Sleep as a Remedy for Stress:
the Sleep Response at a Cellular Level

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science in Biology

by
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Sleep as a remedy for stress: The sleep response at a cellular level

by

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Master of Science in Biology

We spend about a third of our lives asleep, but the function of sleep remains a great mystery. Many hypotheses for the function of sleep have been put forward based upon the cellular perturbations associated with sleep deprivation. It has yet to be shown which of these perturbations drives sleep. All metazoans studied have been found to sleep, including invertebrate model organisms such as the nematode roundworm Caenorhabditis elegans. C. elegans is an exceptionally cost effective and tractable system and has been established as a model for sleep in several laboratories. We have previously shown that sleep in C. elegans occurs in response to environmental stresses such as heat, cold, hyperosmolarity and toxicity. We wish to understand what specific aspect of these environmental stressors is driving sleep. To address this, we have focused on heat-induced sleep. Here we show that thermosensation via the major thermosensory neurons is not required for heat-induced sleep, raising the possibility that heat-induced disruption of protein homeostasis may drive the sleep response. Consistent with this, we find that animals with impaired protein homeostasis networks show enhanced sleep after stress, and conversely, conditions that enhance protein homeostasis reduce the duration of heat-induced sleep. Intriguingly, we find that activation of the endoplasmic reticulum unfolded
protein response by the drug tunicamycin does not affect sleep behavior, revealing that ER stress alone is not sufficient to activate the sleep response. Together these results indicate that cellular stress drives sleep behavior in *C. elegans*, and point to an aspect of cytosolic stress as the sleep-inducing signal.
Introduction

Why we sleep

We spend about a third of our lives sleeping and sleep has been the subject of intense study. However, the question of why we sleep remains one of the most puzzling in science. Sleep disorders affect 40 million Americans, and sleep problems are either a symptom or a contributing factor in many other diseases (www.ninds.nih.gov/disorders/brain_basics/understanding_sleep.htm). Lack of sleep in humans impairs concentration and memory, reduces immune function, and increases the risk of developing obesity, diabetes and cardiovascular diseases (reviewed by Orzel-Gryglewska, 2010; Banks and Dinges, 2007). Many genes that affect sleep have been identified from microarray expression data and genetic screens (reviewed in Crocker and Sehgal, 2010), but the identity of the essential genes required for regulation of sleep remains a mystery.

There are two broad categories of existing hypotheses for the function of sleep. One category of hypotheses is brain-centric, suggesting that sleep specifically enhances brain function. In the other category are the somatic hypotheses, which state that sleep functions to enhance the cellular and/or metabolic processes of the whole organism. Some of these brain-centric hypotheses state that sleep enhances learning and the formation of memories (reviewed in Rasch and Born, 2013). In support of these hypotheses, sleep disruption causes defects in both memory and learning (Karni et al., 1994; Van Der Werf, 2009). A related hypothesis states that sleep is needed to restore synaptic homeostasis. According to this hypothesis, synaptic potentiation strengthens certain synapses activated while awake and synaptic depotentiation reduces the strength of all synapses while asleep, thus restoring a baseline from which new connections can be made (reviewed in Tononi and Cirelli, 2006). Evidence to support this hypothesis is provided by microarray data that shows higher expression of genes involved in synaptic depotentiation during sleep (Cirelli et al., 2004).

In the somatic category of hypotheses, it has been proposed that that sleep is integral to the immune system (reviewed in Zielinski and Krueger, 2011). Supporting data shows that loss of sleep reduces the numbers of natural killer cells in humans (Irwin et al., 1996), and abrogates the immunization of mice (Brown et al., 1989). Additionally, genes that function in the immune system such as the cytokines interleukin-1 β and tumor necrosis factor α are somnogenic when
injected into the brains of rabbits (Krueger et al., 1984; Shoham et al., 1987). These immune factors may modulate sleep, but do not appear to be strictly required for it, as mutant animals still sleep (Baracchi and Opp, 2008). A different somatic hypothesis states that the function of sleep is to maintain metabolic balance (reviewed in Sharma and Kavaru, 2010). According to this hypothesis, a metabolic signal such as adenosine builds up during the day and triggers sleep at a certain threshold (proposed by Benington and Heller, 1995). Indeed, stimulation of the adenosine receptor promotes sleep in rats (Satoh et al., 1996), suggesting that sleep could be stimulated by adenosine, the end product of ATP consumption.

Defining a sleep state
Showing that sleep deprivation results in perturbations of normal physiology helps us understand how sleep improves physiological function. However, without showing which perturbations of normal physiology will cause sleep, we cannot understand why we sleep in the first place. Disruption of human cognitive and physiological function would clearly be unethical and can easily be avoided by using a model system as a proxy to study sleep. Sleep in mammals has many characteristics that are shared between species. Mammals such as mice and rats have long been used to investigate sleep, but are relatively expensive to work with and difficult to manipulate genetically. Determining which other animals can be used as models for the study of sleep requires the ability to accurately identify a sleep state and distinguish it from behaviors that may be superficially similar. The definition of sleep has relied on electroencephalogram (EEG) measurements of cortical activity to validate sleep states. However, EEG is not applicable to non-vertebrates, because these patterns are in part produced by the structure of the vertebrate brain (review by Siegel, 2005). The role that these patterns play in sleep is not yet well understood and they could be an attribute of sleep rather than a distinguishing characteristic. These issues necessitate the specification of alternate criteria for recognition of a sleep state.

Sleep is essentially a behavior and as such, behavioral criteria might be used to define it. The specification of such behavioral criteria is possible if care is taken to distinguish it from non-sleep quiescent states such as resting, paralysis, hibernation and incapacitation. The accepted behavioral criteria to assess sleep are: (1) a state of inactivity with a species-specific rest posture, (2) increased arousal threshold (reduced responsiveness to external stimuli), (3) quick reversibility to wakefulness with sufficient stimulus, (4) homeostatic regulation—a need for
rebound sleep, (5) interaction with the circadian clock or clock genes (reviewed by Zimmerman et al., 2008).

The use of the above behavioral criteria has allowed identification of sleep states in established model organisms including the zebrafish *Danio rerio* and most recently invertebrate model organisms such as the fruit fly *Drosophila melanogaster* and the nematode roundworm *Caenorhabditis elegans*. Following the identification of sleep states in these animals, all of them have been established for the study of sleep (reviewed by Crocker and Sehgal, 2010). In addition to sharing behavioral characteristics with human sleep, these sleep states appear to be regulated by some of the same genes that regulate sleep in humans. These include epidermal growth factor receptor, cAMP-dependent kinase A and the circadian genes Period and Clock (reviewed in Zimmerman et al., 2008). This evidence indicates a possible primitive origin of sleep, and suggests that sleep in these invertebrates may share a function with higher animals (reviewed in Zimmerman et al., 2008). These links help to establish relatively simple model organisms as tools for the study of sleep.

Of these model organisms, *C. elegans* is the simplest in which a sleep-like state has been identified (Raizen et al., 2008). Several states of quiescence have been found in this nematode, though many have not been shown to fulfill the behavioral criteria outlined above. Behavioral quiescence in *C. elegans* is defined as a cessation of feeding and locomotion. Quiescence is seen under at least five conditions, three of which have not been shown to represent a sleep-like state:

- One of these quiescent states is known as satiety. Satiety occurs when animals are fed a higher quality food than their normal laboratory food source, and is most pronounced after fasting. Satiety occurs in cyclical bouts that can last for several minutes (Gallagher and You, 2014). It has not yet been shown whether or not satiety fills any of the behavioral criteria for sleep besides quiescence.
- When *C. elegans* are transferred to a liquid environment they exhibit behavior known as thrashing. Thrashing behavior resembles movement on solid media, but at a greatly accelerated rate. After extended periods in liquid, behavior cycles between thrashing and periods of quiescence (Ghosh and Emmons, 2008). This quiescence is quickly reversible in response to mechanical stimulation of the tail, but not the head. It has yet to be shown
whether there is reduced responsiveness to stimuli during quiescence, or consequences to deprivation of quiescence.

- Diapause is another state of quiescence during which metabolism and growth are slowed or arrested. The most well studied diapause state is the dauer larva, an alternate developmental stage that juvenile animals can enter in harsh environmental conditions such as lack of food, overcrowding and high temperatures (reviewed by Fielenbach and Antebi, 2008). These animals can remain motionless for extended periods, but when stimulated even with a light mechanical stimulus resume movement immediately (Cassada and Russell 1975). Thus, dauer animals do not appear to exhibit reduced sensory responsiveness. Due to the fact that this state is accompanied by developmental arrest it is thought to represent a state akin to hibernation rather than to sleep (Gaglia and Kenyon, 2009).

In contrast to these non-sleep-like quiescent states, there are two events that trigger a documented sleep-like behavior in this nematode:

- The first sleep state, lethargus is a period of cuticle restructuring and quiescence that occurs just prior to each larval molt which fits all of the behavioral criteria for sleep (Raizen et al., 2008).

- The second sleep-like state was established in part by the work presented in this thesis, and is termed stress-induced sleep, because it occurs in response to environmental stress.

*C. elegans* is proving an excellent model for the study of sleep (reviewed in Nelson and Raizen, 2013) and three phylogenetically conserved signaling pathways have so far been identified to regulate sleep in this animal. The first pathway is mediated by the second messenger cGMP. Excessive quiescence in a cGMP-dependent kinase (PKG) gain of function mutant suggests that increased levels of cGMP promote both lethargus and stress-induced sleep (Raizen et al., 2008). This mechanism appears to be conserved in mice, as loss of function PKG mutants are unable to sustain a normal sleep/wake cycle (Langmesser et al., 2009). Second, deletions of cAMP response element binding proteins increase quiescence in mice, implicating cAMP signaling as a component of waking behavior (Graves et al., 2003). Consistent with this role, increased cAMP signaling in *C. elegans* increases responsiveness to stimuli during lethargus quiescence (Raizen et al., 2008). Third, it has been shown that overexpression of epidermal growth factor (EGF)

**Epidermal Growth Factor Signaling**

It has long been known that infusion of cerebrospinal fluid from sleep-deprived animals induces sleep in mammals, suggesting that somnogens are secreted in the brain (Legendre and Piéron, 1913). One class of secreted factors that have been shown to act as somnogens are members of the epidermal growth factor family; EGF induces sleep when infused into the brain of rabbits (Kushikata et al. 1998) and the EGF family ligands transforming growth factor α and neuregulin-1 inhibit activity in rodents (Kramer et al., 2001), suggesting that EGF signaling regulates sleep. Epidermal growth factor receptor (EGFR) mutant mice show abnormal activity during periods of rest (Kramer et al., 2001), providing evidence that this effect is mediated by EGFR. Overexpression of the EGF homolog LIN-3 also induces behavioral quiescence in C. elegans, indicating that the somnogenic effect of EGF is deeply conserved. Genetic manipulations in C. elegans and the fruit fly show that mutations in EGFR suppress quiescence induced by EGF overexpression indicating that behavioral suppression is not due to off-target effects of ligand overexpression (Van Buskirk and Sternberg, 2007; Foltenyi et al., 2007). It is not known what the physiological significance of EGF signaling during sleep is. The developmental sleep state, lethargus is weakly impaired in let-23/EGFR mutants (Van Buskirk and Sternberg, 2007) suggesting that EGF signaling contributes to lethargus quiescence, but in a minor or partially redundant manner. The fact that EGF overexpression alone triggers a striking behavioral quiescence and that this effect is deeply conserved through phylogeny indicate that EGF-dependent quiescence may have an additional role beyond developmentally timed quiescence.

In this thesis I describe our identification of a non-developmental role for EGF: a sleep-like behavioral quiescence induced by environmental stresses such as heat, cold, ethanol and hyperosmolarity. We have found that this sleep-like state fulfills some behavioral criteria for sleep, namely inactivity, reduced responsiveness and reversibility (Hill et al., 2014). While homeostasis has yet to be shown for stress-induced sleep, animals that are unable to sleep show reduced survival following severe stress. This demonstrates that there are consequences of an inability to sleep, paralleling the negative consequences of sleep deprivation in other organisms.
Finally, the independence of this sleep-like state from circadian regulation may be evidence that this behavior represents a primitive form of sleep. As such it presents an interesting opportunity to determine non-circadian factors in the regulation of sleep.

Of the many stressors that we identified as causing sleep-like behavior in *C. elegans*, heat stress is the one that I focus on in my thesis. Specifically I aim to understand what specific aspect of stress drives sleep and how this sleep benefits *C. elegans*. In Chapter I, I describe the development of an automated locomotion tracker that is optimized for analysis of sleep behavior. In Chapter II, I characterize heat stress-induced behavioral quiescence and I identify some of the genes underlying it. In Chapter III, I test the possibility that known thermosensory circuits are responsible for translating environmental stress into a sleep-inducing signal. I determined that thermosensation was unlikely to be responsible and I found evidence that proteostasis networks may contribute to the sleep-inducing signal. In Chapter IV I test the possibility that an aspect of cellular damage by heat, namely proteotoxicity, can induce sleep. While I provide evidence in support of this idea, I also provide evidence that proteotoxicity is not the primary mechanism linking environmental stress to EGF-induced sleep. Finally, in Chapter V, I start to address the question of how sleep may redress the physiological disruption caused by heat stress. My investigation of changes in transcription and protein aggregation indicate that the benefits of sleep may not lie in the enhancement of protein homeostasis.
Chapter I: Worm Locomotion Tracking

Worm trackers and their characteristics
An obvious defining characteristic of a sleep-like state is an absence of locomotion. Locomotion can be observed manually, but it is tedious and susceptible to bias. Video capture and computer processing facilitate unbiased quantification of movement. An automated locomotion tracking system or “worm tracker” allows digital video capture from a microscope and analysis of movement parameters using software tools. There are two main approaches taken to tracking animals, depending on the goals of the project and cost (reviewed in Husson et al., 2013). The first approach is to simultaneously track multiple animals, extracting coordinates of individuals to derive speed and direction. This strategy is well suited to assays of spontaneous movement, chemotaxis, and habituation responses (Swierczek et al., 2011). The second approach is to follow a single animal, often plotting a curved “skeleton” and deriving more detailed characteristics such as amplitude and turning velocity. This strategy requires more complex equipment such as a motorized microscope stage, and is better suited to tasks such as non-invasively measuring evoked behaviors in optogenetics experiments (Stirman et al., 2011). Lethargus quiescence has been quantified in individual C. elegans using single animal trackers (Raizen et al., 2008; Van Buskirk and Sternberg, 2007). However, tracking multiple animals increases the throughput of experimental comparisons. To quantify locomotion during heat-induced quiescence, we turned to the Parallel Worm Tracker developed by the Goodman lab at Stanford University (Ramot et al., 2008). Advantages offered by this system include (1) the use of a numerical computing environment, namely MatLab®, that allows adaptation of any component of the tracker code, (2) an ability take videos at regular intervals, allowing animals to be tracked over the duration of recovery from heat shock, and (3) operability with firewire cameras.

Capturing “worm tracks” and C. elegans quiescent behavior
The Parallel Worm Tracker processes data frame-by-frame following image acquisition, and a series of steps are taken to identify animals in each video frame. In the first step, objects are distinguished from the background by converting all the pixels in the image to either black or white, based on an appropriate pixel intensity threshold. This yields a white background with black objects of various shape and size, some of which are animals, others of which are imperfections in the agar. For this step, high contrast images with little variation are desirable to
accurately distinguish animals from background. Animals tend to be a particular size, i.e., ~1.2 mm long and ~0.1 mm wide. Objects that greatly exceed or fall far below these dimensions can be excluded, allowing the tracker to separate worms from imperfections in the agar. Minimum and maximum size limits are used to define objects as animals. A MatLab® function REGIONPROPS derives a central position or “centroid” from the shape of each of these animal objects to allocate coordinates (Fig. 1). Differences between the current and prior coordinates are used to track animals from one frame to the next, and the collection of these coordinates for a single animal constitutes a “track”. The algorithm to make tracks from these coordinates assumes that the closest point in the next frame is the next coordinate in the track. To ensure that animals are correctly tracked from frame to frame, another round of processing places limits on the distance an animal can move within a given time frame for a valid track. Tracks are terminated on the last video frame, when animals leave the field of view, or when animals collide to produce an object that exceeds the single-worm size limits. Individual track coordinates and velocities are stored as raw data in MatLab® files. I calibrated the tracker parameters to our microscope and the chosen magnification by visual confirmation and used >60 tracks to check for errors. I also wrote a script, “Draw tracks”, that plots the calculated tracks over the video to make assessment of track accuracy easy and repeatable. This is now incorporated into the graphical user interface so that any user of the tracker can perform this check in the future. Having confirmed the accuracy of tracks, I wished to derive a quiescence statistic from these simple data.

<table>
<thead>
<tr>
<th>A</th>
<th>Object centroid method</th>
<th>B</th>
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<td>frame</td>
<td><img src="image2.png" alt="Frame sequence" /></td>
</tr>
<tr>
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<td>coordinate</td>
<td><img src="image4.png" alt="Initial coordinate" /></td>
</tr>
<tr>
<td>combined coordinates</td>
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<td>coordinate</td>
<td><img src="image6.png" alt="Combined coordinate" /></td>
</tr>
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Figure 1. Comparison of methods for determining the precise coordinates for an animal. Nematodes move relatively smoothly across the surface of an agar plate along a sigmoidal path. (A) Using the relative center of the object by determining the median point, or “centroid”, a good approximation of both the location and speed of the animal is obtained. (B) Compare this method to the simplest possible alternative; instead of estimating the center of the animal, the first encountered coordinate could be used as its location. To understand were this comes from, understand that a computer reads an image the same way you would read
To directly quantify behavioral quiescence it is necessary to distinguish quiescence from other behaviors of *C. elegans*. In the lab, *C. elegans* are usually raised on plates of agar seeded with lawns of the bacteria *Escherichia coli* strain OP50. The animals are lying on their sides and move across the agar surface in a sinusoidal fashion, see Fig. 2 (Brenner, 1974). Waking movement includes forward travel, turns, reversals, and momentary pauses (Croll, 1975). These pauses are rarely more than a few seconds long unless in response to aversive stimuli (Gray et al., 2005). As unprovoked pauses are extremely short, they more likely represent a restful waking state than a sleep state. To accurately identify locomotor behavior that is indicative of sleep, a minimum time window should be employed that will exclude these brief pauses associated with waking behavior.

Figure 2. Normal *C. elegans* movement. N2 adults move in a sinusoidal fashion across the surface of agar plates. Animals move almost continuously forward with occasional reversals and turns. They leave sinusoidal trails that can be seen in their *E. coli* food source.
In addition to selecting the duration of inactivity that defines a sleep bout, it was also necessary to set a velocity threshold that would define inactivity vs. movement. The video processing that determines an animal track is susceptible to random variation that produces spurious movement for motionless objects (Fig. 3). To find out precisely how a motionless animal would be characterized by the tracker, I used a plate of animals paralyzed with the acetylcholine receptor agonist levamisole. To define a motionless animal, I selected the maximum velocity that was recorded for these paralyzed animals and set this value as a threshold for immobility/movement. Having set a definition of immobility, I then set the duration of immobility that would define a sleep bout. Since we know that brief periods of immobility are unlikely to represent true quiescence, each track was divided into a sequence of windows, and I defined animals as quiescent if they were immobile for an entire 5-second window. The fraction of quiescence for each track was calculated as the number of quiescent windows over the total number of windows. The mean fraction of quiescence for a particular video was determined as the mean of these quiescence values for all tracks. A final extension of the software saves this data, as well as mean velocity data, in text files for analysis outside of MatLab®. A text file is created for a single video and includes overall statistics and track-by-track data. For experiments with serial video recordings taken at multiple time points, this extension also compiles the overall statistics for each time point in the experiment.

