 15 worms per strain. At day 1 of adulthood, worms are heat stressed at 33 ℃ for 6 hours and then allowed to recover at 20 ℃ for 48 hours. Motility is characterized as either normal or abnormal. Both BWM HSF-1 O/E strains and neuronal-effector #3 showed the greatest percentage of normal movement compared to controls (WT and HSF-1 O/E).

A second thermorecovery experiment was performed with the same strains in Figure 12 but with the inclusion of two additional HSF-1 O/E strains: neuronal-effector #1 and BWM-effector #3. The WT worms showed 60% normal movement

after heat stress with recovery, and the whole worm HSF-1 O/E control showed 90% normal movement (Figure 13). Contrary to the results from the first experiment, all tissue specific HSF-1 O/E strains showed lower percentages compared to the HSF-1 O/E control. The tissue specific HSF-1 O/E strain that showed the highest percentage of normal movement was the BWM-effector #3 (60%), and the lowest percentage of normal movement was neuronal-effector #2 and neuronal-effector #3 (20%) (Figure 13). In the first thermorecovery experiment, the neuronal-effector #3 strain showed 53% normal movement which was the highest percentage out of all the neuronal HSF-1 O/E strains tested (Figure 12). The BWM-effector #2 had the largest percentage of normal movement (67%); however, in the second experiment it decreased to 30%, being the lowest percentage of normal movement recorded from all three BWM HSF-1 O/E strains (Figure 13). These experiments would need to be performed in triplicate in order to show an accurate average representation of the data.