Figure 3. Generation of spurious movement during video processing. The image taken by the camera is a digital approximation of the animal in the real world. Random differences in the amount of light that reach the camera during recording result in a pixel having slightly different colors in two consecutive frames. An example of such a pixel is circled in red. A predetermined shade of grey represents the threshold for determining whether a pixel is part of the background or an object. In the pixel highlighted, this variability
causes the virtual object to lose a pixel in the second frame. To determine the location of an animal, the median point or “centroid” of the virtual object is used, so when the virtual object changes, the centroid changes, even though the animal does not move. When a comparison is made between frames the virtual object appears to have moved. As a result, a non-zero value is calculated for movement of the quiescent animal.

Validating quiescence algorithms

I wished to validate the results obtained when using the quiescence algorithm that I had created. I wished to initially confirm that the velocity data from which quiescence values are derived was comparable with that in the literature. The velocity of N2, a standard strain of wild-type animals (Brenner, 1974), is variable in the published literature even among data collected from labs using the same Parallel Worm Tracker software from the Goodman lab (Table 1). However, experimental conditions differed between labs, such as methods for transferring animals or adding food to agar plates. Our velocity data falls within these data, and is very close to other results using this software. Though the velocity data seemed adequate, I still needed to validate my quiescence data. We have observed a sleep-like behavior in response to heat shock and I used this method to elicit quiescence. To provide corroboration of our quiescence algorithm, I compared visual assessments of this quiescent behavior with the tracker data. Automated tracking of locomotion after heat shock was indeed able to detect locomotory quiescence, but there were significant differences from the data produced by visual inspection (Fig. 4A,B). Automated tracking shows locomotor quiescence that steadily declines in the hour following heat shock (Fig. 4A), while visual inspection detected a peak of quiescence at 20 minutes after heat shock (Fig. 4B). This difference may be due to shortcomings in our quiescence algorithm, or it may be due to experimental differences. For example, visual inspection was always performed in the presence of an \textit{E. coli} food source, while automated tracking was performed in the absence of food.
Table 1. Velocities of wild type animals from different sources. There is considerable variation in published data, and our results fall within this variation. These differences are likely attributable to variation in experimental conditions. Data are for N2 animals cultivated on nematode growth media (NGM) with or without a food source. Methods of seeding plates differed greatly, presumably resulting in different densities of food. So far as could be determined, all experiments were performed at room temperature and used young adult or adult animals raised at ~20°C.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Food</th>
<th>Cultivation Temperature</th>
<th>Velocity (µm.sec⁻¹)</th>
<th>Reference</th>
</tr>
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<tbody>
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<td>NGM</td>
<td>—</td>
<td>20°C</td>
<td>170 †</td>
<td>This study</td>
</tr>
<tr>
<td>NGM</td>
<td>—</td>
<td>20°C</td>
<td>219 †</td>
<td>Ramot et al., 2008</td>
</tr>
<tr>
<td>NGM</td>
<td>—</td>
<td>20°C</td>
<td>16</td>
<td>Dernovici et al., 2007</td>
</tr>
<tr>
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<td>—</td>
<td>19–24°C</td>
<td>152</td>
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<tr>
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<td>20°C</td>
<td>173</td>
<td>Valdes et al., 2012</td>
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<tr>
<td>NGM + food</td>
<td>Overnight OP50</td>
<td>20°C</td>
<td>30 †</td>
<td>This study</td>
</tr>
<tr>
<td>NGM + food</td>
<td>Fresh concentrated OP50</td>
<td>20°C</td>
<td>31 †</td>
<td>Ramot et al., 2008</td>
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<tr>
<td>NGM + food</td>
<td>Overnight OP50</td>
<td>?</td>
<td>40 †</td>
<td>Macoskci et al., 2009</td>
</tr>
<tr>
<td>NGM + food</td>
<td>OP50</td>
<td>20°C</td>
<td>~40</td>
<td>Hawasli et al., 2004</td>
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<td>~120</td>
<td>Karbowski et al., 2006</td>
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<tr>
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<td>Swierczk et al., 2011</td>
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<tr>
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<td>OP50 + animals for ≥72h</td>
<td>?</td>
<td>~30</td>
<td>Yemini et al., 2011</td>
</tr>
</tbody>
</table>

† Velocities obtained using software derived from the Goodman Tracker.
The standard food source for *C. elegans* in the laboratory is the *Escherichia coli* strain OP50. When tracking animals, thin, fresh lawns of OP50 are preferable. Animals leave conspicuous trails on plates with thick OP50 lawns that the tracking algorithm does not reliably eliminate. My subsequent updates to the tracker interface and video initial processing, discussed below, have drastically reduced though not eliminated this problem. If using agar plates without a bacterial food source, or with a thin culture this problem is effectively eliminated. However, using this method, if significant amounts of bacteria are introduced when transferring animals, they tend to congregate at the site of transfer, which significantly impedes tracking—*C. elegans* are usually transferred between plates by use of a platinum wire “pick” coated in bacteria. Without this coating of bacteria, it is very difficult to transfer an animal. To counter this effect, I attempted to smooth out the site of transfer using a heated wire pick. This method resulted in aberrations in the surface of the agar that the tracking algorithm could not resolve. A final change to the method solved this problem: I transferred animals using a pick made from horsehair. This material is perfect for sliding under animals and lifting them without food. In hindsight, a combination of the two techniques would have worked well, that is, using a horsehair pick to transfer animals to plates with thin lawns of *E. coli*. In future experiments I will test and likely use this method.

In addition to eliminating problems with the techniques involved in setting up experiments, I wished to ensure that the calculations were sound. A potential shortcoming in our quiescence algorithm may lie in our method of calculation. The fraction quiescence is calculated as an average of the fraction quiescence of individual tracks. If the fraction quiescence for a short track is treated the same as for a long track, this leads to an overrepresentation of short tracks in the mean. Animals that are moving are more likely to have short tracks when they leave the frame or intersect with another animal. Thus there could be a bias towards moving animals in the mean fraction quiescence. A better alternative may be to calculate the mean across segments regardless of which track they belong to. To investigate this possibility, I rewrote the algorithm to interpret data in an interval-centric rather than an animal-centric manner. The new algorithm takes the fraction quiescence of all windows of all tracks in a video. An analysis of the same data using the new algorithm did not change the overall results (Fig. 4C). The level of significance and overall trends are identical to the algorithm used in Fig. 4A. The mean standard deviation was similar for the old and new algorithms; mean standard deviations for the old and new algorithms were 0.11
and 0.12 respectively in heat shock treatments. As the newer algorithm seems less susceptible to unintentional bias, I elected to keep the newer algorithm.

Figure 4. Heat-induced quiescence scored by automated tracking software is similar to that scored visually. Both heat shock treatments show quiescence for the first hour after heat shock. (A) Using the automated tracking software, quiescence was calculated every 10 minutes from a 1 minute video. A track window was classed as quiescent if there was no movement above 5µm.sec⁻¹ during a 5 second window. There was no food on plates. (B) Quiescence scored by eye. An animal was scored as quiescent if there was no movement below the pharynx for 5 seconds. Plates were seeded with OP50, which results in higher quiescence for the control. (C) Changing from an animal-centric to an interval-centric algorithm does not
greatly affect data analysis. This uses the same video and data files as in (A), but calculates a mean of windows rather than a mean of tracks. At least 5 trials of 20 young adult animals raised at 20°C were used in each trial. Heat-shocked animals were scored after a 30 minute incubation at 35°C, and control animals were scored after a 30 minute incubation at room temperature (~24°C). Error bars indicate standard error. *P<0.05, t-test (two-tailed).

**Goodman tracker software setup**

In addition to creating a quiescence algorithm, I had to make other changes to the original Parallel Worm Tracker software code before it was useable. Despite the openness of the software to alterations and different hardware configurations, a few years have passed since its release without updates. I made updates to the code to keep up with the latest version of MatLab®. Also, the newer specification of our camera (Unibrain Fire-i 580b) did not integrate smoothly with the software. The camera was operable, but required a careful selection of other settings to ensure that the frame rate was correct. The amount of data in a single frame is constant, and the camera and computer exchange data at a relatively constant speed, so these factors determine the frame rate. The frame rate in the functioning mode of our camera is set indirectly by adjusting the data rate. Frame rate could be affected if the bandwidth is reduced, and this is more likely to happen in the event that the shutter speed is close to or slower than the frame rate. I ensured that shutter speed was set well below the maximum time allowed for a given frame rate to obviate this effect. To further ensure that these settings were appropriate, I directly measured the frame rate by timing video acquisition using a stopwatch and confirming that it matched video playback time.

**Microscope setup**

With the software setup complete, I then wished to address complications with the microscope setup. Our microscope (Leica S6D) is a stereomicroscope, which gives us depth of field and allows us to manipulate specimens. To achieve a stereo image on this microscope, there are two ocular image light paths situated at a slight angle from the illumination light path. The illumination light path is brightest at its center. On many stereomicroscopes equipped with cameras, including the S6D, the camera shares one of the imaging light paths used by the eyepieces (Fig. 5). This results in an image that has an off center gradient in the brightness across its area. This gradient would be minimal if the light path was centered, but as it is not, the range of brightness levels is increased. Thresholding of brightness is a key step in processing recorded video to identify worms. Each video frame is a collection of pixel values representing grayscale colors. The gradient results in a range of pixel values for the background that overlaps the range
of pixels representing worms. This confounds worm tracking and so I had to overcome this issue. One possible solution might be to use a higher quality camera that possesses a greater dynamic range to reduce this effect. This solution would be costly and may not be sufficient for tracking. Similarly, purchasing a microscope designed to ensure that the lighting was as even as possible with a camera in the center of the illumination path would be costly. I came up with some alternative solutions to work around this problem. First, I added a “crop video” option that trims off the area of video with unacceptably low brightness before analysis. To keep animals within this more restricted field of view, they were placed on the agar surface enclosed by square copper corrals made in-house from 1/8” copper plate.

Figure 5. Simplified illustration of the microscope setup. The illumination light path is the path of light from the source through the specimen and would ideally match the image light path. The image light path is the path followed by light ending up in the ocular lenses or the camera. On stereomicroscopes like the Leica S6D, these are angled to accommodate stereo vision.

**Background correction**

A second approach that I took to address the problem with background brightness was to add a background correction function. Robust image processing algorithms exist, but tend to be proprietary and as such are a part of expensive software packages that cannot be easily integrated
with MatLab. They also require a significant amount time to process a video frame. For these reasons I wrote my own subtractive algorithm. The advantage of this subtractive algorithm is that it is fast enough to be applied to a live video feed. For recorded video all of this processing is performed at the completion of video to ensure uninterrupted recording at the widest possible range of conditions. The background gradient changes at different magnifications, so to perform correction, this function relies on either stored data at a predefined magnification or a background image provided by user input. To achieve this, I used several agar plates without animals and recorded images for magnifications of 1x and 2x. I used an average of images (n>5) at a particular magnification to determine what a background image is expected to be like. If desired, the new interface allows the user to capture their own background image, which will temporarily replace the predefined matrix until the worm tracker software is closed. Using a custom image is critical if a magnification is used other than those for which stored data exist (1x and 2x). The background correction function uses the stored background matrix as an assumption of the image background and subtracts it from video. In this way, each video frame can be normalized before thresholding begins. The effect of this correction function is displayed in the preview window.

**Creating an effective user interface**

Finally, I redesigned the user interface to be more informative to the user and give more direct input to the functions of the tracker (Fig. 6). The original Parallel Worm Tracker has three completely separate graphical windows to perform the tasks required to track animals. The new tracker has a single window, and most of the functions of the old interfaces are now performed automatically when tracking is complete. The menu will still bring up the older windows for any functions that may be needed for backward compatibility or other unforeseen purposes. The main window can be used to record video, track, analyze data and reanalyze old files. The preview window initially just showed a raw video feed from the camera. I replaced this with a window that will show the user exactly what the tracker will use to find animals. This new window will resize to the final video file size, apply the background correction function and show the effect of the black/white threshold that will be used to separate animals from the background. Single images can also be captured using this window.
Figure 6. Updated worm tracker interface with core functions accessible from one window. A new preview window shows what the end product will look like, saving time in setting up an adequate quality tracking video.

I trust that this new user interface is much more intuitive, and the tracker is now a useful tool. However, I would recommend exploring other options that may overcome some of the major limitations of the Parallel Worm Tracker. One option is the “WorMotel”, a system that has been used to show the quiescence of many individual animals simultaneously following heat shock. This system differs from the current tracker in two major aspects: (1) it incorporates a device to keep animals confined and separated, and (2) it uses a different computational strategy to detect movement. Rather than examining animals on a petri dish, they are placed in 48 individual compartments in a custom made silicon-based device. The device then allows tracking of individual animals, unlike the Parallel Worm Tracker. Quiescence is then determined via frame subtraction. Using this computational approach, differences in background conditions are not a limitation. Possible drawbacks of this system compared to the Parallel Worm Tracker are: (1) it
requires time-consuming user input to confirm that animals have not escaped their wells, (2) some amount of programming knowledge may be necessary to operate the device (Nelson et al., 2014 and personal communication).

**Scientific review for tracking systems**
As exemplified by the creation of our own video processing and quiescence functions, the MatLab® environment provides the tracker software with flexibility. This flexibility is an asset when transferring it to different laboratories and performing diverse assays. However, setting up the tracker was challenging and could be simplified. Collaboration between labs would allow for a more readily useable and user-friendly tracker, saving on setup time. However, I feel that a central archive or website, updateable by several users in the worm community would be necessary to facilitate these changes in a way that makes them sufficiently accessible to justify the time investment required. It has recently been suggested that published scientific code be submitted to a peer review process, similar to journal articles (Hayden, 2013). This type of review would ensure best coding practice is used, and that the logic in core algorithms is complete. If this approach is used from the outset, it saves time for future programmers, and makes code more easily understood. I feel that my experience with the tracker adds support to the argument for a peer review process in scientific computing.
Chapter II: Characterizing the Acute Response to Heat Stress

Heat shock induces quiescence
We wished to study behavioral quiescence in *C. elegans*. Behavioral quiescence occurs during development as part of the molt between larval stages, and also in response to LIN-3 overexpression. Overexpression of LIN-3 was achieved by placing a copy of *lin-3* under the promoter region of the heat-induced gene *hsp-16.2*. During the investigation of LIN-3 overexpression it was apparent that heat shock itself caused a short period of transgene-independent quiescent behavior. We wished to perform a more detailed characterization of this behavior, in part to assist in designing proper controls for behavioral assays involving heat-inducible gene expression. Previous observations of *C. elegans* behavior following heat shock found that exposure to temperatures above about 32°C triggers avoidance, escape responses and a cessation of feeding (Glauser et al., 2011; Liu et al., 2012; Jones and Candido, 1999).

Testing the effect of different heat shock methods on subsequent quiescence behavior
To understand exactly how heat shock affects animals, we systematically tested different heat shock conditions. We first examined feeding behavior, as movement of the pharyngeal grinder is easily seen under a low power microscope and adult wild-type wakeful animals do not cease pumping in normal laboratory conditions (Avery and You, 2012). Heat shock causes complete cessation of the pharyngeal grinder. First, we used this method to test the effect of different incubation methods on behavior. Young adult animals on agar plates were incubated for 30 minutes at 35°C. Plates were incubated either directly on the shelf of the incubator, or in a bead bath, or in a water bath. The bead and water baths were kept in the 35°C incubator at all times. Immediately after heat shock, <30% animals were feeding, while 60 min after heat shock >95% of animals are feeding. This was true for all three treatments and the recovery curves are similar, but there is a significant difference in the quiescence behavior of animals between treatments ($P=0.009$; Fig. 7A). We notice a trend, with incubation in water inducing the greatest quiescence on average, followed by incubation in beads and incubation on the shelf. This trend is likely to be a result of the greater conductivity of water and beads than that of air, and the greater surface area of water in direct contact with the plate than beads. As water seemed the most stringent method, we selected water as the media for use in all further experiments.
Next, we assessed the effect of incubation time on behavioral quiescence. We expect that more severe heat shock may result in an increased period of feeding quiescence. Consistent with this, we observed increasing behavioral quiescence following 35°C heat shock with durations of 15, 30 and 45 minutes respectively ($P=0.0004$; Fig. 7B). We chose a 30-minute heat shock for further observation of behavior.

**Locomotion is suppressed following heat shock**

Once we had selected conditions for our heat shock experiments, we wished to quantify the locomotor activity of animals following heat shock. We quantified locomotor quiescence using the automated locomotion tracker and algorithm described in Chapter I. Animals showed mildly inhibited quiescence for an hour following heat shock (Fig. 3A). We would expect that if this quiescence were a state of general malaise, then speeding recovery by cooling plates on ice would diminish behavioral quiescence. In contrast, we later found that cooling plates rapidly to room temperature enhances the inhibition of locomotion (Hill *et al.*, 2014). This observation suggested that heat-induced quiescence may not be due to general malaise but rather is reflective of a quiescence program, an indication supported by analyses described below.

**Egg laying is suppressed following heat shock**

In addition to cessation of feeding and locomotion, we wished to see if any other behaviors were affected by heat shock, and we chose to investigate egg-laying behavior. We expected that following a 35°C heat shock there might be a cessation of egg-laying concomitant with the inhibition of other behaviors. Our data indicate that this is indeed the case (Fig. 7C), with egg laying rate not significantly changed during heat shock, but decreasing after heat shock. Egg laying rate remains suppressed until 1 hour, though the difference at this time point is not significant due to the small sample size.
Figure 7. Characterizing heat-induced quiescence. (A) Comparing heat shock incubation methods. There is a trend that is consistent across time points, with incubation in water producing the strongest behavioral effect. Two-way ANOVA, $P=0.009$, $n \geq 7$. Except at 30 minutes, there was no significant difference between treatments at individual time points. (B) Comparing different incubation times. The effect of incubation time is highly significant, $P=0.0004$, two-way ANOVA, $n=5$. (C) The effects of heat shock on egg-laying behavior. The number of eggs laid per worm was counted at 30 minute intervals before, during and after a 30 minute 35°C heat shock. $n=4$, except at 60 minutes after where $n=2$. *$P=0.003$ student's t-test. Bars indicate minimum and maximum values, boxes indicate quartiles, the center line indicates the median, and + indicates the mean.

**Is a brief temperature exposure sufficient to induce quiescence?**

We wished to determine whether a very brief noxious temperature exposure was sufficient to trigger behavioral quiescence. To achieve this we observed animals on a heated microscope stage. Animals were observed as the temperature increased, and seen to stop pharyngeal pumping
abruptly at 28–30ºC (Fig. 8A). If animals were removed from the heated stage as soon as they stopped pumping, they resumed within 1-2 minutes, and the plate temperature at the time of resumed feeding was between 23–28ºC (Fig. 8B). While we did not quantify locomotor behavior during these brief heat exposures, it was clear that animals were quite mobile as the temperature increased, an observation consistent with previously described heat-induced escape responses (Liu and Baumeister, 2012). The fact that animals continue to move indicates that the full suite of quiescent behaviors cannot be induced by brief exposure to heat.

Figure 8. The effects of a brief temperature exposure. Animals were observed on a heated microscope stage and quiescence was scored as absence of pharyngeal pumping. Each line represents one trial with 10–20 animals; three separate trials are shown. (A) Animals were observed while the temperature was increasing. Animals become quiescent rapidly as the temperature rises above 28–30ºC. (B) Animals were also observed while the temperature was decreasing and recover as the temperature returns below 23–28ºC.

Heat-induced quiescence is regulated by neuronal activity
If heat-induced quiescence is a state of general cellular dysfunction, then we would expect all mutant animals to behave similarly to wild-type, but if it is a programmed response, then animals lacking the required genes for the response would remain active following heat shock. A controlled response may be mediated by neuronal signaling. If such a signal exists, then animals with impaired neuronal signaling would be expected to be less quiescent than wild type following the same heat stress treatment.
Neurons can communicate with other cells through release of signaling molecules such as neurotransmitters and neuropeptides. These signaling molecules are stored in specialized vesicles, allowing for their regulated release from the cell. Pre-synaptic terminals contain two major classes of vesicles. Synaptic vesicles contain neurotransmitters, which carry relatively fast signals across the synapse to adjacent cells. In contrast, dense-core vesicles are responsible for the release of neuropeptides, which modulate post-synaptic responses and generally act on a longer time scale (reviewed by Mains and Eipper, 1999). Dense-core vesicles can be situated away from the synapse and their secretion may be triggered by different conditions than synaptic vesicles (reviewed by Scalettar, 2006). Many of the genes specifically required for the regulation of the release of these vesicles are known. UNC-13 is required for synaptic vesicle release at both GABAergic and cholinergic synapses (Richmond et al., 1999). UNC-18/munc-18 is a chaperone that ensures the docking of the vesicle to the membrane by UNC-64/syntaxin and may also transport UNC-64 to the synapse (reviewed by Ramakrishnan et al., 2012). UNC-31/CAPS is required for the calcium-dependent exocytosis of dense-core vesicles (Speese et al., 2007). To test for a requirement for neuronal signaling in heat-induced behavioral quiescence, we examined three reduction-of-function neuronal signaling mutants, unc-13(e51), unc-18(e234) and unc-31(m68) for quiescence following heat shock.

Animals with “unc” or uncoordinated phenotypes show abnormal locomotion, so we continued to use absence of pharyngeal pumping to score quiescence in these mutants. unc-13 and unc-31 animals also have abnormally slow pharyngeal pumping, but still continue to pump in standard laboratory conditions. We assessed the behavior of these three mutant strains following a 35°C heat shock. We observed a striking reduction in quiescence in unc-13 and unc-31 animals (Fig. 9A-B). As both UNC-13 and UNC-31 are known to play a role in dense-core vesicle release, these data strongly implicate neuropeptide/endocrine signaling in heat-induced quiescence. By contrast, we observed no quiescence defect associated with strong reduction-of-function of UNC-18 activity (Fig. 9C). As UNC-18 has been shown to play a role in synaptic vesicle release but not dense-core vesicle release, we conclude that synaptic transmission does not play a significant role in heat-induced quiescence.
To further test the idea that dense core vesicle release is required for behavioral quiescence, we examined \textit{pkc-1} mutant animals. PKC-1/PRKCE is a serine/threonine protein kinase C. PKC-1 has been shown to be required for the release of neuropeptides by dense core vesicles (Sieburth \textit{et al.}, 2007). If neuropeptide release is required for quiescence, then \textit{pkc-1} mutant animals should become active more quickly than wild type following heat shock. Consistent with this idea, we find that \textit{pkc-1} mutant animals are less quiescent than wild type (Fig. 9D).

Since dense core vesicles are known to release neuropeptides, we wished to examine the possibility that quiescence is controlled by these neuropeptides. If so, quiescence behavior in animals with impaired processing of neuropeptides should be reduced. Neuropeptides are produced as pro-peptides consisting of multiple neuropeptides that are catalytically processed to produce mature neuropeptides. EGL-3/KPC-2 and EGL-21/carboxypeptidase E are two such enzymes that cleave pro-peptides (Li and Kim, 2008). If any of the neuropeptides processed by these enzymes is required for quiescence, we expect impaired quiescence. Surprisingly, \textit{egl-3} and \textit{egl-21} animals are much more quiescent than wild type (Fig. 9E–F). A possible explanation for this is that EGL-3 and EGL-21 participate in an inhibitory mechanism regulating heat-induced quiescence. If our interpretation of the role of PKC-1 in sleep is related to neuropeptide release is correct, then perhaps there are both activating and inhibitory neuropeptides involved in heat-induced quiescence. If such activating neuropeptides exist, then the EGL-3 and EGL-21 results indicate that enzymes other than those tested process some of the neuropeptides required for quiescence.

We observed a reduction in quiescence in many neuronal signaling mutants, i.e. mutant animals were more likely to resume pumping sooner, but we did not observe continued pharyngeal pumping at the end of heat shock in any of these animals. The failure to find any mutant that was continuously active implies that the initial cessation of pharyngeal pumping is not part of the subsequent quiescence. This cessation may be a cell-autonomous effect of heat shock, perhaps as a result of some cellular disruption such as protein misfolding. However, together with the fact that animals quickly resume feeding and do not cease locomotion with a brief exposure to heat indicates that the initial inhibition of feeding may be part of a separate avoidance response in addition to cell-autonomous effects.
Figure 9. Neuronal signaling mutants fail to show behavioral quiescence. Animals were subjected to a 30 minute 35ºC heat shock and then quiescence was determined by observing pharyngeal pumping. (A) UNC-13 required for vesicle release and (B) UNC-31, required for dense core vesicle release, are also required for normal heat-induced quiescence. (C) UNC-18, required for synaptic transmission, is dispensable for heat-induced quiescence. (D) PKC-1 is also required for normal quiescence. (E–F) EGL-3 and EGL-21 are dispensable for quiescence, and may act in an inhibitory pathway. *P<0.05, student’s t-test.
37°C heat shock causes two bouts of quiescence

Thus far, we have characterized heat stress at 35°C and seen all animals recover within about 60 minutes. When animals are heat shocked at 37°C for 30 minutes they exhibit a similar initial period of quiescence immediately following heat shock. However, wild-type animals show an extended second bout of quiescence not seen after a 35°C heat shock. This second bout starts at about 3 hours, with a peak at 4 hours and full recovery between 6 and 12 hours, (Fig. 10). Since the second bout is so striking, and this bout of quiescence is clearly distinct from the competing effects of avoidance responses or malaise immediately at the end of heat shock, we performed many of our experiments at this more stringent temperature.

An additional transcription factor required for ALA development ceh-14 is required for sleep

In addition to the requirement for neuronal signaling, we found that the transcription factor CEH-17 is required for heat-induced quiescence (Hill et al., 2014). CEH-17 is a Phox-2-like homeodomain protein that is a transcription factor required for the development of the ALA and the 4 SIA interneurons (Pujol et al., 2000). EGF-induced quiescence dependent on the ALA neuron has previously been shown (Van Buskirk et al., 2007). ALA differentiation is controlled by at least three transcription factors: ceh-17, ceh-14, and ceh-10 (Van Buskirk and Sternberg, 2010). ceh-14 is not known to be expressed in the SIAs and thus if both ceh-14 and ceh-17 mutant animals are sleep defective following stress we can conclude that this signal is most likely mediated by ALA and not some other cell. As expected, ceh-14 animals show a severe sleep defect (Fig. 10). In addition to the ALA, ceh-14 contributes to the differentiation of the thermosensory neurons AFD and is required for thermosensation (Cassata et al., 2000). Since we have shown that AFD-mediated thermosensation is not required for sleep, we can rule out a role for thermosensory neurons in heat-induced sleep. However, ceh-14 is expressed in 15 other neuronal cell types so we cannot definitively rule out a role for these neurons in sleep behavior even though it seems unlikely (Kagoshima et al., 2013). Both ceh-17 and ceh-14 mutants were found to be resistant to the somnogenic effects of EGF overexpression (Van Buskirk and Sternberg, 2010). Together these data suggest that heat-induced sleep requires EGF signaling within the ALA neuron.
Figure 10. Multiple ALA transcription factors are required for normal sleep. Quiescence behavior of wild type, ceh-14(ch3) and ceh-17(np1) mutant animals following a 30 minute 37°C heat shock. ceh-14(ch3) animals show a defect in quiescence similar to ceh-17(np1), indicating a requirement for ALA in sleep behavior. *P<0.05, Fisher’s test ceh-14 vs. N2, error bars represent standard error.

Do other nematodes sleep?

Since stress-induced sleep in *C. elegans* shares genetic components of sleep with higher organisms, we might expect to find a stress-induced sleep state in other animals. Circadian control of sleep may mask this effect in many animals, but we do not expect this to be the case in other nematodes. *C. elegans* is a free-living nematode in the family Rhabditidae found on decomposing plant material (Félix and Duveau, 2012). We tested *Pristionchus pacificus*, a free-living and facultatively necromenic nematode in the family Diplogastridae for quiescence following a 35°C heat shock (Fig. 11A). Pharyngeal pumping is more difficult to observe in *P. pacificus* than *C. elegans* due to the lack of a pharyngeal grinder. For this reason we used locomotion to assay *P. pacificus* quiescence. We found that following heat shock, *P. pacificus* nematodes indeed exhibit sleep-like behavior, and display even more pronounced locomotory quiescence than *C. elegans* (Compare Figs. 4 and 11). In *C. elegans*, a more stringent heat shock of 37°C produces two bouts of quiescence, with the second bout peaking at about 4 hours after heat shock (Fig. 10). In *P. pacificus*, we found that a 37°C heat shock elicited a single prolonged quiescent bout (Fig. 11B). The lack of mutants for EGF signaling components in *P. pacificus* makes it impossible to say whether this sleep-state is EGF-dependent, as it is in *C. elegans*. However, the existence of stress–induced sleep in *P. pacificus* reveals that this phenomenon is not
unique to *C. elegans*. This notion has recently been supported with the observation of stress-induced sleep in *Drosophila* (Lenz et al., 2015).

Figure 11. Wild-type *Pristionchus pacificus* PS312 also shows heat--induced quiescence. Locomotion was assayed using computer software. Quiescence was defined as no movement above 10µm.sec⁻¹ for a minimum of 6 seconds. Non-heat-shocked *P. pacificus* animals are shown as a control. (A) Quiescence following a 30 minute 35°C heat shock. n=1. (B) Quiescence following a 30min 37°C heat shock, n=1 or 2 as indicated on the figure. Where shown, error bars represent standard error.

**The break between sleep bouts is not attributable to limiting amounts of LIN-3 or ADM-4**

As mentioned briefly above, after a heat shock at 37°C or above, there are two sleep bouts. The first is very similar in duration to the single sleep bout observed in response to a 35°C heat shock, but following recovery from this sleep bout, animals enter an additional prolonged bout of quiescence (see Fig. 11 for an example of this phenomenon). Why is it that animals experience two separate sleep bouts, rather than simply remaining continuously quiescent for a prolonged period? We reasoned that some component of the sleep-promoting pathway may be present in limiting amounts, such that the duration of the initial sleep bout is limited to approximately one hour, regardless of heat shock stringency. If the transcription of this component were stimulated by heat stress, this would render animals competent to enter a second sleep bout if required. Any component of the sleep-promoting pathway could be potentially limiting (Fig. 12). For example,
it is known that activation of EGF receptors leads to their internalization (Goh and Sorkin, 2013), creating a 'refractory period' of signaling. Similarly, sleep-promoting neuropeptides released by the ALA neuron may not be replenished quickly enough to support continuous sleep behavior. If any of the genes required for sleep are transcriptionally upregulated in between sleep bouts, this might provide a partial explanation for the appearance of the second, prolonged peak of quiescence. Accordingly, we wished to test whether any of the known components of stress-induced quiescence are transcriptionally upregulated in response to stress.

Figure 12. Known components of stress-induced sleep in C. elegans. An unknown signal triggers the cleavage of the pro-EGF homolog LIN-3. We know that a metalloproteinase, ADM-4/ADAM17, is required for stress-induced sleep and suspect that it is the protease responsible for cleaving LIN-3 (unpublished observation). The LIN-3 ligand binds to the LET-23 receptor, a homolog of EGFR, on the ALA neuron. Several neuropeptides, presumably released by the ALA neuron, induce sleep behaviors (personal communication from the Sternberg lab).

Our lab has identified a metalloproteinase, ADM-4, that is required for heat-induced sleep behavior (unpublished data) and we wished to test whether this gene was transcriptionally upregulated following heat shock. We performed RT qPCR of adm-4, but were unable to detect
any difference in expression (Fig. 13). Thus it seems unlikely that increased transcription of \textit{adm-4} occurs preceding the second bout of sleep. We then turned our attention to the next component of the pathway for sleep behavior, LIN-3/EGF.

![Figure 13. The level of \textit{adm-4} mRNA does not increase 2 hours after a 37°C heat shock. Fold induction of \textit{adm-4} was determined relative to non-heat shocked, age-matched controls with expression normalized to the housekeeping gene \textit{tba-1}.](image)

LIN-3/EGF is a diffusible signaling molecule that activates EGFR on the sleep-inducing neuron ALA. We wished to examine changes in \textit{lin-3} expression and first focused on changes in alternative splicing, rather than overall levels of \textit{lin-3} transcripts, as there is evidence that a particular isoform, LIN-3C, may be the sleep-promoting isoform (Van Buskirk \textit{et al.}, 2007). To determine if there were any such changes in isoform-specific expression, we performed RT PCR of whole animals. No differences in expression between isoforms were found (Fig. 14A). \textit{lin-3C} is expressed at levels undetectable in whole animals (Fig. 14A), but has been shown to be expressed in the head (Van Buskirk \textit{et al.}, 2007). We therefore examined transcripts from isolated animal heads. Even this more stringent analysis failed to pick up any expression differences between \textit{lin-3} isoforms (Fig. 14B). As this analysis relied on the brightness of bands on an electrophoresis gel, it was only semi-quantitative. We further quantified \textit{lin-3C} levels by RT qPCR and found no change (Fig. 14C). We have yet to perform non-isoform specific analysis of \textit{lin-3} levels, but given that we see neither increase in \textit{lin-3C} nor any relative change in the isoforms, these results together suggest that \textit{lin-3} transcription is not increased following heat shock.
A

B

C

fold induction of mRNA

tba-1 lin-3C
Figure 14. There is no apparent difference in expression between *lin-3* isoforms 2 hours after a 37°C heat shock. (A) *lin-3* RT PCR of whole animals using primers that flank the alternatively spliced region, to examine changes in the relative intensity of isoforms following heat shock. Heat shocked animals were compared to non-heat shocked controls. (B) RT PCR of RNA isolated from dissected heads, using the same primers as in panel A, to examine potential pharynx-specific differences in expression between *lin-3* isoforms. No differences were detected. (C) The levels of *lin-3C* mRNA do not increase 2 hours after a 37°C heat shock. Fold induction of *lin-3C* was determined relative to non-heat shocked, age-matched controls with expression normalized to the housekeeping gene *tba-1*.

While our analysis of *lin-3* and *adm-4* expression was limited to 2 hours after heat shock, it seems unlikely that any transcriptional changes underlying the behavioral dynamic would not have occurred by this time, given that the second peak of quiescence can be seen as early as 3 hours after heat shock. If there are transcriptional changes that underlie the appearance of the second sleep bout, they must be in other genes. Of the known components of the pathway for stress-induced sleep, the remaining candidates are the EGF receptor homolog LET-23 and ALA-expressed neuropeptides. We plan to examine expression of LET-23 in the near future. The identity of the full suite of ALA-expressed neuropeptides is an area of active study, and one neuropeptide-encoding gene, *flp-13*, has been identified. Interestingly, transcription of *flp-13* increases following heat shock (Nelson *et al.*, 2014), so a limitation in sleep-promoting neuropeptides may explain the break between bouts of quiescence.
Chapter III: Heat Stress Does Not Initiate Sleep via a Thermosensory Pathway

Wild-type animals exhibit quiescence immediately following removal from a 35°C heat shock. We have found that components of EGF signaling and the ALA neuron mediate this quiescence (Hill et al., 2014), but we do not know how stress triggers EGF signaling. In response to heat, *C. elegans* initiates an immediate thermal avoidance response consisting of turns and reversals (reviewed by Aoki and Mori, 2015). The animal also exhibits an escape response delineated by accelerated movement. A well-characterized thermosensory circuit controls these behaviors. In this circuit, a pair of left and right bilateral thermosensory neurons AFDL/AFDR (AFD left and AFD right) sense temperature and process thermal information. This information is relayed to a pair of interneurons, AIYL/AIYR (AIY left and AIY right) that mediate thermophilic or cryophilic drive in navigational decision-making. The end result of which are the escape and avoidance behaviors (reviewed by Aoki and Mori, 2015). Do known sensory mechanisms initiate both thermotactic responses and sleep, or are there novel mechanisms that trigger sleep in response to environmental or cellular stresses? If thermosensation is required to drive sleep, this would most likely use known thermosensory mechanisms. Animals lacking these components of thermosensation would be expected to be deficient in their sleep response. Conversely, if sleep is triggered directly by cellular stress, we would expect none of the thermosensory mutants to be deficient in their sleep response (Fig. 15). Thus, we looked for sleep in mutant animals deficient in their ability to detect thermal stimuli.
Figure 15. Proposed model of alternate mechanisms for heat-induced quiescence. Heat is known to cause cellular stress and also to trigger acute thermotactic behaviors via the AFD and AIY neurons. I aim to determine whether the behavioral quiescence observed in response to prolonged heat exposure is driven directly by cellular stress, or whether it may rely on prolonged activation of the same thermosensory circuit that mediates the acute avoidance response to heat. If thermosensation triggers the sleep response, known thermosensory-defective mutants should be impaired for heat-induced quiescence. If direct detection of cellular stress drives sleep behavior, then mutants that are defective in chaperone responses should show enhanced quiescence.

**Initial data indicates that AFD sensory neurons are required**

A pair of sensory neurons called AFD is responsible for the thermotactic response of *C. elegans* between 15–35°C and contributes to perception of noxious temperatures above 35°C (Liu and
Baumeister, 2012). If this heat-sensing mechanism is required for heat-induced sleep, animals lacking functional AFD neurons would be expected to be quiescence-defective. TTX-1, an Otd/Otx homeodomain transcription factor is required for all differentiated characteristics of the AFD neuron (Satterlee et al., 2001). Consistent with a role for the AFD neurons in stress-induced sleep, we initially observed that the reduction-of-function mutants ttx-1(p767) and ttx-1(oy29) are each associated with defects in stress-induced quiescence (Fig. 16A&B).