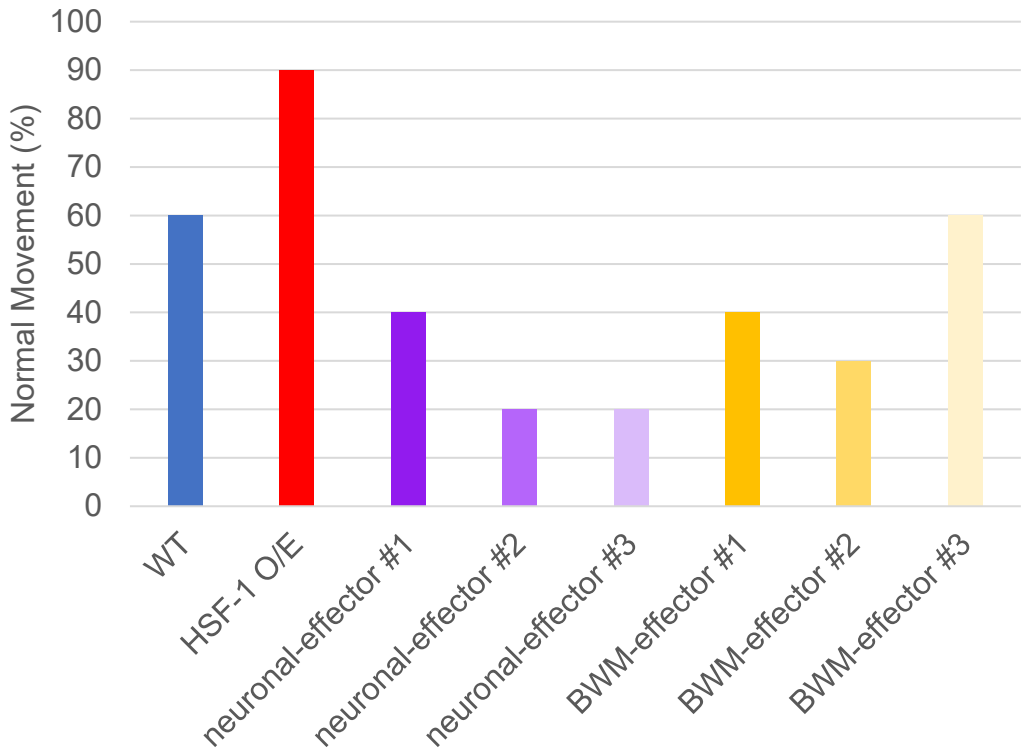

Figure 13. Thermorecovery of six *C. elegans* transgenic tissue-specific HSF-1 O/E strains. Integrated effector lines (effector #1, #2 and #3) were crossed with neuronal and body wall muscle (BWM) driver lines to generate neuronal and BWM tissue specific HSF-1 O/E strains. Percent of normal movement is determined for neuronal HSF-1 O/E strains (purple), BWM HSF-1 O/E strains (yellow), WT; N2 (blue) and whole worm HSF-1 O/E; EQ140 (red), n = 10 worms per strain. At day 1 of adulthood, worms are heat stressed at 33 ℃ for 6 hours and then allowed to recover at 20 ℃ for 48 hours. Motility is characterized as either normal or abnormal. Compared to the HSF-1 O/E control, all tissue specific HSF-1 O/E strains showed a lower percentage of normal movement after 48-hour recovery of heat shock at 33 ℃.

Due to the inconsistency of results from the thermorecovery experiments, two additional tissue specific HSF-1 O/E strains were generated with effector #4 and the neuronal driver and BWM driver. A thermorecovery experiment was performed, and normal movement was recorded after heat shock at 33 ℃ for 6 hours and 48-hour recovery at 20 ℃. For this experiment, five controls were included: WT, whole worm HSF-1 O/E, effector #4, neuronal driver and BWM

driver. Both the effector and driver strains alone showed a higher percentage of normal movement compared to the WT. The neuronal HSF-1 O/E strain showed 27% normal movement and the BWM HSF-1 O/E strain showed 40% normal movement (Figure 14). Both showed higher percentages of normal movement compared to the WT (20%); however, they were lower when compared to the whole worm HSF-1 O/E strain (47%). This experiment would need to be replicated in triplicate to increase accuracy in the results. The data from the tissue-specific HSF-1 O/E strains suggests that the lines may be correct based on the larger percentage of normal movement compared to the WT control.