**AIY interneurons are not required**

The sole downstream synaptic partners of the AFD neurons are the AIY neurons. These AIY neurons are part of the thermosensory circuit required for normal thermotaxis (Hobert et al., 1997) (Fig. 15). AIY cell fate is controlled by the Lim homeodomain transcription factor TTX-3 (Hobert et al., 1997). We analyzed ttx-3 mutant animals, expecting to them to be deficient for heat-induced sleep behavior. Unexpectedly, these mutant animals were not significantly different from wild type (Fig. 16C). One possible explanation is that the ttx-3 reduction-of-function ks5 allele that we tested was incompletely penetrant and thus failed to produce a detectable phenotype. To rule out this possibility we examined three additional alleles of ttx-3, including a 1.8kbp deletion. All of these alleles are known to be thermosensation defective, however, none of them showed a sleep-defective phenotype (Fig. 16D–F). Our observation that TTX-1, but not TTX-3, appears to be required for heat-induced sleep suggests that that AFD triggers sleep via a novel AIY-independent thermosensory mechanism.
Figure 16. AFD may be required and AIY is not required for heat–induced quiescence. Fraction of quiescent animals, as assayed by feeding inactivity following a 30 min 35°C heat shock. The AFD-defective genotypes ttx-1(oy29) (A) and ttx-1(p767) (B) show reduced quiescence relative to wild type following heat shock. C–F, The AIY neuron does not appear to be required for heat-induced quiescence. AIY-defective ttx-3 alleles ks5 (C), mg158 (D), ot22 (E), and the 1.8kbp deletion allele ot358
(F) show no sleep deficit. This assay is highly susceptible to day-to-day variability so the time taken for wild-type animals to recover differs between trials. Each point represents the mean of at least 3 trials. Error bars represent standard error. *$P<0.05$ as determined by a two-tailed student's t-test. $P$ values are given in Tables 2 and 3.

The AFD-specific gene NHR-38 is not required
ttx-1 is expressed not only in the AFD neurons but in pharyngeal marginal cells as well, and its role in heat-induced sleep may not reflect a function of AFD neurons. In order to address whether the AFD neurons are indeed required for heat-induced sleep, we examined additional AFD-defective mutants. nhr-38 is a nuclear hormone receptor exclusively expressed in the AFD neurons, and appears to control a subset of AFD characteristics (Camon et al., 2003). We examined the behavior of nhr-38(gk299) reduction-of-function mutant animals following heat shock, predicting that these animals may also show defective quiescence. However, these animals showed slightly enhanced quiescence compared to wild type (Fig. 17A). This result does not rule out a role for AFD, as nhr-38 appears to control fewer aspects of AFD function than the upstream regulator ttx-1 (Satterlee et al., 2001).

There is only a weak requirement for intracellular components of AFD thermosensation
Thermosensation by the AFDs utilizes a cGMP-gated calcium channel TAX-2/TAX-4 and at least four guanylyl cyclases GCY-8, GCY-12, GCY-18 and GCY-23. The molecular mechanism for heat sensation by AFD is summarized in Figure 18. Interestingly, tax-4(ks11) reduction-of-function mutants are more quiescent than wild type (Fig. 17B). tax-4 is expressed in at least 9 neuron types in addition to AFD (Komatsu et al., 1996). Therefore tax-4 may have a sleep-inducing role in AFD that is masked by a role in the negative regulation of sleep in other neurons. Thermosensation by the guanylyl cyclases is at least partially redundant and loss of three of the guanylyl cyclases, GCY-8, GCY-18 and GCY-23 was shown to abolish thermotaxis (Inada et al., 2006). We find that gcy-23(nj37) gcy-8(oy44) gcy-18(nj38) mutant animals are impaired in their sleep response (Fig. 17C), consistent with a role for at least some of the thermosensory components of AFD in initiating sleep behavior.
Figure 17. TRPV channels and AFD neurons are not required. Fraction of quiescent animals, as assayed by feeding inactivity following a 30 min 35°C heat shock. *ceh-17 animals are genetically ablated for the ALA sleep-inducing neuron and are included as a negative control. (A) Animals lacking the AFD-specific gene *nhr-38* show enhanced quiescence. (B) Animals lacking the cGMP-gated calcium channel component *TAX-4* also show enhanced quiescence. (C) Animals lacking three of the guanylyl cyclases required for thermotaxis are mildly sleep-defective. (D) A triply mutant strain that lacks TRPV channel activity shows strongly enhanced quiescence. (E) Animals genetically ablated for only AFD neurons show a robust heat-induced sleep response. (F) A repeated series of trials with the AFD developmental transcription factor mutant allele *ttx-1(oy29)* show enhanced quiescence, in contradiction of the original trials in Figure 16A&B.
Each point represents the mean of 3 or more trials. Error bars represent standard error. *$P<0.05$ vs. wild type as determined by a two-tailed student's t-test. $P$ values are given in Tables 2 and 3.

Figure 18. A model of the molecular mechanism of heat sensation by the AFD sensory neurons (adapted from Inada et al., 2006). An increase in temperature is detected by an unidentified thermoreceptor, which may be a G-protein coupled receptor or another molecule upstream of the guanylyl cyclases. Another possibility is that the guanylyl cyclases detect heat directly. Of the 29 identified guanylyl cyclases in *C. elegans*, four have been identified that are required for normal sensation of temperature in the AFD: GCY-8, GCY-12, GCY-18, GCY-23 (Inada et al., 2006; Liu and Baumeister, 2012). Stimulation of the guanylyl cyclases causes an increase in levels of intracellular cGMP. The elevated levels of cGMP then activate a calcium channel formed from two subunits, TAX-2 and TAX-4. The resulting influx of calcium changes the membrane potential of the AFDs.
Noxious heat detecting TRPV channels are not required

Under noxious heat conditions, other neurons are important contributors to thermosensation in addition to AFD. Specifically FLP and PHD neurons are required for noxious heat avoidance (Liu and Baumeister, 2012). These neurons may contribute to the sleep response immediately after heat stress. Unfortunately, no specific genetic mutants are available for FLP or PHD neurons to directly test for this requirement. However, noxious heat sensation by these neurons utilizes the *C. elegans* TRPV (transient receptor potential vanilloid) genes *ocr-2* and *osm-9* (Liu and Baumeister, 2012). We examined animals harboring reduction-of-function mutations in both *ocr-2* and *osm-9*, and found a robust heat-induced sleep response (Fig. 17D). This result indicates that noxious heat sensation by FLP and PHD neurons is dispensable for heat-induced sleep.

Genetic ablation indicates that AFDs are not required

Given that the only strong evidence for the role of AFD in quiescence was a sleep-defective phenotype in *ttx-1* mutants, we wanted to conclusively investigate AFD involvement by examining animals in which AFD was the only cell type ablated. We obtained a strain expressing the “executioner” protein of cell death Caspase-3 under the AFD specific promoter *gcy-8* (Luo et al., 2014). These AFD(-) animals showed a robust heat-induced sleep response (Fig. 17E). We confirmed that this caspase transgene was indeed present in this strain, by examining fluorescent reporters that mark the transgene insertion: mCherry under the *gcy-8* promoter, and GFP under the *gcy-8*, *unc-112* and *ttx-3* promoters. As expected for *unc-11p:gfp* we observed strong expression in the coelomocytes of all adult animals indicating that the insertion was still present. We observed no expression of mCherry in any animals indicating that the AFD neurons were indeed not present in adult animals. Our data from the *gcy-8p:Caspase-3* strain strongly suggests that the AFD neurons are not involved in heat-induced quiescence. Since this result contradicted our original *ttx-1* data, the question is whether there is another tissue/cell required for sleep, or a problem with our original assay. To test this we repeated the assay for *ttx-1(oy29)*, but were unable to replicate the original sleep-defective phenotype (Fig. 17F). Thus, the most likely explanation is that there was a problem with our original assay. Perhaps the age of animals was poorly matched.

Interestingly, locomotory quiescence during lethargus is abolished by a loss-of-function mutation in *npr-1*. Normal quiescent behavior during lethargus is restored by double mutations in both
and either *tax-4* or *osm-9*, indicating that NPR-1 mediates quiescence by inhibiting the activity of sensory pathways (Choi et al., 2013). Since lethargus quiescence defects can be rescued by impaired sensory function, this suggests that quiescence is achieved by inhibiting wakeful sensory functions such as thermosensation. In this case, the enhanced quiescence we observed in thermosensation defective animals may be due to inactivation of circuits that contribute to wakefulness. In the future, it would be interesting to ascertain whether the function of NPR-1 is specific to lethargus, or also functions to mediate stress-induced quiescence.

Taken together, our data indicate that thermosensation is not required for heat-induced quiescence. It could be that cellular stress (i.e., protein misfolding) drives sleep directly. Interestingly, it has been shown that thermosensation by AFD and AIY enhances the systemic transcriptional response to heat shock (Prahlad et al., 2008), with thermosensation-defective mutants having reduced induction of chaperone activity relative to wild-type animals. Thus the prevalence of enhanced quiescence phenotypes that we have observed in certain thermosensation mutants (Figs. 16&17) could be explained by an inability of these animals to efficiently recover from stress.
Table 2. Statistical tests of thermosensation data, t-test results \( (P\) values) for assays at 35ºC. Green shading indicates \( P<0.05\).

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<th>15</th>
<th>20</th>
<th>30</th>
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Table 3. Statistical tests of thermosensation data, Fisher’s exact test results (*P* values) for assays at 35ºC. Green shading indicates *P*<0.05.

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Chapter IV: Can Disruption of Protein Homeostasis Drive Sleep?

We have characterized a state of sleep-like quiescence following heat shock, and we have shown that known thermosensory mechanisms are not required for this sleep state. In fact, we have observed the opposite: thermosensory-defective animals show slightly prolonged sleep bouts following heat shock. Thermosensation in *C. elegans* enhances the transcriptional response to stress, which is referred to as the heat shock response or HSR. Since thermosensory mutants have a weakened HSR, and therefore have impaired restoration of protein homeostasis, the prolonged-sleep phenotype that we have observed in thermosensory mutants suggests that an imbalance in protein homeostasis may drive sleep. Proteins require a specific conformation for optimal function, and when proteins are in a non-native conformation, they lose normal function and may even acquire toxic function. Many factors contribute to the appearance of these non-native proteins, but especially stressors such as heat, cold and toxins. Maintenance of protein homeostasis, or proteostasis, by clearing these non-native proteins is critical for cell function and viability. Protein homeostasis is achieved by maintaining a balance between a network of processes such as transcription, protein folding, protein degradation and sequestration of proteins by chaperones (reviewed by McClellan *et al.*, 2005).

Changes in the proteostasis network facilitate recovery from stress-induced damage and allow adaptation to changing environmental conditions. A key part of this system are the global changes in gene expression mediated by HSF-1- and DAF-16-dependent stress responses (Sugi *et al.*, 2011). HSF-1 is a transcription factor that is sequestered in the cytoplasm. Upon exposure to heat and other environmental stresses, HSF-1 is released to activate transcription of molecular chaperones and other factors aimed at restoration of proteostasis, collectively called heat shock proteins or HSPs (reviewed by Richter *et al.*, 2010). DAF-16 mediates dauer formation, longevity, stress resistance and immunity in response to various environmental conditions. DAF-16 activity increases the transcription of chaperones crucial to stress resistance, and genes required for resistance to oxidative stress (Volovik *et al*, 2014). DAF-16 is controlled by the insulin-signaling pathway, but heat and oxidative stressors can induce DAF-16 activity independently of insulin signaling (reviewed by Landis and Murphy, 2010). A class of chaperones, the small HSPs, all have both HSF-1 and DAF-16 binding sites in their promoter regions and are induced by the activity of both transcription factors (reviewed by Landis and Murphy, 2010). If the quiescence
we have characterized is an effect of these known transcriptional responses, then we would expect *hsf-1* or *daf-16* mutant animals to be defective for heat-induced quiescence. If, however, the sleep response is triggered by proteostatic defects in an HSF-1 and DAF-16-independent manner, we would expect to see exaggerated sleep in these mutants due to their inability to restore proteostasis.

**Quiescence is independent of the stress induced transcription factors HSF-1 and DAF-16**

Activation of the heat shock response via HSF-1 is a multi-step process requiring both DNA binding and transcriptional competence (reviewed by Åkerfelt *et al.*, 2007). The *hsf-1* reduction of function allele *sy441* has a premature stop codon and lacks the transactivation domain, thus the protein can still bind DNA but not activate transcription (Hajdu *et al.*, 2004). We examined *hsf-1(sy441)* mutant animals for their ability to undergo heat-induced sleep. We found that, in response to a 35°C heat shock, these animals have a phenotype that is almost identical to wild type. Thus sleep behavior is not dependent on the heat-induced transcription factor HSF-1 (Fig. 16A). The *daf-16* allele *mu86* is a large deletion likely to be null (Lin *et al.*, 1997). We examined *daf-16(mu86)* animals for a sleep defect and found that these animals in fact have the opposite phenotype—they are more quiescent than wild type (Fig. 16B). Thus stress-induced sleep does not require DAF-16 activity, and further, as *daf-16* null mutants are severely impaired in restoration of proteostasis following heat shock, this evidence suggests that proteostatic disruption may promote sleep behavior following heat stress.

In response to a more stringent 37°C heat shock, which produces two peaks of quiescence, we observe enhanced quiescence during the first peak and decreased quiescence during the second peak in the *hsf-1(sy441)* mutant relative to wild-type (Fig. 16C). The enhanced quiescence seen in *hsf-1* mutant animals during the first peak is consistent with a role for proteostatic disruption promoting sleep in an HSF-1-independent manner, and further confirms our initial observation that the first sleep bout following stress is not affected by the activity of this transcription factor. The decreased peak of quiescence observed during the second sleep bout suggests that HSF-1 may promote the transcription of some quiescence-inducing gene that is required during the second bout. Interestingly, increased transcription of one such gene, the quiescence-inducing neuropeptide FLP-13 is induced by heat shock, consistent with the possibility that it may be regulated by HSF-1 (Nelson *et al.*, 2014). We therefore examined the flp-13 promoter region for
potential HSF-1 binding sites. Two regulatory sequences are known to enhance transcription in *C. elegans* following heat shock, (1) the heat shock element, or HSE (consensus sequence TTCTAGAA), a DNA sequence to which HSF-1 binds, and (2) the heat shock associated site, or HSAS (consensus sequence GGGTGTC). The factor that binds HSAS is not known, but it is possible that this factor is HSF-1 or one of its cofactors (GuhaThakurta et al., 2002). Consistent with the possibility that *flp-13* induction upon heat shock is dependent on HSF-1, we identified two putative HSE sites and one putative HSAS in the promoter region of *flp-13*, and an additional two HSE sites within the gene itself (Fig. 19).

![Figure 19](image.png)

Figure 19. Heat-induced quiescence is not dependent on the classic transcriptional responses to stress regulated by HSF-1 and DAF-16. Animals were subjected to a 30 min 35°C/37°C heat shock and then quiescence was determined by observing pharyngeal pumping. (A) *hsf-1(lf)* animals exhibit no difference in quiescence from wild type after a 35°C heat shock. (B) *daf-16(lf)* animals exhibit enhanced rather than defective quiescence. (C) *hsf-1(lf)* animals exhibit enhanced quiescence immediately following a 37°C heat shock, but less quiescence during the subsequent peak of quiescence. *P<0.05, student’s t-test.
Figure 20. Putative heat shock regulatory elements for *flp-13*. Vertical lines indicate locations of regulatory elements and arrows indicate their orientation. Four putative HSEs were found (yellow) −1163, −415, 517, 1018 bases from the *flp-13* translational start site, and one putative HSAS was found (red) −29 bases from the translational start site. The HSEs matches 7 of 8 positions of the consensus sequence, and HSAS is an exact match to the consensus sequence. Exons are shown as boxes, with coding sequence in blue and non-coding sequence in dark grey. *flp-13* exons connected by dotted lines. The gene identifiers and names are shown below. F33D4.11 is a short non-coding RNA.
Pre-exposure results in decreased quiescence following heat shock

Neither thermosensation nor the transcriptional responses to stress appear to drive sleep, and the enhanced sleep phenotypes seen in the chaperone response-defective mutant animals examined thus far suggest that proteostasis may trigger sleep. If disruptions of proteostasis constitute the signal for sleep need, then we expect the duration of quiescence to be proportional to the magnitude of proteostatic disruption following heat shock. It has been shown that pre-exposure to stress can confer thermotolerance by triggering up-regulation of chaperone networks (Xia et al., 1999; Kourtis et al., 2012). This thermotolerance likely reflects decreased proteostatic disruption. Therefore, if proteostatic disruption is driving sleep, we expect to see reduced quiescence in animals that have been pre-exposed to heat (Fig. 21).

Figure 21. Proposed model of heat–induced sleep behavior in normal and preconditioned animals. An initial heat shock creates tolerance to subsequent heat exposure through the heat shock transcriptional response. These preconditioned animals are then protected from proteostatic disruption. If proteostatic disruption is driving sleep and we predict to see reduced quiescence in preconditioned animals.

To investigate the possibility that thermotolerance confers a reduced sleep need, we heat shocked one set of animals, then 2 hours later we heat shocked them again alongside a cohort that had never previously experienced heat shock. Consistent with our model above (Fig. 20), we find that pre-exposed animals do exhibit reduced quiescence relative to animals that are experiencing their first heat shock (Fig. 22A).

We next compared *hsf-1* mutants to wild type to investigate whether induction of the transcriptional response following the first heat shock could indeed explain this result. Since *hsf-1* reduction-of-function animals cannot mount a robust transcriptional response, they are not likely to experience proteostatic benefits from pre-exposure to heat. We therefore expect
preconditioning to have a lesser effect on sleep behavior in hsf-1 mutants compared to wild-type animals. Only a single trial was performed, so the data are hard to interpret reliably. At 15 minutes after heat shock, hsf-1(sy441) reduction-of-function animals remain quiescent, which may reflect a restoration of sleep need with a loss of thermotolerance. However, there is a significant reduction of quiescence in preconditioned hsf-1(sy441) animals 30 minutes after heat shock indicating some effect of pre-exposure on sleep duration (Fig. 22B). hsf-1(sy441) is not a null mutation and may still have some residual activity, which may explain why we still see a reduction in sleep amount.

Figure 22. Pre-exposure results in decreased quiescence following heat shock. (A) Quiescence is reduced 15 minutes after heat shock in animals that are subjected to a second heat shock 2 hours after the first. *P<0.05, student’s t-test, n=5. (B) Quiescence is not reduced 15 minutes after heat shock, but is reduced at subsequent time points in hsf-1 animals that are subjected to a second heat shock 2 hours after the first. *P<0.05, fisher’s exact test, n=1.

Restoration of proteostasis in any tissue appears to reduce sleep need

We have presented evidence that impaired proteostasis can prolong quiescence, but how is proteostasis is monitored for the assessment of sleep need? Circumstantial evidence hints at two enticing possibilities for this site of action:

1) Stress-induced sleep depends on EGFR activation within the single neuron ALA, but the source of the EGFR ligand LIN-3 responsible for this activation remains unknown. A large amount of lin-3 mRNA has been detected in the pharynx of the animal, a tissue in which it has no known function (Saffer et al., 2011). Auspiciously, the pharynx is an
organ that interfaces with the outside environment and as such, is an attractive candidate to sense the stresses that induce sleep. One of the stressors we identified is the *Bacillus thuringensis* toxin Cry-5B. Cry-5B induces EGF–dependent quiescence when consumed by the animal and the quiescent response to Cry-5B is striking in its immediacy (Los *et al*., 2013; Hill *et al*., 2014). We reason that if the relevant source of LIN-3 were pharyngeal cells, then the sleep response may be quite rapid upon exposure to a toxin like Cry-5B that directly affects tissues of the pharynx.

2) Neurons may be the relevant tissue for monitoring proteostasis. Neurons consume far more energy than other tissues; in humans the brain consumes ~20 of the body’s oxygen, and in the fly 10% of the ATP consumption takes place in the retina (reviewed by Harris *et al*., 2012).