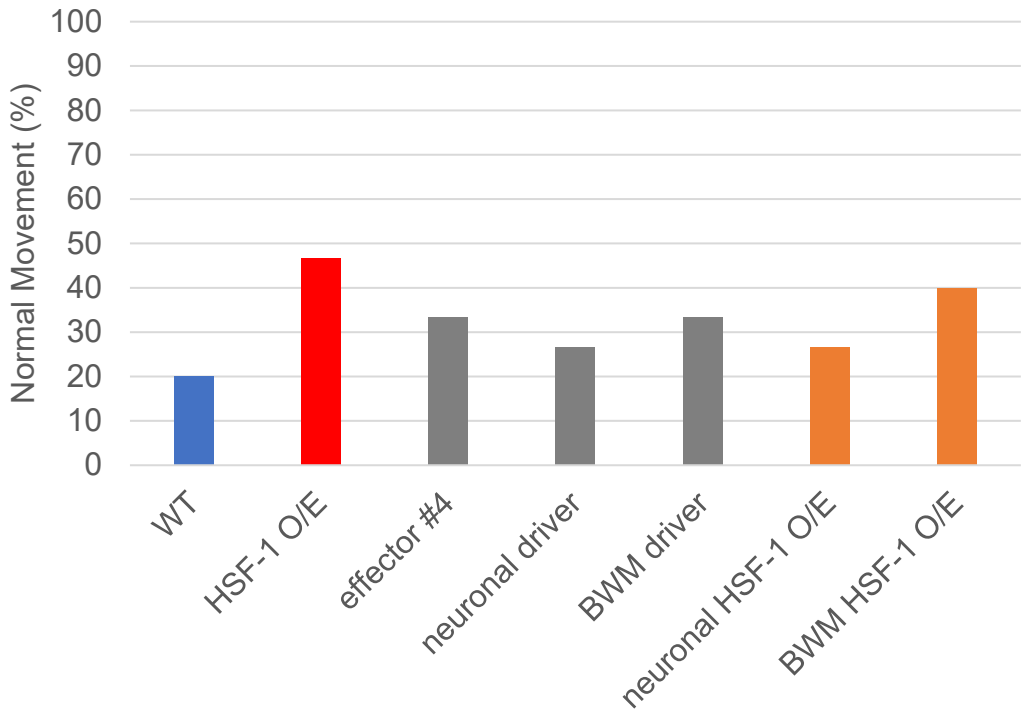

Figure 14. Thermorecovery of two *C. elegans* transgenic tissue-specific HSF-1 overexpression strains using effector #4. Effector #4 line was crossed with the neuronal and body wall muscle driver lines to generate tissue-specific HSF-1 overexpression strains. Controls include WT; N2 (blue), whole worm HSF-1 O/E; EQ140 (red), effector #4 (grey), neuronal driver (grey) and BWM driver (grey). Transgenic lines generated from the cGAL system include neuronal specific HSF-1 O/E line; and BWM specific HSF-1 O/E line (orange); n = 15 worms per strain. Both tissue specific HSF-1 O/E strains show lower percentages of normal movement compared to the HSF-1 O/E control however greater percentages of normal movement compared to the WT.

As an alternate verification method to thermorecovery, a neurological disease worm strain was utilized to verify the neuronal HSF-1 O/E strain. Worms expressing the tau protein in the neurons have impaired neurological function and a distinct motility defect upon observation. Previous work from the Guisbert lab showed that whole worm HSF-1 overexpression can alleviate this movement phenotype. It was hypothesized that if the neuronal HSF-1 O/E strains were correct

then this motility defect would be reversed. The BWM HSF-1 O/E should have no effect on the defect as the tau protein is found specifically in the neurons not the muscle.

This tau worm strain was backcrossed four times with N2 worms to remove the RNAi sensitivity, and the strain was determined homozygous based on 100% transmission efficiency of the RFP marker in the muscle. Hermaphrodite worms from the backcrossed tau strain were crossed with neuronal-effector #4 males. Worms were selected based on visualization of GFP in the pharynx, RFP in the coelomocytes and RFP in the muscle. Hermaphrodite worms were also crossed with BWM-effector #4 males, and visualization using confocal fluorescence showed GFP and mCherry in the pharynx and RFP in the muscle. Both the tau-neuronal-effector #4 and tau-BWM-effector #4 strains showed movement defects similar to the original tau strain. These strains were determined heterozygous based on the low percentage of the worms expressing the three fluorescent markers.

A motility assay was performed to validate the neuronal HSF-1 O/E strain, and the number of thrashes or body bends from a single worm per minute was recorded for each strain (Figure 15). The WT strain recorded an average of 193 thrashes with effector control showing slightly higher with 199 thrashes. This difference was not significant between the two worm strains. The neuronal driver had significantly fewer thrashes per minute compared to both WT and effector (p-value = 0.05 and p-value = 0.01, respectively). The neuronal HSF-1 O/E strain had approximately 76 thrashes per minute which is a significant decrease compared to

the values obtained from the three controls. This suggests that HSF-1 O/E in the neuronal tissue has a negative impact on worm motility. The tau worm strain had on average 38 thrashes per minute which showed a dramatic motility defect when compared to WT worms. This strain also had a visible slow movement defect with some worms having a hooked tail phenotype. This phenotype is where the tail of the animal is curved and no longer demonstrates the typical thrashing movement but drags behind the worm. Interestingly, the tau- neuronal HSF-1 O/E strain showed a similar phenotype to the tau worm control but had a more negative effect on motility (approximately 8 thrashes per minute, *p*-value = 1.51E-05). The reduced number of thrashes from the neuronal HSF-1 O/E line compared to the WT could suggest that HSF-1 in the neurons may be toxic at high levels, and therefore, when expressed in the tau worm strain, there is an even greater decrease in worm motility. Further verification of this worm strain by genotyping and immunohistochemical analysis would identify whether HSF-1 is overexpressed in the neuronal tissue of the worm.