In response to a 37°C heat shock, *hsf-1* mutant animals show enhanced sleep during the first hour, and a lower peak of quiescence during the second bout, but a longer perdurance of quiescence as compared to wild type (Fig. 16C). As sleep is more pronounced during the first hour, we focused on this part of the time course. We attempted to determine the site of action for this effect by rescuing *hsf-1* selectively in either the pharynx, neurons, body wall muscle or intestine. Interestingly there was a significant rescue of the wild-type sleep phenotype in all strains examined. The phenotype for any given tissue-specific rescue of *hsf-1* was extremely variable between trials: in some trials no phenotypic rescue was observed, while in others a strong phenotypic rescue was seen. Time courses seem to indicate a more significant rescue in the pharynx (Fig. 23A vs. B–D). As variability was high, we further repeated the experiment. We chose to focus our extended analysis in the first bout, at a point when the difference between wild-type and *hsf-1(sy441)* controls was greatest, specifically 30 minutes after heat shock. When all the data is analyzed together, we see a partial rescue in all of the transgenic strains (Fig. 23E), *i.e.* a normal sleep phenotype is partially restored by HSF-1 expression in any of the tissues tested. Thus, rather than supporting either of our initial hypotheses, the data support a third model in which quiescence–inducing stress is monitored systemically. It could be that a diffusible signal can come from any cell in response to stress and this signal somehow causes release of EGF. And, perhaps the variability in our data could be explained as a stochastic effect of the levels of this signal coming from individual tissues.
It has been found that systemic HSF-1 activity mediates thermotaxis behaviors. It does so by regulating hormonal signaling that is monitored by an estrogen responsive nuclear hormone receptor, NHR-69 in thermosensory neurons (Sugi et al., 2011). It is possible that this signaling event also contributes to sleep behavior. However, the thermotaxis effect was dependent on transcription controlled by HSF-1, hence such a signal would be most likely to contribute to the restoration of normal sleep seen in the second bout rather than constitute a major sleep–inducing signal.
hours after 37ºC heat shock
fraction of animals quiescent
wild type (N2) hsf-1(sy441) myo-2:hsf-1 PHARYNX

hours after 37ºC heat shock
fraction of animals quiescent
wild type (N2) hsf-1(sy441) ges-1:hsf-1 INTESTINE

hours after 37ºC heat shock
fraction of animals quiescent
wild type (N2) hsf-1(sy441) myo-3:hsf-1 BODY WALL

hours after 37ºC heat shock
fraction of animals quiescent
wild type (N2) hsf-1(sy441) unc-14:hsf-1 NEURONS

53
Figure 23. *hsf-1* tissue-specific rescue indicates that the stress signal comes from all tissues. (A–D) Individual time course of sleep behavior following a 37°C heat shock for each tissue specific rescue. The x-axis is expanded for the first hour after heat shock where disruption of the transcriptional response is expected to have the most direct affect on sleep behavior. No significant difference was detected in the first peak for the initial rounds of this experiment. To the left of the axis, sleep behavior around 3–6 hours after heat shock is also shown. This interval represents the second peak of quiescence where *hsf-1* animals are less quiescent than wild type. In an *hsf-1*(*sy441*) reduction-of-function background, *hsf-1* cDNA was expressed (A) in the pharynx, (B) in neurons, (C) the body wall muscle and (D) in the intestine under the respective promoters indicated below the graph. *P*<0.05, fisher’s exact test, *n*=4. (E) The assay was repeated at least 6 times for each genotype and a significant restoration of normal sleep behavior was found with expression of *hsf-1* in any tissue. *n* is indicated by the white numbers within bars. *P*<0.05, student’s t-test, see table 4 for individual *P* values.

Table 4. *P* values versus *hsf-1*(*sy441*) at 30 min after heat shock; two-tailed t-test.

<table>
<thead>
<tr>
<th><em>hsf-1</em>(sy441) vs.</th>
<th>myo-2p:<em>hsf-1</em> (pharynx)</th>
<th>myo-3p:<em>hsf-1</em> (body wall)</th>
<th>ges-1p:<em>hsf-1</em> (intestine)</th>
<th>unc-14p:<em>hsf-1</em> (neurons)</th>
</tr>
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<tbody>
<tr>
<td><em>P</em> values</td>
<td>0.02</td>
<td>0.0006</td>
<td>0.0008</td>
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Does aging affect either the ability to sleep or the need to sleep?

Humans sleep for much longer during infancy than as adults. This increased sleep need is likely to be associated with developmental changes. As adults continue to age sleep disruptions increase and the amount of nighttime sleep decreases. Some studies report increased daytime sleep to account for sleep lost at night, so it seems that the ability to maintain sleep declines even though sleep need persists (reviewed by Vitielo, 2006). These effects have also been seen in mice and flies, with older animals sleeping more during the day and showing less ability to sleep at night (reviewed by Roberston and Keene, 2013). Interestingly, a failure of the unfolded protein response is correlated with these changes. Specifically, levels of the protein BiP/GRP78 increase with sleep deprivation, but in older mice this increase in BiP expression disappears (Naidoo et al., 2008). We might expect similar changes in C. elegans; the ability of older adults to sleep following heat stress may be decreased.

To test this hypothesis, we examined adults of varying age for heat-induced sleep responses. Contrary to our hypothesis, we observe a clear trend of increasing quiescence with age. While the period of sleep during the initial bout of quiescence after heat shock does not show any notable change with age, there is an increase in the duration and intensity of the second bout of quiescence (Fig. 24A). We hypothesize that this increase in quiescence may reflect a decreased ability of older animals to restore protein homeostasis. This hypothesis is supported by observations of increased clumping of aggregation reporter proteins, and decreased function of metastable (temperature sensitive) proteins in older C. elegans adults (Ben-Zvi et al., 2009). As these studies were performed on the scale of days rather than hours, we wished to examine whether proteostasis may already be declining during the first 30 hours of adulthood, possibly explaining the rapid changes in sleep behavior that we observed. To this end, we examined the survival of these animals in response to noxious heat. If the proteostasis machinery is less efficient with age, then we expect the survival of animals following extreme heat shock to be impaired, driving the increased sleep need. We found that, in the days following a 37°C heat shock, older animals are mildly impaired for survival (Fig. 24B).

We also examined the level of HSP-16.2 induction following heat shock in adult animals from 0–30 hours old. We observed a decrease in HSP-16.2 induction in adults as they age (Fig. 24C). The reduction may reflect a decline in the ability of animals to mount a response to cellular stress as
they age. However the change between 6 hour adult and 30 hour adults is less than two-fold, and does not show a severe reduction in the ability to mount the heat shock response.

It should be noted that the ages examined here are well within the period for which animals are reproducing and these animals could be considered to be relatively young. In *C. elegans* there is an age-associated breakdown of proteostasis with observable protein aggregation by day three of adulthood and a sudden collapse of the ability to maintain proteostasis by day seven of adulthood (Ben-Zvi *et al.*, 2009). In the future it would be interesting to test *C. elegans* for a greater sleep need following this event. At the same time it would be informative to test for a loss HSP-4/BiP induction correlated with a major change in the patterns of sleep consistent with that seen in fruit flies (Naidoo *et al.*, 2008).

Since the intervals examined for the effects of age on heat-induced sleep behavior are all so close together, perhaps the difference in sleep behavior is merely correlated with age, and another factor causes enhanced sleep over time. For instance, adult *C. elegans* exhibit a cyclical variation of survival in response to environmental stress and in expression of the stress resistance genes GPX and GPDH-1 (Kippert *et al.*, 2002; Simonetta *et al.*, 2008). These responses can synchronize with or “entrain” to light/dark cycles and temperature cycles (Kippert *et al.*, 2002; Simonetta *et al.*, 2008). Locomotion behavior in adult *C. elegans* also exhibits cyclical changes that will synchronize with to light/dark cycles and therefore may be under the control of the same circadian mechanism. However, this locomotor behavioral cycle has been found to be linked not only to the circadian cycle, but also to the expression of the developmental cycle gene LIN-42/Period (Simonetta *et al.*, 2009). In our experiment animals were isolated at specific times shortly after the L4/adult molt, an event locked to the cyclical expression of LIN-42 (reviewed by Monsalve and Frand, 2008). Thus it is conceivable that there are residual cyclical effects in the sensitivity to heat stress linked to changes in gene expression following the final molt. Since we have seen that animals sleep more following higher temperature stressors (Hill *et al.*, 2014), such cycling in the sensitivity to heat stress might explain the differences in sleep response of different age adult animals. This idea could be tested by examining animals at several intervals extending for at least two days beyond the L4/adult molt and also by entraining animals to different stages in the daily cycle.
Figure 24. Older animals sleep more. (A) Animals were subjected to a 37°C heat shock and quiescence was scored as absence of pharyngeal pumping. Different age animals respond significantly differently to heat shock, two-way ANOVA with repeated measures, $P=0.027$. Bars represent standard error. $n=2$ trials. (B) Younger worms show slightly better survival than older animals in response to the same 37°C heat shock as shown in A. See table 5 for individual statistics. (C) Fold-induction was determined by fluorescence intensity of an $hsp-16.2p:gfp$ reporter in animals 24 hours after a 37°C heat shock relative to
non-heat-shocked controls of the same age. Bars represent standard error. 0-6hr and 6-12hr animals are not significantly different, all other comparisons $P<0.01$ using a one-way ANOVA. One trial was performed, the number animals examined is indicated by white numbers within bars.

Table 5. Survival test $P$ values determined by the log rank test (Mantel-Cox). 0–6 hour adults have significantly enhanced survival over adults >12 hours old. $P$ values <0.05 are highlighted in green.

<table>
<thead>
<tr>
<th></th>
<th>0-6 hour adults</th>
<th>6-12 hour adults</th>
<th>12-18 hour adults</th>
<th>24-30 hour adults</th>
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<td>0-6 hour adults</td>
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<td>0.1</td>
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<tr>
<td>6-12 hour adults</td>
<td>0.007</td>
<td>0.2</td>
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<tr>
<td>12-18 hour adults</td>
<td></td>
<td>0.007</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>24-30 hour adults</td>
<td>0.003</td>
<td>0.1</td>
<td>0.07</td>
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Over a longer timescale than we examined here, it is possible that there is a breakdown in the ability of aging animals to sleep, similar to mice and humans. This breakdown may be a sudden loss in the ability to fall quiescent, or it may be marked by the inability to maintain a quiescent state for sufficient periods of time. Fragmented quiescence or ‘microhomeostasis’ has been observed during a developmentally linked sleep-like state in *C. elegans* following sleep deprivation (Nagy et al., 2014). Such a fragmented sleep pattern in aging animals may be detected by analyzing the dynamics of sleep in individual animals.

Since there are changes in the sleep patterns of animals both with aging and during early development, we might also predict increased stress-induced quiescence in *C. elegans* larvae. Preliminary data does indicate that larvae exhibit stress–induced quiescence, and may sleep more than adults (Fig. 25). The reliability of this particular data set is undermined by the occurrence of lethargus, a sleep-like period that coincides with each larval molt. Future experiments need to be performed with animals that are sufficiently synchronized to resolve stress-induced from lethargus.

We examined the level of HSP-16.2 induction in larval stage 4 (L4) animals vs. adults, although not other larval stages. Intriguingly L4 animals have a decreased induction of HSP-16.2 relative to young adults (Fig. 23C). As fold expression depends on target levels before and after heat shock, there are two possible explanations for this result. (1) The level of HSP-16.2 expression
after heat shock is less in larvae than it is in adults, and (2) the baseline levels of HSP-16.2 expression are higher to start with. Constitutively high chaperone levels have been observed in larval stages relative to adults in the insect *Belgica antarctica* (Rinehart et al., 2006), which protects animals prior to heat shock and would explain a reduced need to mount a transcriptional response in larvae. If this were also the case for *C. elegans* we might expect a higher baseline expression of chaperones. However, a similar difference was not detected in *C. elegans*, with baseline *hsp-16.2p:gfp* fluorescence of L4 within the range of baseline fluorescence in different ages of adults (mean of mean pixel intensities 370.6 for L4 vs. 253.3–405.9 for adults). Hence explanation (1) is the most likely. This result seems to indicate a correlation between levels of HSP-16.2 induction by heat shock and the amount of sleep, supporting the idea that sleep is driven by disruptions to proteostasis.

Figure 25. Preliminary data indicates that larvae experience stress induced sleep. It appears that they may even sleep more than young adults, but analysis is complicated by insufficient synchrony of these animals to avoid confusion with developmental quiescence that occurs at the end of each larval stage.
Chapter V: How Does Sleep-like Behavior Benefit C. elegans?

How is sleep beneficial to C. elegans?
There is clearly a physiological benefit to sleep, as we have previously observed a survival defect in sleepless, ALA(-) C. elegans animals following a severe 40°C heat shock (Hill et al., 2014). What does sleep do at the cellular level to protect these animals? Heat shock and other environmental stresses are known to interfere with cellular function, causing a state of cellular stress including an imbalance in proteostasis. Cells can respond to such proteostatic imbalance both by downregulating the production of new proteins, and by repairing and degrading existing proteins (Fig. 26). These cellular processes were once thought to be regulated cell-autonomously, but recently it has been found that they are modulated at the organismal level (reviewed by Taylor et al., 2014). Since behavioral quiescence in C. elegans is triggered by the same kinds of environmental stress that cause proteostatic imbalance, perhaps quiescence is an additional mechanism to enhance the repair and degradation of misfolded proteins.

Premature protein aggregation was not detected in sleepless animals
We hypothesize that sleep enhances the restoration of proteostasis following heat shock. A failure of proteostasis can result in the formation of insoluble aggregates of unfolded proteins within the cytoplasm (Fig. 26). Following our hypothesis, sleepless ALA(-) animals may be defective in restoration of protein homeostasis. In this case we predict that protein aggregation would be detected earlier and more often in ALA(-) animals than wild type after a 40°C heat shock.
Figure 26. A model of the potential fates of misfolded proteins in a cell. Disruption of the environment—i.e. by stressors like heat—leads to an accumulation of proteins folded in an intermediate or non-native state. These misfolded proteins lack their normal function and can even become toxic to the cell (reviewed by Gregersen et al., 2007). Unfolded proteins can be refolded by chaperones or degraded by the ubiquitin proteasome system. If these processes fail, proteins can clump together into insoluble aggregates in the cytoplasm.

We chose to exploit a system that has been established in *C. elegans* for the study of protein misfolding disorders that affect humans, in an effort to observe the effect of sleep on proteostasis (reviewed by Alexander et al., 2014). Proteostasis is disrupted in some human diseases, in particular diseases linked to genes with long repetitive elements. A set of protein conformational disorders is caused by stretches of trinucleotide repeats, such as poly-CAG (glutamine) within a gene. These polyQ proteins may be sensitive to proteostatic imbalance, as they have been shown to form insoluble aggregates under certain conditions such as aging and heat shock, that are associated with proteostatic breakdown (reviewed by Gregersen et al., 2007; Wyttenbach et al., 2000). The appearance of these protein aggregates increases with the length of these repeats. *C. elegans* strains carrying fluorescent reporters fused with trinucleotide repeats allow *in vivo* visualization of protein aggregation.
In *C. elegans*, expression of polyglutamine (polyQ) repeats fused to a yellow fluorescent protein (YFP) reporter manifests as either diffuse fluorescence of soluble protein in cells, or as punctate insoluble protein aggregates. The formation of these aggregates occurs during aging and with overexcitation of the muscle tissue in which they are expressed; this was also found to be concomitant with a breakdown in proteostasis (Garcia et al., 2007). We reasoned that sleeplessness may exacerbate protein aggregation following heat shock, and we therefore chose to examine polyQ reporters with little or no baseline aggregation. In a wild-type background, a 24 glutamate repeat reporter (Q24) does not show aggregation, and a 35 glutamate repeat reporter (Q35) shows age-dependent aggregation at ≥1 day of adulthood (Fig. 27). We wished to move these reporters into a sleepless *ceh-17* mutant background. However, as the Q35 reporter is integrated on the same chromosome as *ceh-17*, we selected the Q24 reporter for our mutant analysis. We then exposed Q24 and Q24; *ceh-17* animals to a 40°C heat shock and examined these animals for differences in reporter aggregation. We observed heat-shock-dependent aggregation in both strains, but were unable to detect a difference between wild type and *ceh-17* (Fig. 28; Fig. 29A).

![Figure 27](image-url)

**Figure 27.** Phenotypes of untreated young adult polyQ animals. Three representative images are shown of each phenotype. Neither Q24 nor Q35 strains show aggregation as young adults (<24 hours old) and so may be appropriate to test for a breakdown of proteostasis in heat-shocked sleepless ALA(-) animals. Untreated animals with the *unc-54p:Q24::YFP* reporter show diffuse fluorescence (A–C). Similarly, untreated young adult animals with the *unc-54p:Q35::YFP* reporter also show diffuse fluorescence (D–F). Fluorescence in the Q35 strain appears slightly brighter and is likely to reflect a higher copy number insertion of this reporter than the Q24 strain.
We are able to detect the onset of aggregation in both strains and if there is a difference this reporter system should be sufficiently sensitive to detect it. Perhaps sleep does not enhance proteostasis, but some other aspect of physiology. Another potential explanation is that the primary function of sleep is not to enhance proteostasis in muscle tissue, where the Q24 reporter is expressed. One alternative explanation for our inability to detect differences in the aggregation between these strains might be that the presence of the Q24 reporter somehow interferes with the beneficial effects of sleep. However, even among Q24-carrying strains we still observe a survival advantage of wild-type over sleepless ceh-17 mutants after a 40°C heat shock (Fig. 29B).
Figure 28. Images of *unc-54p:Q24::YFP* animals after a severe 20 minute 40°C heat shock. Three representative images are shown of each phenotype. 24 hours after heat shock little to no aggregation is observed and Q24 animals (A–C) appear similar to *ceh-17(np1)* animals. 96 hours after heat shock aggregation is observed, though both Q24 animals (G–I) and *ceh-17(np1)* animals (J–L) appear to show similar levels of aggregation. An additional, higher magnification, image of protein aggregation from the animal from panel J is shown (M).
Figure 29. There is no difference in polyQ24 aggregation between wild type and ceh-17. (A) Quantification of aggregation in Q24 animals in the days following a severe 20 minute 40°C heat shock. n≥3 trials for days 1–3, n=1 trial for day 4. An animal was scored as showing aggregation if 1 or more cells exhibited any aggregation. Bars represent standard error. (B) Transgenic lines show impairment in survival of ceh-17 mutants.

The mRNA level of heat shock proteins is not significantly reduced in ALA(-) animals

The HSF-1-dependent transcriptional response to heat shock is a well-characterized mechanism to restore proteostasis (reviewed by Richter et al., 2010). The transcriptional targets of HSF-1 are predominantly heat shock proteins (HSPs), many of which are chaperones that serve to refold aberrant proteins. If sleep enhances the restoration of proteostasis following heat shock, it may do so by enhancing the transcriptional response to heat shock. We hypothesize that sleep may conserve resources for engagement of a robust and prolonged transcriptional response, thereby facilitating recovery of cellular homeostasis. A potential long term consequence of such a failure to induce robust HSP expression would be a prolonged but sub-optimal expression of HSPs in ALA(-) animals (Fig. 30). To test for this effect we quantified the expression of HSPs following heat shock at both the RNA and protein levels.
Figure 30. Hypothetical graph of differences in HSP expression between wild-type and sleepless animals following heat shock. HSPs transcript levels start to increase upon heat shock and peak some time after heat shock. If sleep enhances the transcriptional response to stress, then the response may be deadened in sleepless ALA(-) animals. Since this response assists the recovery from stress, continued physiological stress may also cause an increased perdurance of these transcripts in these sleepless animals.