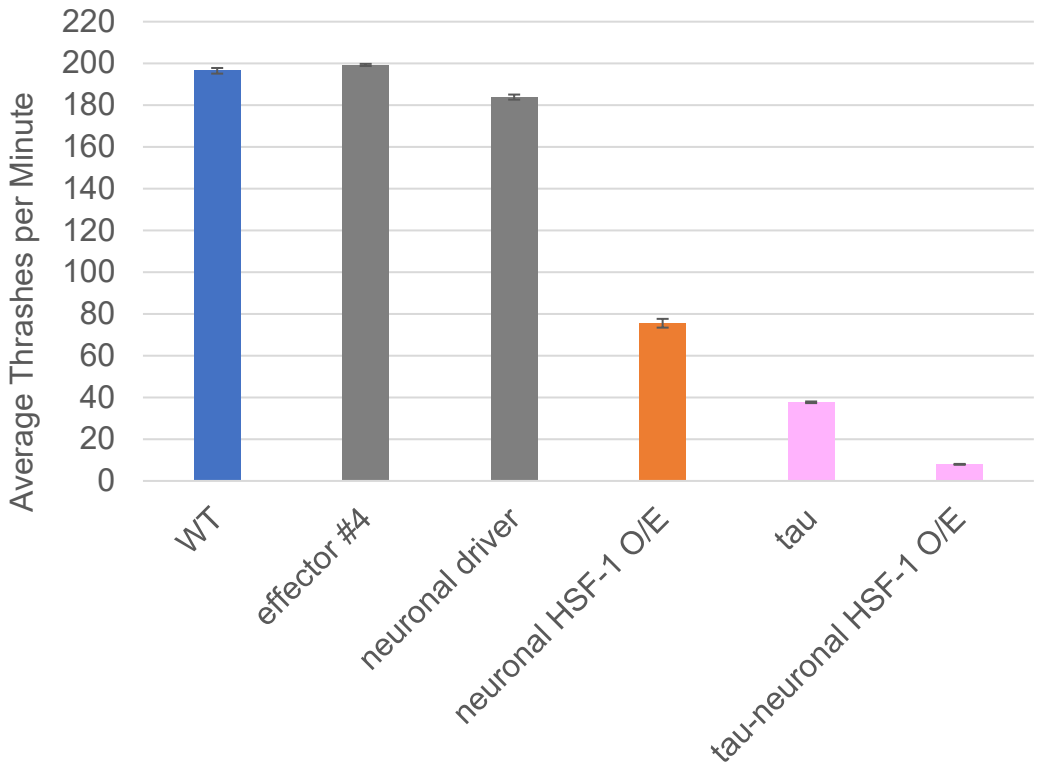

Figure 15. Thrashing assay of neuronal HSF-1 overexpression on *C. elegans* tau disease model. Day 1 adult worms were placed in M9 buffer and allowed to equilibrate for 30 seconds before thrashes were counted for a minute. Data represents mean ± SE of n=30 worms, collected across 3 independent trials. Compared to the WT; N2 (blue), the neuronal driver strain (grey) had significantly fewer thrashes on average (*p*-value = 0.05). The neuronal HSF-1 O/E strain (orange) compared to the WT, neuronal driver and effector #4 (grey) had significantly fewer thrashes (*p*-value = 5.033E-05, *p*-value = 6.97E-05, *p*-value = 2.56E-05, respectively). The tau-neuronal HSF-1 O/E strain (pink) had significantly fewer thrashes per minute compared to the tau strain alone (pink) *(p*-value = 1.51E-05).

A second motility assay was performed to analyze the BWM HSF-1 O/E expression strain on the tau motility defect (Figure 16). The WT strain showed 188 thrashes per minute, and both the effector #4 strain and BWM muscle driver showed approximately 194 thrashes per minute. This difference was not significant between the WT and the effector and driver strains. The BWM HSF-1 O/E showed

more thrashes per minute compared to the neuronal HSF-1 O/E strain (Figure 16); however, it still had significantly reduced motility compared to the WT strain (*p*-value = 7.20E-06). This suggests that HSF-1 O/E in the BWM has a negative effect on overall motility. The tau worm strain had on average 46 thrashes per minute which was a dramatic reduction in motility compared to WT worms. The tau-BWM-HSF-1 O/E strain had an average of approximately 17 thrashes per minute which was significantly reduced when compared to the tau strain alone (*p*-value = 5.85E-07). This strain performed better than the neuronal HSF-1 O/E strain which had an average of approximately 8 thrashes per minute (Figure 15); however, both tissue specific HSF- O/E strains showed decreased motility compared to the tau disease model.

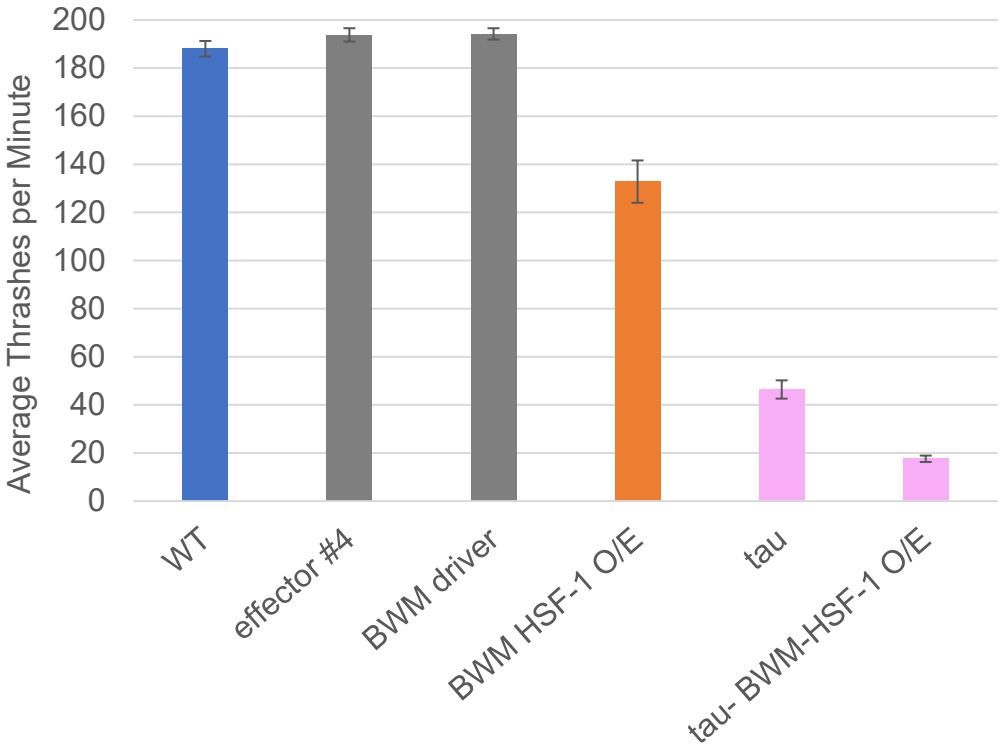

Figure 16. Thrashing assay of body wall muscle (BWM) HSF-1 overexpression on *C. elegans* tau disease model. Day 1 adult worms were placed in M9 buffer and allowed to equilibrate for 30 seconds before thrashes were counted for a minute. Data represents mean ± SE of n=30 worms, collected across 3 independent trials. Compared to the WT; N2 (blue), the BWM driver strain (grey) and the effector #4 strain had no significant difference in average number of thrashes. The BWM HSF-1 O/E strain (orange) compared to the WT showed on average significantly fewer thrashes per minute (*p*-value = 7.20E-06). The tau-BWM HSF-1 O/E strain (pink) had significantly fewer thrashes per minute compared to the tau strain alone (pink) (*p*-value = 5.85E-07).

Chapter 4

Conclusion

The research described in this thesis characterizes the organismal responses to temperature stress in *C. elegans*. The discovery that cessation of reproduction during chronic temperature stress is a rapid and reversible in conditions that do not affect worm lifespan suggest that this cessation is a programmed event designed to preserve fecundity during conditions of temporary stress. Moreover, the HSR pathway, a molecular response to acute temperature stress was implicated in this reproductive maintenance, indicating a coordination between molecular and organismal effects.