It has been shown that hsp-4 mRNA levels peak at about 10-fold after 60 minutes into a 33°C heat shock, though mRNA levels start to decline by 90 minutes after the start of heat shock (Rodrigues et al., 2011). We performed RT-qPCR of hsp-4 at 9 and 12 hours after a 30 minute 37°C heat shock. We observe only a 3-fold increase in hsp-4 mRNA levels in response to heat shock and we observe no significant difference between wild-type and ceh-17 mutant animals (Fig. 31A). This result indicates that sleep does not enhance transcription of hsp-4. The lower induction in our assay than previously observed, 3-fold vs. 10-fold, suggests that peak expression of HSP-4 occurs earlier after heat shock than we examined. If this is the case we might expect to detect any differences in the perdurance of expression between wild-type and ceh-17 animals. However, we do not detect sustained expression of hsp-4 at 9 or even at 12 hours after a 37°C heat shock (Fig. 31A).

We also examined the transcription of hsp-16.11 following heat shock. HSP-16.11 has been found to be induced 100-fold after a 30 minute, 32°C heat shock (Ben-Zvi et al., 2009). We performed RT-qPCR of hsp-16.11 following a 30 minute, 37°C heat shock. Consistent with previous observations we see a 64-fold induction of hsp-16.11 9 hours after heat shock. 12 hours after heat shock there is still a 24-fold induction of hsp-16.11 (Fig. 31B). We do not find that ceh-17 animals have a significantly lower level of hsp-16.11 mRNA than wild type (Fig. 31B). Though none of the differences are statistically significant, the qPCR data taken together may indicate a trend with sleepless animals having a diminished capacity to upregulate transcription of a subset of HSPs. We might expect the induction of expression following heat shock to be greater
than previously observed, given the greater temperature of the heat shock here (37°C) than in the publication (32°C). We attribute this discrepancy to other differences in our experimental procedure such as the greater sampling time after heat shock and as cooling plates on ice subsequent to heat shock treatment.

**Transcription of HSPs is not significantly enhanced in ALA(-) animals**

As described above, we predict that sleepless mutants may have a lower peak expression of HSPs, but a longer perdurance due to sustained defects in proteostasis. Consistent with a longer perdurance of HSP expression, our lab has observed a small but significant increase in *hsp-16.2:gfp* and *hsp-4:gfp* expression in *ceh-17* mutants relative to wild-type at 24 hours after heat shock (Hill *et al.*, 2014). However, we wished to examine earlier time points to determine whether the peak of HSP expression was indeed reduced as predicted by our model. We therefore examined a time course of reporter expression following heat shock. We found the greatest induction to be around 9 hours after heat shock, but saw no significant difference between wild type and *ceh-17* mutant animals (Fig. 31C).

Thus our data do not support the model in which sleep promotes a maximally efficient transcriptional response to cellular stress. It is possible, however, that sleep does enhance the transcriptional response of many chaperones but to such a small degree for each gene such that our assays were not sensitive enough to detect the change. In total such small changes may be enough to protect the animal and explain enhanced survival. Or, perhaps quiescence enhances many different systems that together may be sufficient to explain the survival defect in *ceh-17* animals. Finally, it could be true that sleep is serving a different purpose, perhaps conserving energy for post-transcriptional mechanisms such as chaperone activity.
Figure 31. There is no significant difference in HSP expression. RNA was prepared for RT qPCR at the indicated times after a 30 minute 37°C heat shock. (A) qPCR analysis of *hsp-4* mRNA. Though a 3-fold induction of *hsp-4* was detected relative to non-heat shocked controls, no significant difference in *hsp-4* mRNA induction is seen between wild type and *ceh-17* mutants. (B) A greater induction of *hsp-16.1* mRNA was detected following heat shock relative to non-heat-shocked controls, and *ceh-17(np1)* animals show reduced induction compared to wild type, but this difference was not significant. White numbers within bars indicate the number of biological replicates. Each of these biological replicates represents three technical replicates. *P* >0.05 student's t-test. (C) Fold induction in expression of an *hsp-16p:gfp* reporter following a 30 minute 37°C heat shock. No significant difference in reporter expression was detected between wild type and *ceh-17* mutants. Fold induction was determined by quantifying relative fluorescence intensity between heat shocked and non-heat-shocked, age-matched animals. Bars represent standard error.
Methods

Strains
Strains were raised at 20°C on NGM plates with *E. coli* OP50. The following strains were obtained from the *Caenorhabditis* Genetics Center (CGC) unless otherwise noted:

**WILD TYPE STRAINS**

*Caenorhabditis elegans* Bristol N2

*Pristionchus pacificus* PS312 (Gift from Ray Hong)

**MUTANT STRAINS**

CB120 *unc-4(e120) II*

CB234 *unc-18(e234) X*

CF1038 *daf-16(mu86) I*

DR68 *unc-31(m68) IV*

FG125 *ocr-2(ak47) osm-9(ky10) IV; ocr-1(ak46) V*

FK129 *tax-4(ks11) III*

FK134 *ttx-3(ks5) X*

FK311 *ceh-36(ks86) X*

IB16 *ceh-17(np1) I*

IK130 *pkc-1(nj3) V*

IK597 *gcy-23(nj37) gcy-8(oy44) gcy-18(nj38) IV*

IK800 *gcy-8(oy44) IV*

KP2018 *egl-21(n476) IV*

MT150 *egl-3(n150) V*

MT2495 *lin-15B(n744) X*

MT7929 *unc-13(e51) I*

MT8189 *lin-15B&lin-15A(n756) X*

OH161 *ttx-3(ot22) X*

OH8 *ttx-3(mg158) X*

OH9331 *ttx-3(ot358) X*

PR767 *ttx-1(p767) V*

PS3551 *hsf-1(sy441) I*
TB528 ceh-14(ch3) X
VC2324 flp-6 (ok3056) V This allele is a deletion of most of nhr-38 and a neighboring gene. (Provided by the CGC and made by the C. elegans Reverse Genetics Core Facility at the University of British Columbia, which is part of the international C. elegans Gene Knockout Consortium)

**STRAINS WITH EXTRA CHROMOSOMAL ARRAYS AND INSERTIONS**

AM138 rmIs130[unc-54p:Q24::YFP] II
AM140 rmIs132[unc-54p:Q35::YFP] I
CL2070 hsGFP(dvls70)
CVB5 ceh-17(np1) I; rmIs130[unc-54p:Q24::YFP] II
CVB7 ceh-17(np1) I; zSi3001[hsp-16.2p:GFP:unc-54 + Cbr-unc-119(+)] II
IK0982 hsf-1(sy441); njEx393[myo-3p:hsf-1cDNA, ges-1p:nls-gfp]
   (Gift from Ikue Mori)
IK0983 hsf-1(sy441); njEx394[ges-1p:hsf-1cDNA, ges-1p:nls-gfp]
   (Gift from Ikue Mori)
IK0984 hsf-1(sy441); njEx395[unc-14p:hsf-1cDNA, ges-1p:nls-gfp]
   (Gift from Ikue Mori)
OS3062 hsf-1(sy441) I; nsEx1730[myo-2p:hsf-1 + hsp-16-2::GFP + hsp-16-41::GFP + (pRF4) rol-6]
PS302 let-23(sy10) unc-4(e120)/mnC1 dpy-10(e128) unc-52(e444) II
PS4886 plc-3(tm1340)/mIn1[mIs14 dpy-10(e128)] II (tm130 is a knockout allele generated by the National BioResource Project, Tokyo, Japan, which is part of the International C. elegans Gene Knockout Consortium)
PY1283 ttx-1(oy29) oyIs17[gcy-8p:GFP + lin-15(+)] V
TJ3001 zSi3001[hsp-16.2p:GFP:unc-54 + Cbr-unc-119(+)] II
TU3111 uIs60[unc-119p:YFP + unc-119p:sid-1]
Incubation methods

To test different media for heat shock, *i.e.* water, beads and air, a bath of water and a bath of beads (Lab Armor) were kept in a 37°C incubator. Agar plates containing young adult animals were placed agar side down in the respective baths or directly on the shelf. ≥20 animals were scored for quiescence as absence pharyngeal pumping in each trial. Water was chosen as the media for heat shock and, in subsequent treatments, we used a circulating waterbath.

Egg-laying

≥10 adult animals ~24hours after the L4–adult molt were moved to fresh plates. Animals were given 30–60min to recover from being moved. All the eggs on the plate were counted at the beginning of the experiment. To determine the number of eggs laid in a given interval, the number of eggs found at a given time was subtracted from the number of eggs found at the next time point.

Temperature to initiate quiescence

Animals were observed on a heated microscope stage made from a modified Motic microscope in which the bulb was directly under the stage. The temperature of the plate was measured using a generic thermocouple device with temperature sensing probes inserted directly into the agar. Animals were constrained to the field of view using a copper corral made in-house from copper sheet metal. 10–20 animals were scored for quiescence as indicated by an absence pharyngeal pumping.

RT qPCR

RNA was extracted in duplicate from synchronous populations of whole animals either 2 hours after a 30min 37°C heat shock or without heat shock. Animals were lysed in trizol and then RNA was extracted using a column extraction kit following the manufacturer's instructions (GeneJet RNA). Samples in any given trial were normalized to equal nucleic acid concentrations as determined using a Nanodrop spectrophotometer. The RNA was then treated with DNase I following manufacturer's instructions (Promega). A reverse transcriptase reaction was then performed according to the instructions (iScript RT). The resulting cDNA was then used as template in qPCR using the SYBR green master mix (Bio-Rad) and the reaction was quantified using a Bio-Rad CFX96 thermocycler.
qPCR calculations

Expression analysis was performed using the accompanying software package to the Bio-Rad CFX 96 thermocycler. This quantification of expression used the ΔΔC\textunderscore T method. For quantification of *hsp-4* and *hsp-16.11* expression, a combined average of *tba-1* and *ama-1* were used as a reference. For all other experiments *tba-1* was used as a reference gene. Expression of experimental samples was then normalized to similarly staged but non-heat-shocked animals.

RT semiquantitative PCR

RNA was extracted in duplicate from synchronous populations of whole animals either 2 hours after a 30 minute 37°C heat shock or without heat shock. For analysis of worm heads, animals were dissected using a 26 gauge needle and ≥20 heads were placed directly into trizol. Animals were lysed in trizol and then RNA was extracted by the phase separation method following the protocol in Appendix 2. The reverse transcriptase reaction was performed according to the instructions (iScript RT). The resulting cDNA was used in 20\(\mu\)l PCR reactions with DreamTaq master mix (Life Technologies).

Analysis of locomotion in *Pristionchus pacificus*

Plates with thinly seeded lawns of OP50 were made by spinning down 1ml stationary phase cultures and removing the supernatant. Bacteria were resuspended in 100\(\mu\)l of culture media and spread on plates. 2–4 hours later 50 animals were placed on these plates and locomotion was captured using the Goodman tracker described in Chapter I. The tracking algorithm had been updated since writing Chapter I and the new algorithm was used.

35°C/37°C heat shock

Except where noted, all heat shocks were delivered to well-fed young adult worms by placing animals onto 12ml NG plates in a 35°C/37°C circulating water bath for 30 minutes. After heat shock, plates were rapidly cooled to room temperature by placing on ice for 2 minutes, then moved to a stereomicroscope for scoring of heat-regulated behaviors. For all time points, the lid was removed before scoring because condensation obscures the animals.
**Quiescence assays**

Quiescence was assayed as a cessation of movement of the pharyngeal grinder. Scoring of feeding behavior was 'all-or-none'; hence if any pharyngeal pumping was observed the animal was scored as recovered. Animals outside of the bacterial lawn were ignored, as feeding behavior may be reduced in absence of food. Means were calculated from a minimum of 20 worms per trial, with a minimum of 3 trials. At the suggestion of a reviewer, an effort was made to increase the rigor of our quiescence assay by only scoring an animal as quiescent if it both ceased movement of the pharyngeal grinder and locomotion. The more rigorous assay applies only to more recent data sets and is indicated as such in the figure legends.

**RNA interference**

RNAi was performed by feeding as described previously (Klamath et al., 2000). Specifically, *E. coli* was used to express double stranded RNA under the control of the *lacZ* promoter. Clones specific to the genes of interest were selected from the Vidal or Ahringer libraries (see Table 5). These clones were grown in LB broth with 0.6mg/ml carbenicilin at 37ºC for 8 hours. 2mL of these cultures were spun down, and the bacteria plated on NGM plates with 0.6mg/ml carbenicilin and 1mM IPTG. These plates were incubated overnight at 37ºC, then for a further 1-2 days at room temperature (~23ºC). RNAi sensitive animals were then synchronized by bleaching, and eggs were put on RNAi plates. These animals were cultured on these plates at 20ºC and then young adult animals were transferred to new plates just prior to heat shock.

Table 6. *E. coli* strains used for RNAi. The Vidal RNAi library was obtained from Open Biosystems and the Ahringer library from Source BioScience.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Source</th>
<th>Clone name (WS112)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gfp</td>
<td>A gift from the Sternberg lab</td>
<td>—</td>
</tr>
<tr>
<td>adm-4</td>
<td>Vidal RNAi Library</td>
<td>ZK154.7</td>
</tr>
<tr>
<td>hsf-1</td>
<td>Ahringer RNAi Library</td>
<td>Y53C10A</td>
</tr>
<tr>
<td>daf-16</td>
<td>Vidal RNAi Library</td>
<td>R13H8.1</td>
</tr>
</tbody>
</table>

**Double heat shock**

Two groups of young adult animals were staged. Animals to be pre-conditioned were administered a standard 35ºC heat shock as described above, the other group remained at room temperature. 2 hours after the first heat shock both groups were subjected to an identical 35ºC heat shock.
Age
To obtain animals of specific ages, young adult animals were selected within 6 hours of the L4-adult molt. Selection occurred at the specified time intervals preceding the experiment so that all animals were administered the same heat shock at the same time.

Larvae
Larvae were selected by visual identification of the stages. As mentioned in the text, a significant portion of animals may be progressing into lethargus. In the future, larvae could be staged at specific time intervals from hatching to avoid this issue.

Identifying putative transcriptional regulatory sites
To identify potential heat-shock responsive elements controlling flp-13 expression, I examined a 3.6 kb region including flp-13 plus 2.2 kb of upstream sequence for HSE and HSAS motifs. Ideally, a position-specific weighted scoring matrix for each motif would be used to identify regulatory elements, but such a matrix has not been published. Instead I used a combination of consensus sequences and close variations (one mismatch allowed) based on sequence logos. To accomplish this I used the online tool DNA PATTERN FIND, available at http://www.bioinformatics.org/sms2/dna_pattern.html. From these candidates, I accepted as putative regulatory elements only those for which the sequence contained no more than one mismatch from the consensus sequence. The heat shock element, or HSE, consensus sequence is TTCTAGAA, and the alternatives indicated by the sequence logo at each nucleotide position (in brackets) are [TAG][TA][CG][TC][AC][GC][AG][AG] (GuhaThakurta et al., 2002). The heat-shock associated site, or HSAS consensus sequence is GGGTGTC, and the alternatives indicated at each nucleotide position are [GT][GA]GT[GTC][TAG][CT] (GuhaThakurta et al., 2002).

In order to validate my approach, I wanted to ensure that it would neither identify spurious sites, nor miss too many possible regulatory elements. I tested my method against the housekeeping gene tba-1, which is not expected to change expression following heat shock. No heat shock inducible motifs would be expected for this gene. Consistent with this, no sequences matching my definition for putative HSE or HSAS regulatory elements were found in the 1.8 kb of sequence
representing \textit{tba-1} itself and 1.2 kb of upstream sequence. On the other hand if I use only the logo sequence information (\textit{i.e.} allowing more than one mismatch from the consensus sequence), I find comparable numbers of putative motifs for \textit{tba-1} and \textit{flp-13}, indicating that secondary filtering is indeed necessary to identify biologically relevant sites. As a positive control, I also tested F08G2.5, which is a gene of unknown function that is induced by heat shock. I found two HSEs and three HSASs in 2.5 kb of sequence, consistent with previous observations (GuhaThakurta et al., 2002). Thus my approach appears to be sufficiently discriminate to avoid spurious hits and still detect potentially meaningful sequences.

\textbf{Fluorescence microscopy}

Quantification of relative fluorescence intensity and imaging of aggregation were performed using a Zeiss Axio Imager A2. Worms were placed in M9 solution on 1\% agarose pads and immobilized with \textasciitilde1\,\mu M levamisole. Images were captured using Zen software (2011 blue edition).

\textbf{Relative fluorescence intensity used to determine changes in gene expression}

Heat shock evoked the strongest induction of \textit{hsp-16.2p:gfp} reporter expression in nuclei of cells within the foregut, hindgut and spermatheca. We chose to quantify fluorescence of these nuclei. In any given experiment all images were captured as described above, at the same exposure and brightness. ImageJ was used to determine relative fluorescence intensity by drawing a circle around the foremost intestinal nucleus of each animal and obtaining the average pixel intensity. These values were normalized to those of non-heat-shocked animals of the same strain to determine fold change in GFP expression.

\textbf{Aggregation}

Imaging was performed as described above. polyQ::YFP reporters show diffuse fluorescence in the absence of aggregation. With the onset of aggregation, bright granular puncta form in the tissue. We quantified aggregation by counting the number of animals containing any puncta (the number of puncta were not scored?)
**Survival**
An animal was defined as dead if it failed to show any movement, even after a strong stimulus to the head, tail and midbody. If an animal showed any movement it was counted as alive. Dead animals were removed from plates and the fraction of animals that had died out of the total was tracked each day. Animals that were unaccounted for (i.e. animals that may have crawled off the plate and did not die as a result of the experiment) were excluded. When necessary, surviving parents were transferred to new plates to avoid confusion with progeny.

**Statistics**
Prism software (Graph Pad) was used to calculate statistics and create figures. Significance was determined using a Fisher’s exact test for pooled quiescence data, and a student’s t-test for paired samples. One-way ANOVA was used for experiments with more than two groups, two-way ANOVA with repeated measures was used for experiments with time courses for more than two groups. The log rank test was used to compare survival.
References


Appendix A: Supplementary Data

Enhanced sleep in RNAi hypersensitive unc-119p::sid-1 lin-15B animals

Our RNAi hypersensitive strain TU3335 showed enhanced quiescence relative to wild type (Fig. 32A). TU3335 contains a lin-15B loss-of-function mutation that enhances the effectiveness of RNAi (Calixto et al., 2010). lin-15B is also known to repress EGF/Ras signaling, presumably by down regulating expression of some signaling component (Davidson et al., 2010). Since EGF signaling is required for stress-induced sleep, the derepression of EGF signaling in a lin-15B mutant background may explain the increased propensity for these animals to sleep. To test this possibility, we examined lin-15B mutants to see if the enhanced sleep phenotype was replicated. At the same time we examined lin-15B&lin-15A mutants that lack the genes for two redundantly acting pathways regulating EGF signaling (Davidson et al., 2010). Surprisingly we are unable to detect the same enhanced sleep phenotype in either of these strains (Fig. 32B). Neuronal tissue in wild-type animals is resistant to RNAi. The strain TU3335 contains a double stranded RNA transporter expressed ectopically under a neuronal promoter, unc-119p::sid-1, allowing RNAi to function in neuronal tissues. Thus we also examined unc-119p::sid-1 animals to see if this could explain the phenotype. We did not observe an enhanced quiescence phenotype in this strain (Fig. 32B). We therefore attribute the enhanced sleep phenotype seen in TU3335s to an unknown allelic variant in the background of the strain.
Figure 32. The RNAi sensitive strain TU3335 sleep response is different from wild type. (A) Quiescence behavior of wild type animals and the RNAi sensitive strain following a 30 minute 36°C heat shock. (B) Quiescence behavior of wild type and mutant strains following a 37°C heat shock. Quiescence behavior was scored as the fraction of worms that were neither feeding nor moving, n=1.