4.1 Reproduction and lifespan after chronic stress

It was previously established that exposure to 28 ℃ caused cessation of reproduction. The scope of reproduction under chronic stress demonstrated that after 12 hours of heat stress at 28 ℃, there was no phenotypic effect on reproduction; however, between 18 and 24 hours of heat stress there was a dramatic down shift in the number of progeny produced per hour thus suggesting more of a programmed event rather than a gradual accumulation of damage from chronic heat stress in the worm. It will be important in the future to analyze additional timepoints using individual worms to determine how rapidity and stochasticity of the shut off.

With this reproduction defect observed between 18-24 hours, the overall lifespan of WT worms was analyzed to see if chronic heat stress at 28 ℃ impacts the health/aging of the worm. These results showed that after 24 hours of heat stress with recovery, WT worms could live an average lifespan similar to that of an unstressed worm. Therefore, the lifespan was not affected after 24 hours of heat stress, whereas reproduction was negatively affected. If the chronic stress period was longer than 48 hours, then the lifespan was significantly reduced.

Together, this data indicates that the shut off occurs during conditions where there is either minimal organismal damage or that such damage does occur but that responses in the worms are sufficient to counteract this damage. Additional chronic stress timepoints could be analyzed for both reproduction and lifespan to obtain a more accurate representation of the full effects from all durations of chronic stress.

Future analysis could go beyond that of progeny production during chronic stress and more towards the effects of heat stress on fertilization or embryonic development. Progeny recorded for these experiments included both eggs and larvae; however, it would be beneficial to indicate what percentage of the eggs hatch after heat stress for all durations. The presence of larvae for all durations of heat stress at 28 ℃ (data not shown) implies that fertilization and embryonic development still occur; however, there is no indication as to the severity of damage to these biological processes. Spermatogenesis occurs in the hermaphrodite worm during the L4 larval stage. The germline cells differentiate into oocytes during adulthood. Typically, the self-fertilization period is about 4 days for the average

two-week lifespan of a *C. elegans* worm. The worm therefore has a limited number of self-produced sperm that can participate in fertilization and maturation of the eggs. However, if the hermaphrodite worm mates with a male worm, the sperm pool can be replenished (Pazdernik and Schedl 2013). Without sperm, oogenesis is inhibited. If the sperm is damaged due to increased temperature stress, then this will reduce the number of fertilized oocytes laid. High temperatures have been known to affect male fertility in mammals by causing damage to sperm morphology and decreasing fertilization rates (Thonneau *et al.* 1998). Visualization of the sperm using a fluorescent tagged marker after periods of heat stress could indicate whether a dramatic depletion in sperm count was observed compared to unstressed worms. Also, the visualization of matured oocytes inside the worm after periods of heat shock could indicate whether fertilized embryos are affected by heat stress. Uterine morphology after heat stress could also be analyzed for damage or abnormalities. Are the worms retaining their fertilized eggs for longer in the uterus? These are areas that could be further explored.

4.2 Recovery of fecundity after chronic stress

If the reproductive phenotype (cessation of egg laying) was due to a planned decision from the worms to shut off reproduction prior to catastrophic damage from heat stress, then the worms would be able to recover after returning to unstressed temperatures. Recovery of fecundity was observed for worms heat stressed for 24 hours. Even though full recovery of brood size was not obtained, some recovery

was still achievable after 24 hours of chronic stress at 28 ℃. With lower durations of heat stress, more progeny were produced during the recovery period indicating that the heat stress does have some damaging effect on reproduction. Future directions for recovery experiments include having the recovery period last until the worms have laid their last egg and analyzing what percentage of the progeny hatch into viable offspring. This would provide a comparison with the number of larvae that hatched during heat stress versus the recovery period after heat stress.