**Knockdown of the stress response by RNAi**

We have shown that thermosensation by the main thermosensory neurons is not required for heat-induced sleep, suggesting that sleep may be triggered by detection of heat-induced protein unfolding. If this is the case, then animals that are impaired in their ability to maintain proteostasis are expected to show exaggerated sleep responses. Many of the effects of heat shock can be attributed to a disruption of normal protein structure, as most proteins are optimized to fold and function in a narrow physiological range (reviewed by Richter et al., 2010). The presence of unfolded proteins activates the HSR, a conserved transcriptional response that is engaged to restore normal cellular homeostasis. The master transcriptional regulators of the heat shock response in *C. elegans* are heat shock factor 1 (HSF-1) and DAF-16/FOXO (reviewed by Rodriguez et al., 2013), and animals harboring mutations in these factors experience decreased survival (reviewed by Åkerfelt et al., 2007). Our lab has shown that in response to a 37°C heat shock, *hsf-1(sy441)* reduction-of-function and *daf-16(mu86)* null mutant animals exhibit an initial bout of quiescence that is similar to wild type, and a second bout that shows a decreased peak quiescence relative to wild type but of longer duration. One interpretation of this result is that some of the genes required for sleep are under the control of these transcription factors. Animals with reduction-of-function mutations in another stress-responsive transcription factor DAF-16 were also found to exhibit enhanced quiescence (Hill et al., 2014). As each of these results were
obtained using a single mutant allele of each gene, we wished to confirm this result by knocking down the expression of these genes by RNA interference (RNAi).

Double-stranded RNA was administered by the feeding method (Timmons, 2001) and RNAi against gfp was used as a negative control. adm-4i, expected to show impaired quiescence, was used to confirm that we could detect a sleep phenotype. We observed hsf-1i and daf-16i animals to determine whether they exhibit prolonged sleep following heat shock and found enhanced quiescence with hsf-1i at 9–12 hours after heat shock, consistent with our previous mutant analysis (Fig. 33). However, we observed that at 1 hour following heat shock, hsf-1i is less quiescent than the gfp control, an effect not seen with the hsf-1(sy441) reduction-of-function mutant animals which are as or more quiescent than wild-type at this time point (Fig. 19C, Hill et al., 2014). Since the sleep phenotype of gfp does not recapitulate that of wild type, we suspect attribute this difference primarily to changes in the control. To try and address understand this effect we also investigated the effect of mutations in the RNAi strain we were using, but could not attribute this difference to any one known genetic difference (see chapter II). We did not observe enhanced quiescence with daf-16i and hsp-4i (Fig. 33). The sleep phenotype is much less pronounced with RNAi than in daf-16(mu86) reduction of function mutants (Hill et al., 2014), and RNAi may not be sufficient to produce this phenotype for daf-16. In fact these animals show less sleep than the gfp control. Similar to HSF-1, this result could be due to a difference between the sleep behavior of gfp control and wild type.

In the future N2 alone may be more effective to test these phenotypes. However, as neurons in wild type C. elegans are particularly resistant to RNAi, any phenotype that occurs due to an effect on neurons may not be detected by this method.
Figure 33. Feeding RNAi was performed against stress-response genes *hsf-1* and *daf-16*, and against *gfp* as a control. Fraction of animals quiescent at various time intervals after a 30 minute 37°C heat shock. Animals that were neither moving nor feeding were scored as quiescent. (A) 1 hours after heat shock. (B) 9 hours after heat shock (C) 12 hours after heat shock. *P*<0.05, Fisher’s Exact test, n=4. (D) The complete time course for all genes, including the *adm-4i* negative control (Data in A–C was taken from the set shown here). Enhanced quiescence was not detected at other time points. *P*<0.05, Fisher’s Exact test *gfp* vs. *hsf-1i*, error bars represent standard error.
Appendix B: Using the Goodman Lab Multiworm Tracker

Introduction
The Multiworm Tracker was originally developed by David Ramot from the Goodman lab at Stanford university. The version used here has been substantially modified from his original. The aims of these changes are to improve usability and to add features to analyze quiescence. Considerable assistance in the initial setup was given by Chris Cronin at Caltech, and Bayan Parsa at CSUN assisted in writing a more rigorously tested quiescence algorithm. For my specific contributions, and for detailed information on the design and function of the Multiworm Tracker, see Chapter I. This chapter also explains the principals behind the core algorithms of the tracker and may provide a useful background.

Key Requirements
- MATLAB® and the MATLAB® Image Processing Toolbox™
  - This software requires a license and add-on license
- A DCAM compatible camera (usually firewire/1394)
  - These include the Unibrain camera that we use and many others. See the IEEE1394 Camera List by Damien Duchamps for other compatible cameras.
- A C-mount or similar lens-to-microscope adapter
- If possible, a microscope where the brightness is evenly distributed is strongly recommended.
  - Many binocular scopes use a light path designed for the right or left eye. This light path is at an angle to the single beam of light from the light source, resulting in a gradient in brightness across the image taken by the camera. This gradient becomes worse at lower magnifications.
Setting up the Multiworm tracker software and hardware for the first time

The following list gives you the basic steps needed to set up the tracker. Once your microscope and camera are set up, you will need MatLab to recognize the camera. You will also need to tell MatLab where to find the Multiworm tracker code. Additional steps may be necessary, beyond those steps described below.

I. Assemble your microscope, firewire camera and computer.
   1. We used a Mac, but any Windows or Linux computer should work. Some troubleshooting will be necessary on these other systems.
   2. Don’t forget that you need a license for MatLab and its Image Acquisition Toolbox.
   3. Attach the camera to the microscope, you may need to find an adapter.

II. Setting up the software.
   1. First MatLab needs to know where to find the code. Find the file “createPaths.m” and drag it into the MatLab command window. If successful, a message should appear.
   2. Now close and reopen MatLab. You should have a Worm Tracker window.

III. Link the hardware to the software.
   1. Make sure the camera is selected on the microscope (on the Leica S6D a dial with “DOC” and “VIS” toggles the camera and eyepiece). Make sure the microscope light is on and perhaps put a specimen on the stage.
   2. Go to START (a button in the bottom left corner of the MatLab window) → TOOLBOXES → IMAGE ACQUISITION.
   3. In the window that appears click “Start Preview”.
   4. In the “Hardware Browser” panel, click on the entries (video formats) in the list until you find a working mode. Make sure the light is not too bright!
5. Once you find a working video format, enter this into the WormTrackerPreferences.xls file. You can use the button shown below:

   a. Type the entry exactly as seen (excluding any final asterisk) in the Excel preferences file on the “Video Capture Prefs” sheet.
   b. You also need the device name; this is name in parentheses at the top of the list. Enter this in the same worksheet.

6. Close, reopen, and check that the camera is connected using Preview.

IV. Finally calibrate the tracker.

   1. Take an object of known size (say a ruler) to determine how many pixels make up a mm in the tracker videos. Use 1x magnification. Go to the “Worm tracker preferences file” again and enter this conversion factor into the “Analysis Prefs” worksheet.
Notes about the worm tracker preferences file

Though features can be changed on the fly through the software interface, almost all features of the tracker can be permanently adjusted through the preferences file. A brief walkthrough of the preferences file follows. The most useful settings have been moved to worksheet number 6.

There are 6 worksheets:

1. Camera Prefs
   • The settings here are for the camera. Many do not have an effect on the latest firewire cameras.

2. Video Capture Prefs
   • The settings here affect the video recording and defaults. Go to worksheet 6 first.

3. Tracker Prefs
   • The values here determine the performance of the tracker. These values include those that determine the size of a nematode at a given magnification (using worksheet 5)

4. Analysis Prefs
   • The values here are also used to track nematodes. The most important is the value for pixel size.

5. Reference
   • These values determine what limits to place on a tracker object to determine if it is a worm. This sheet is used as a reference by “Tracker Prefs” to allow for use of different magnifications. These have been tested at 1x and 2x, but the other values are interpolated.

6. Useful settings
   • Useful defaults for the tracker are located here.
   • The objective magnification used must be entered here to tell the tracker what settings to use.
Plate setup
Thin lawns work best for tracking. The tracker cannot distinguish large tracks in the bacteria from actual worms. One method is to seed plates a few hours before tracking. Spread the bacteria at least enough to avoid creating an edge in the video frame. Avoid plates that were seeded more than a day before tracking. Note that the thickness of lawns can affect the rate that nematodes move.

An unavoidable factor that affects locomotion is moving nematodes to plates. Nematodes move faster after being transferred, and this effect can last 20 minutes or more. Any experiment should account for this effect.

Microscope setup
The following was written using a Leica S6D microscope with a double-sided mirror below the stage. Many of the following steps would be altered when using different microscopes. The goal is to get an even image with high contrast between the worms and background.

- Slide mirror all the way back, away from you
- Set the light intensity to about 4.25 (and then adjust as necessary)
- Zoom to the desired magnification on the dial.
- You will need to tell the tracker what magnification you chose above. Click the “Quit and edit settings” button.
  - At the time of writing suitable values have been determined for 1.0x and 2.0x
  - For other magnifications values have been interpolated.
  - This setting must ultimately match that shown in the tracker window. More on this under Recording Video.

- Advance the angle slightly to balance the contrast between bright white nematodes, and obscure nematode tracks in the media
Recording video

Now you are ready to start recording, open MatLab.

As you have probably already found out, the tracker opens automatically; give it a minute to start. After MatLab and the tracker itself have loaded, the tracker window appears.

First make sure the dial magnification you wish to use on the Leica scope is the same number as that shown in green. If not use the “Quit and edit settings” button below to change it.
The number to change is the first number of the “Useful settings” worksheet. Make sure to save the excel file before reopening the tracker. To reopen the tracker, you will need to type “WormTracker” in the command window, or restart MatLab.
The Video Capture window requires you to enter in some parameters. First where to save files, and what to call them.
Choose whether you want a single video, or multiple videos. For either, enter the length of video. Multiple video mode is useful for collecting tracking data from a single plate over an extended period of time. The interval is the time between the start of each video. The duration is how long the video capture goes on for. A video is captured at the very beginning and very end, so in the above right example the last video will start at 60 min and end at 70 min.
Video processing and analysis options

Choose the video processing options that you wish to use:

- “Crop video” removes the part of the video with the greatest aberration in background brightness. The effect of this function can be seen with “Preview Video”, though the existing preview must be restarted.

- “Background correction” is a function to correct uneven brightness in the video at particular magnifications. The effect of this function can be seen with “Preview Video”.

- “Peg threshold now” presets the threshold value so that the results of thresholding can be seen and controlled before video capture starts. This step ensures that video quality will be acceptable to accurately track nematodes. The effect of this function can be seen with “Preview Video”.

- “Analyze after capture” will run tracking functions on the video immediately after capture. This creates a .mat file containing data you will need for final analysis. If capturing multiple videos, be careful that there is sufficient time between videos for processing. Video analysis can take a substantial amount of time and cannot be interrupted. If you are recording videos at high frequency you may wish to reformat this step manually later (use the menu with Step 1 and 2 mentioned above).
Preview and record

• “Preview video” allows you to see what the camera will record, and shows the effects of processing options (above).
  o The image shown is will become video during recording.
  o The green numbers in the bottom left corner show the computer’s time to indicate whether the preview is still active.
  o “Take a picture” will store the current frame as a .png image. A prompt will ask where to save the image.
  o “Close” will exit the preview.

• “Record Video” closes preview and starts the video recording sequence.
Optimizing video

When capturing video the most important thing to have is an even white background with distinct nematodes. The preview window is an indispensable too to accomplish this—It lets you see what the video will be like. To correct the image, adjust the lamp intensity and the mirror position on the microscope. The video processing functions explained above also help optimize video quality. Examples of acceptable and unacceptable videos are shown here.

Preview window with acceptable video quality. This preview window is using “peg threshold”. Background aberrations are clearly visible, but are be smaller than nematodes and will be excluded during processing.

An ideal image with a clear white background—no aberrations.

Acceptable image with a thicker lawn of bacteria — there will be only small background aberrations, similar to the example above.

Acceptable image with a thicker lawn of bacteria.
Menu options
At the top of the screen several functions are available in a drop-down menu.

Video processing options allow you to prepare raw video for the tracker. If you allow the tracker to process video automatically you don’t need these options. The following three functions are made available as options to reprocess video if desired.

It is likely that you might need to call the tracker algorithms after video capture. To do this Use the menu options under “Worm tracker core functions” labeled “Step 1” and “Step 2” in order.
Nematode behavior analysis gives access to the same functions as the buttons in the tracker window. In detail:

- **Quiescence analysis (Inst_Speed_param)** is an algorithm to calculate the portion of quiescent nematodes in a video.
- **WormSpeeds** will show the mean speed from selected .mat files.
- **Draw tracks** creates an annotated .avi video with the tracks drawn over the video. This video is useful as a check that the tracker worked as expected for your video.

**Old functions** opens an older analysis GUI. This feature is considered outdated, but may be useful in some circumstances and thus has been placed here.
Troubleshooting

LICENSE MANAGER ERROR:

If the computer has just booted, wait a minute or two till it has time to connect to the internet via the Ethernet cable. — Without this connection MatLab license manager will fail. Make sure MatLab to closes completely. Look at the icon in the dock, if it has a white dot next to it, MatLab is still open. Try again when the computer has connected.

If the above solution does not work, contact the IT department and have them troubleshoot the problem. They may need to restart their license server.
MATLAB OPENS, BUT THE TRACKER SOFTWARE DOES NOT:

Locate the MatLab “Command Window”, type VideoCapture and press ENTER. If you get the error “UNDEFINED FUNCTION OR VARIABLE ‘VIDEOCAPTURE’.” Make sure the tracker files are on the computer and there is a file under /Users/CURRENT_USER/MatLab/startup.m. If you open this file with a text editor, it should contain the paths to all tracker functions.
“OBJ HAS ALREADY STARTED ERROR” WHEN CAPTURING MULTIPLE VIDEOS.

This particular error presents the following output:

```
Beginning recording | video_n1.avi | 12:55:43
Recording: 00:00:14 Error while evaluating TimerFcn for timer 'timer-1'
OBJ has already been started.

[1x26]
Finished recording | video_n1.avi | 12:55:57
FramesAcquired = 60

Error using VideoReader.getFullPathName (line 390)
The filename specified was not found in the MATLAB path.

Error in VideoReader/init (line 429)
    FullName = VideoReader.getFullPathName(fileName);

Error in VideoReader (line 132)
    obj.init(fileName);

Error in trimVideo (line 137)
    themov = VideoReader(NAME);

Error in StopVideoCapture (line 60)
    trimVideo(MovieName);
```

If the line “OBJ has already started” appears at the beginning of the error This indicates that analysis takes longer than the time between videos. Try to reduce analysis time (uncheck some of the “Analysis and editing options”). Reduce the length of the video itself. Or, increase the time between videos.
**SINGLE VIDEO “OBJ HAS ALREADY STARTED” ERROR.**

The same error as above may appear if you try to start video capture before the analysis for the previous video has completed. To avoid errors, wait for the “Video analysis complete” window to appear before starting a new video recording. This window will automatically close, but you can confirm by looking for two rows of dotted lines in the command window too.
NO IMAGE IS DISPLAYED:

1. Check the microscope shutter
2. Check MatLab Image Acquisition Toolbox. If 3 camera show up with various formats, and the macvideo format works but dcam format F7_Y8_656x488_mode0 doesn’t, close MatLab, unplug the camera, and start over.

TIMER NOT FUNCTIONING CORRECTLY:

At the time of writing there are known issues with the graphical timer. These issues do not affect video capture and tracking.
Further reading

http://damien.douxchamps.net/ieee1394/cameras/


Appendix C: Bio-Rad qPCR Software Guide

Introduction
This guide was written for the Bio-Rad CFX96 Thermocycler with the Bio-Rad CFX Manager 3.1 software.

The Bio-Rad CFX96 is a thermocycler with real-time fluorescence detection. Samples can be quantified either with a single probe like SYBR green or FAM, or using up to five TaqMan probes.

The machine requires low profile reaction tubes, compatible plastics available from Bio-Rad CFX96 website under “plastics”.

If you require more specific information than this guide provides try the program’s built in help, and Bio-Rad Tech Support on 1(800) 424-6723.

This guide was written with two goals in mind. Principally, to make running reverse transcriptase quantitative PCR, RT-qPCR experiments using the Bio-Rad machine and software straightforward. Also, to help address issues that may be faced in the design of qPCR experiments and focus on the important aspects of data analysis and validation. This guide is a starting point. Any advice given here should be approached critically, with your own experimental goals in mind. Helpful and more comprehensive material, including links to pdfs can be found under 0 Further reading.

1 Before you start
a Checklist

- Before doing any qPCR you will need to decide whether you will be using TaqMan probes or SYBR Green/FAM.
- Decide how you will chose your primers (and potentially probe sequences), see 2d Primer design.
- Make sure you have suitable reference genes to compare your target against! Publications assessing the validity of reference gene are available for many organisms.
- It is a good idea to plan the experiment in advance.
- Plan to do assays with a minimum of 3 biological replicates, and each of these repeated in PCR to at least technical triplicate.

Of course when you are ready, evaluate your experimental design. See 6b Melt curves and 6c Standard curves.
b Terminology

Common symbols used in the Bio-Rad software and what they mean:

- **qPCR**, quantitative PCR. An assay to determine the relative expression of a target gene. This is quantified by the number of cycles of PCR required to produce enough copies of the target to reach a fluorescence threshold.
- **C_{T},** cycle threshold. The number of cycles required to cross the fluorescence threshold. This threshold is a horizontal line set on the “Quantification” graph. Cycle threshold is also referred to as C_{q}, which is the preferred nomenclature for publication.
- **ΔC_T,** relative expression, the expression of the target relative to reference gene(s).
  \[ \Delta C_T = C_T \text{ target} - C_T \text{ reference} \]
- **ΔΔC_T,** normalized expression, the expression of the target relative to reference gene(s) normalized to a control sample.
  \[ \Delta \Delta C_T = \Delta C_T \text{ sample} - \Delta C_T \text{ control} \]
- **E,** the efficiency of the reaction. This is calculated from the results of qPCR with a dilution series of a sample.
  \[ Efficiency = -1 + 10^{-\frac{1}{\text{ΔΔC_q}}} \]
- **R,** the ratio of expression in samples vs controls.
  \[ R = 2^{-(\Delta \Delta C_q \text{ sample} - \Delta \Delta C_q \text{ control})} \]

c Determining the recipe for a reaction

If you haven’t read the product insert for your chosen reagent, do so now. This is essential background on what parameters are appropriate for your PCR reaction. This includes some information about appropriate primers. A link to the BioRad SYBR Green Supermix document is given in **Further Reading.**

d Primer design

There are two ways to find your primers:

1. **Finding published primers in the literature.** Primers found in rigorous publications, should help save you time in finding primers. However, you should still test these first to make sure they work as expected in your hands. The tests and bioinformatics tools below can help.