4.3 The HSR in reproduction & lifespan after chronic stress

Recovery of fecundity indicates that a molecular pathway may be involved in protecting the organism from damage after heat stress. Acute temperature stress has been previously known to initiate the heat shock response (HSR) in *C. elegans*. The main transcription factor and regulator of the HSR, heat shock factor 1 (HSF-1) was both overexpressed and mutated in worms to determine whether reproduction, recovery of fecundity and lifespan were affected in these worm strains. Results from these experiments showed that a loss of function mutation in HSF-1 (*sy441*) significantly impaired progeny production during chronic temperature stress. This indicates that even at lower chronic stress timepoints such as 12 hours, HSF-1 is necessary to produce progeny. An RNA interference (RNAi) experiment where HSF-1 is knocked down could be used to validate these results.

As HSF-1 is considered necessary for progeny production, does overexpression of HSF-1 improve progeny production during chronic temperature

stress? Data indicates that overexpression of HSF-1 did not maintain progeny production similar to WT during heat stress. Across all timepoints, HSF-1 O/E worms showed a reduction in the number of progeny produced per hour. This indicates that this strain is more sensitive to temperature stress at 28 ℃ compared to the WT.

This could indicate that perhaps HSF-1 O/E is specifically needed in the germline cells in order to improve progeny production during chronic heat stress. Therefore, generation of a tissue specific HSF-1 O/E strain could prove beneficial in testing the importance of HSF-1 in germline cells. This can be quite challenging as foreign genes tend to be silenced in the germline more frequently than in somatic cells. CRISPR can be used to generate single copy transgenes that are resistant to silencing for germline overexpression.

Interestingly, overexpression of HSF-1 did play a role in enhancing the recovery of fecundity after chronic temperature stress. Activation of the HSR therefore plays an important role in recovery of fecundity after chronic heat stress. As whole worm HSF-1 O/E improves recovery of reproduction after heat stress, could HSF-1 O/E in specific tissue types help to improve specific phenotypic defects? This would also identify which tissue types were important for overexpression of HSF-1 to alleviate defects observed from chronic heat stress.

4.4 Tissue specific HSF-1 O/E strains

Generation of tissue specific HSF-1 O/E strains using the bipartite cGAL system of gene expression was performed, and several resulting strains were obtained. Two HSF-1 O/E strains were generated from the extrachromosomal line which was randomly integrated (effector #4) to produce a neuronal HSF-1 O/E strain and a BWM O/E strain. Other neuronal and BWM HSF-1 O/E strains, produced using different effector lines, were also generated. An attempt to produce an intestinal HSF-1 O/E strain was made. However, the low percentage of worms observed to have the two fluorescent markers was significantly reduced upon maintenance of the strain, and eventually the strain was lost. Effector lines #1, #2 and #3 were characterized as heterozygous after multiple attempts to produce a homozygous line. Once these effectors were crossed with driver strains, the resulting HSF-1 O/E strains were also not homozygous. This could be due to leaky gene expression of the transgene or gene silencing. Tissue specific HSF-1 O/E strains were verified based on thermorecovery and the ability to reverse a motility defect driven by neuronal expression of the tau protein. Whole worm HSF-1 O/E was previously identified to reverse this motility defect observed from the tau worm strain (Stanley & Guisbert, unpublished). Therefore, neuronal HSF-1 O/E was predicted to alleviate the defect. The results from a thrashing assay showed that the neuronal HSF-1 O/E strain did not reverse the motility defect seen from the tau worm strain. This result could indicate that the neuronal HSF-1 O/E strain is incorrect or that overexpression of HSF-1 is needed in multiple tissue types in order

to alleviate the movement defect. Data from the BWM HSF1- O/E showed similar results to the neuronal HSF-1 O/E strain. Another possibility for the reduction of motility in the HSF-1 O/E strains is that too much HSF-1 expression is toxic to the worm. Immunofluorescence as well as a progeny counting experiment under chronic stress + recovery would help to indicate whether these strains are correct. More research using the cGAL transgenic system is needed; however, the overall work in this thesis has provided more valuable information on how *C. elegans* respond to chronic temperature stress.

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