2. **Designing your own primers.**
   - Amplicon sequence should generally be between 70–150bp, 100bp is ideal.
   - It is helpful but not necessary to have amplicons that span the ends to two exons, so that only the spliced form will be amplified and the effect of gDNA contamination is minimized.
   - The last five 3’ base in your primers should contain at least one but no more than two G+C bases. This is reported to promote specific binding.
• Overall G+C content close to 50-60%. This is for product stability.
• Avoid short repeats and runs of identical bases within the primer sequence.

Test these primers first with bioinformatics tools:
• Test for primer issues via multiple bioinformatics approaches before ordering.
• The most successful approach for me is to find primers using NCBI Primer-BLAST [http://www.ncbi.nlm.nih.gov/tools/primer-blast/]. Entering a genbank accession here allows you to easily find exon-spanning primers. Check your parameters, I use some advanced features including “Thermodynamic Oligo Alignment” and “Thermodynamic Template Alignment”. The thermodynamic algorithms seem to be much better at detecting primer interactions. I look through the hits to prioritize the best candidates and then run them through ThermoScientific’s Multiple Primer analyzer [http://www.thermoscientificbio.com/webtools/multipleprimer/] to screen for primers least likely to form cross- and self-dimers. I prefer primers with no detectable dimers, even when the sensitivity of this tool is set below 3.

Now test with semiquantitative PCR. Make sure you have a band of the expected size. Some DNA at the bottom of the gel, especially in no template controls is not necessarily a concern. Conversely, detectable primer-dimers and non-specific amplification should be addressed.

efficiency — how it is used

To calculate the relative expression of a target gene using qPCR, the $C_T$ of the target is compared to one or more reference genes. There are two possible approaches to this calculation. The simplest is to assume that the reaction takes place with 100% efficiency, and that the quantity of target doubles with each cycle of PCR. The second, and preferred method is to calculate the efficiency. Ideally, efficiency is calculated as part of the validation of your assay, and for every subsequent plate in your PCR assay. However, doing this every time is costly in terms of resources and space on the plate, so it is often omitted. It is up to you to gauge whether you can still persuade your critics of the validity of your conclusions when assuming 100% efficiency.

To calculate efficiency, you start by generating a standard curve. The point at which the threshold of fluorescence is crossed in a qPCR reaction is earlier with higher quantities of starting material. If you know the relation between different samples, you can predict when you expect to cross the threshold in each sample if the reaction is 100% efficient.

Standard curves can be absolute or relative. Absolute curves are generated using a standard of known concentration. Relative curves use a sample where the true starting concentration is unknown. Absolute quantification of RNA cannot be made using measurements from the Nanodrop. Relative curves are the most often used, and will be used in determining efficiency in the PCR reactions here. In both cases a dilution series
of the starting material is made. These are then used in qPCR reactions. As the relative quantity is known, the difference in Ct values can be predicted. A difference of 1 cycle between samples indicates a two-fold difference in the quantity of the target.\(^1\) On a graph of log\(_{10}\)(concentration) against, Ct the slope is expected to be -3.322. Deviation below this indicates a reaction that is less than 100% efficient and vice versa.\(^2\)

Efficiency (E) should be between 90-105%.\(^3\) Lower indicates a problem with the assay. You could address this by several means. Ensure that no template and no reverse transcriptase controls indicate low levels of genomic DNA, and no significant contamination. Use primers that show high specificity for the target, and do not exhibit base pairing with one another. A non- or semi-quantitative PCR should be performed first to avoid these issues. Use melt peak and efficiency analysis to confirm validity primers. Changing annealing temperatures or addressing the presence of PCR inhibitors in the reaction can also correct low efficiency.

There should be 3 dilutions of the sample at minimum, or up to 7 dilutions. Each of these dilutions should then be in triplicate. A pool of all samples is often used to make the initial undiluted stock. The plate setup, if complete, will allow the Bio-Rad software to create the curve for you (see 4e Setting up efficiency curves). The R\(^2\) is a linear regression of the data points. This statistic gives an indication of how close the replicates are to one another, and so is a measure of confidence in the efficiency calculated. An R\(^2\) value of 0.950 or higher indicates sufficient precision in replicates.\(^4\) An R\(^2\) value below this indicates that there is pipetting error in replicates. The replicates should cluster close together, within 1 cycle. If there are individual replicates that are clearly outliers, these can safely be excluded from the analysis. So long as ≥3 dilutions are detected by the assay, dilutions that fall below the detection threshold of the assay can also be excluded. Pipetting errors can also occur in the dilution, and show in the data as skew of samples away from the slope.

Pipetting error can be reduced by:

- Using master mixes wherever possible.
- Pipetting larger volumes, and reducing repeated pipetting of small samples.
- Making sure pipette tips don’t go all the way to the bottom of the vessel.
- Making sure extra sample is not transferred on the outside of tips.

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\(^1\) If one cycle represents a doubling of the quantity of template, then Ct will change as the log base 2 of the difference in quantity. Then, for a 10-fold difference: \(\log_2 10 = 3.322\).

\(^3\) IDT and MIQE state 90–105% as acceptable, whereas Thermo Scientific state that 90–110% is acceptable.

\(^4\) Many sources state that an R\(^2\) ≥ 0.990 is required. However, this may be unnecessary. See PCR Troubleshooting and Optimization. Chapter 8: The MIQE Guidelines Uncloaked. Shipley (2011). My efficiencies have been ~0.98±0.01
• Using multi-channel or serial pipettors to reduce the number of steps necessary.
• Making sure pipettes are calibrated.
2 Getting started

Plug the machine’s USB cable into the laptop.

Turn on the machine with the on/off switch at the back right side.

Important note: The lid of the machine is motorized and must be opened either by pressing the button on the front, or from the software. Do not attempt to lift or lower the lid manually!
Load your plate or samples. A1 is in the top left corner.

Find the Bio Rad software.

Log in.
Click on “user defined”.
3 Protocol

You will almost always “Select an existing” file for your experiment. If this is the first round, you can set up your PCR conditions with “Create New”. Namely these conditions are temperatures and times for initial denaturation, denaturation, annealing, and extension. Don’t forget to include plate reads!

When done click next. This will bring you to the plate setup.
4 Plate setup
a The basics

Again you can load an existing plate setup, or make a new one. This can be done before or after the reaction, and is readily editable. The next part of this guide will show you how to set up a plate for the first time. More information about each of these steps can be found in the program’s own help under “Create a New Plate”. To complete after the reaction, just hit next and follow the prompts; go to 5 Running your PCR experiment.

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For this part of the setup, the software is particular about the order in which you label the contents of a particular well. Use the built in guide to help you (shown below).
Confirm that the settings in the “Settings” drop down menu are correct (plate size = 96, plate type = clear).

b Choosing fluorophores
Select the appropriate scan mode; “SYBR/FAM Only” for SYBR Green or “All Channels” for TaqMan. Follow up by selecting your fluorophores as appropriate.
Then you must define a sample type. The abbreviations NTC stands for no template control and NRT for no reverse transcriptase control.
c Labeling targets

Now label the targets, this label can be the name of the gene. For example, “ama-1”. Check the box after writing the label, not before. The target label will be applied to your wells when you press enter or click the box.
You only have one box for SYBR. If you are doing TaqMan analysis, and you set up fluorophores correctly there will be labels and boxes for each. Fill in for your targets as appropriate.
d  **Labeling samples**

You can now label samples. Something like: “wt untreated”, “wt heat shock”, etc. Check the box afterwards.

e  **Setting up efficiency curves**

“Replicate number” is not necessary for a general experimental setup, but it is required when creating efficiency curves.

To create an efficiency curve, you will need a dilution series. For example a 4 step dilution series $10^0$, $10^{-1}$, $10^{-2}$, and $10^{-3}$.

Replicates in the program case do not refer to strict technical replicates. Make sure you first increase the “Replicate #” to be the number of dilutions $\times$ the number of targets. If you are making standard curves for 2 genes with 4 different dilutions, replicate # is 8.
When you click “replicate series”, the display will change. Here “Replicate size” is the number of technical replicates for each dilution for a dilution series with 4 dilutions done in triplicate, this will be 3. Starting replicate number is one for the first gene. If you are looking at more than one gene, the second gene will start where the first leaves off, in this example it would be 5, then 9, then 13 and so on. Choose the direction your dilutions have been loaded, and click apply.

If you receive the following error message, make sure you have filled in everything it asks for. If you are doing an experiment without standards, make sure you have not labelled your unknowns as standards!
f Marking reference genes

Use “Experiment Settings” to select the reference gene(s). Also exclude the no template control and no reverse transcriptase control for Gene Expression analysis later on. Though this is not necessary if you are not looking at relative expression; when producing a standard curve for instance.
If you make a mistake, or wish to leave wells empty, select them and use “Clear Replicate #” and “Clear Wells”.

**g  Separate data for different targets — Well groups**

Defining well groups helps during data analysis as it allows you to separate particular groups in the experiment. This is most useful when looking at quantification curves, melt peaks and/or standard curves for particular genes.

To use well groups, Press “Add” to create a group, name it, then select all samples you want in the group. It is helpful to do this for all samples with the same target and give them a group.
h Select different colors for targets or samples

You can adjust the colors of your quantifications curves too. Do this from the “Plate editor” window by clicking on the “Trace styles” button.

Or from the “Data analysis” window under the “Settings” menu.
Then select the samples you want to differentiate using the dropdown and select a color.

Alternatively you can select wells to change directly. The color scheme will be shown in this same window.
When you go to data analysis, the color selections enable you to see different groups together in the quantification curves, melt curves, etc.
5 Running your PCR experiment

Complete plate setup, or at a minimum selected which channels you wish to capture during PCR. Then select “Next” to get to “Start Run”. You can control the machine from here with buttons to open the lid, close it, then start the run.

![Start Run setup interface]

Load your samples, the orientation is the same as in the plate setup, A1 is in the top left corner.
6 Analyzing the data

If you set up “Well Groups”, you can look at all the samples at once, or select particular groups. If you wish to make changes, you can do so now by clicking the Well Groups icon.

At the end of the reaction you will in Data Analysis mode. First make sure your data is saved, and take your samples out of the machine.

Different data and analysis are shown under different tabs. I will cover the most pertinent elements below.

a Quantification data

The threshold should be placed where the precision of the replicates is highest. This will be toward the middle of the geometric phase. The software will automatically set the threshold, usually in an appropriate spot, but this can be changed by clicking and dragging the threshold line. Checking the “Log Scale” box can help to find the linear phase, in this situation chose the lowest consistently linear position you can.
Under the “Quantification Data” tab, it is possible to look at the CT values in different formats. Choose the most helpful with the dropdown.
b Melt curves

You can look at melt peaks for your targets in this tab. Ideally you should have a single clear melt peak for each target. Lack of a clear peak is fine in controls where no amplification is expected, for example no reverse transcriptase and no template controls. A double peak in the same reaction indicates a problem with the primer design.
c Standard curves
For help interpreting standard curves see 1e Efficiency — How it is used and 0 Further reading: A practical approach to RT-qPCR and IDT qPCR Application Guide.

When doing an efficiency experiment, the standard curve will show up automatically next to the quantification curves. Individual replicates are plotted on the graph. Efficiency (E) should be between 90-105%. Check your RNA quality on the NanoDrop if you have problems with efficiency but not melt-curves. An R² value of 0.950 or higher indicates sufficient precision in pipetted replicates.

![Image of standard curve graph]

d Custom views
You can set up which data is displayed under the “Custom Views” tab. Select the desired output with the dropdowns.
e Gene expression analysis

In the following section, only relative expression is covered specifically and not absolute expression. If you completed all the necessary steps for the plate setup, this will show up automatically. A message will tell you if you are missing anything. However, if you have unreasonably high error bars, you may have forgotten to exclude the controls from your results. This is done with checkboxes under “experimental settings”. Note: if using software version 3.0 you may have to go back to the plate setup see 4f Marking reference genes.

You are free to choose how you wish to display your data depending on your goals. For fold changes in expression in our lab, we wish to look at normalized expression relative to an untreated control. Unfortunately it is not possible to select different controls for each genotype. One approach is to select one control and copy the relevant values to another program like excel or prism. Select the other control and do the same again. Alternatively you can export all the raw CT values and calculate fold expression yourself.
f Gene expression analysis revisited — Reaction efficiency

If you included standard curves for efficiency calculation on your plate, the efficiency will be automatically included in the calculation. Efficiencies calculated separately can be included manually. Click the “Experiment settings” button and change the value in the “Efficiency” column.
g  **Excluding anomalous data**

In some circumstances it is necessary to exclude a particular well from analysis. Perhaps you know that the well was pipetted incorrectly. Perhaps an anomalous result in the well is indicated by the other two replicates. To exclude such results, click on “Plate Setup”, then “View/Edit Plate”.

![Diagram showing plate setup and view/edit plate options](image_url)
Select the problematic well(s), then check the box to “Exclude wells in analysis”.

**Statistics — Technical and biological replicates**

The statistics you use are dependent on your experimental design. However, you should not treat technical replicates and biological replicates equally. Technical replicates allow you to increase the precision of PCR results. These combined results form one of your
biological replicates to be used in the statistical test for your biological hypothesis. Statistics provided by the software are usually based on the technical replication in the plate. Thus these values may not be appropriate for assessing biological variability. Talk to your advisor if you are unsure what this means.

A last note about the BioRad software and statistics: to combine all your data from different experiments, it is easiest to export the final gene expression values. You can then work on the statistics to test your biological hypothesis with your favorite software. Assistance with exporting data can be found in the next section.
7 Exporting data
a Copy/Paste

Any tables viewed in “Data analysis” windows can be copied to spreadsheets, and it is often best to select what you want and copy/paste it.

Any graphs can be copied to an office document. On some computers you can drag the green arrow to you destination document. On other computers you need to right click the graph and select “copy”.

![Diagram of green arrow pointing to graph]
b Export menu

Whole datasets can also be exported using the export menu. “Custom export” gives you the greatest control over what to export. The other options allow you to quickly export large portions of data. **The currently selected well group will dictate what is exported.** “All wells” allows you to export everything, otherwise the selected subset will be exported. Standard spreadsheet formats are supported. RDML is a standard format for PCR data if you have an alternative analysis program you want to use for qPCR analysis.

8 Further reading


Appendix D: Protocols

RNA Extraction Protocols

Introduction

There are two main methods of RNA extraction: phase separation and column extraction. Both can be used to extract RNA from *C. elegans*. Column extraction generally uses a kit and seems to be the more common method. The column extraction kits are quick and easy to use, but yields lower (it can be 50-85% efficient according to Zymo) and the kits are more expensive than non-kit methods.

In my hands the biggest challenge is genomic contamination. I have detectable genomic contamination with either method. In-column DNase treatment is insufficient to remove it. However a subsequent in-solution treatment with a DNase kit (containing a stop buffer) is sufficient to remove detectable contamination from either sample type.

A side-note on quantities. Using approximately the same number of young adult animals in one test to compare the methods, approximately equal amounts of oligonucleotides with good purity were shown by the Nano-drop. However, for the same sample I saw a 3 cycle (16-fold) difference for my target. However this target was not a reference gene, and this data is not very sound.

Pay attention to $xg$ vs. RPM when using centrifuges. Some will give you both, others will require that you calculate the RPM for your desired $xg$. The RPM depends on the diameter of the centrifuge.

Sample prep

1. Wash animals from plates with M9 (I use 2–4 plates per sample with ~120 animals on each plate). Be careful not to disturb the lawn of *E. coli* to minimize the presence of bacterial RNA. Worms are usually removed easily as they start thrashing. If not try a plate drop, rather than disturbing the lawn.

2. Collect worms + liquid in a 1.5ml centrifuge tube spin down briefly at $\leq 6,000xg$. Worms should sink, optionally wash once more with fresh M9. Remove as much liquid as possible without removing worms.

3. In the hood, add 200–250µL of Tri-reagent (should be $\geq 10x$ the volume of sample)

4. Freeze in liquid nitrogen, thaw in room temperature nanopure water, vortex repeatedly.

   repeat 3x (additional freeze thaw cycles do not yield more RNA in my hands, even though worm cuticles are visible)

   a. Check on worms to see that they are lysed. Note that excessive freeze thawing can degrade RNA, whilst insufficient will not break open the
cuticle. Remaining cuticles must be spun down before RNA purification.

b. Hypothetically bead bashing improves RNA yield, I was never able to try this, though Summers’ lab has a bead-beater.

5. When your tube is frozen, you can store it at -70°C —stopping point—

6. Go to RNA extraction, either column based (1) or phase separation (2).

(1) Column based RNA Purification

Adapted from the Direct-zol™ RNA MiniPrep Kit instructions*
All centrifugation steps should be performed between 10,000-16,000 x g.

1. Add 100% ethanol to the TRI-reagent sample homogenate in a 1:1 ratio (see volume in step 4).
   —Vortex well.

2. Load the mixture into a Zymo-Spin Column with a Collection Tube and centrifuge at 12,000 x g for 1 minute.

3. Discard the flow-through.

4. Add 400 µl RNA PreWash to the column and centrifuge for 1 minute. Discard the flow-through. Repeat this step.

5. Add 700 µl RNA Wash Buffer to the column and centrifuge for 1 minute. Discard the flow-through. Centrifuge for a further 2 min to ensure complete removal of buffer.

6. Transfer column to a new collection tube.

7. Add 50 µl of DNase/RNase-Free Water4 directly to the column matrix and centrifuge for 1 minute.
   a. Your nucleic acids stick to silica in the appropriate buffer, and are rinsed out by water.

8. Check the quantity and quality of RNA on the Nanodrop
   b. Remember this is total RNA

____________________________________

* Modified to add DNaseI step as recommended by Zymo technical support.
(2) RNA purification by phase separation
Adapted from the Morimoto lab and Invitrogen protocols

1. Grab your lysate samples and thaw them
2. Spin at ≥12,000xg to remove any remaining tissue
3. Transfer the supernatant to a new RNase free tube
4. In a fume hood, add 50µl of chloroform (or  of the quantity of trizol)
5. Vortex for 30 seconds
6. Leave at room temp form 3 minutes
7. Centrifuge at ≥12,000xg for 15 minutes at 4°C
8. Transfer the clear ~125µl upper layer to a new tube (discard the lower red stuff, avoid any white stuff near the interphase, if the top layer doesn’t look clear, spin for even longer)
9. Add 125µl of 2-propanol (isopropanol) and invert to mix (equal to volume in the tube)
10. Leave at room temp for ~10 minutes
11. Centrifuge at ≥12,000xg for 10 minutes at 4°C
12. Try to locate a transparent–white pellet in the tube. Decant the supernatant. Be sure that the pellet is not disturbed
13. Wash the pellet with 75% ethanol, tap the tube to dislodge the pellet
14. Spin down at ≥12,000xg for 5 minutes at 4°C
15. In a laminar flow hood, again decant the supernatant
16. Spin down any remaining liquid and carefully remove with a pipette
17. Leave the tube open to air dry the pellet. This should not take more than 10 minutes, depending on the amount of liquid remaining. Take care not to over-dry the pellet as it is hard to redisolve and may degrade the RNA.
18. Add the desired volume of nuclease free H₂O. Optionally heat to 60°C for 10 minutes to help dissolve the pellet.
19. Treat with DNase or move on to your next procedure.
cDNA synthesis reaction protocol

Make sure you have finished with all DNase treatments and deactivated or removed any DNase.

1. For one 20µL sample, add:
   a. 4µL 5x iScript mix
   b. 1µL reverse transcriptase enzyme
   c. 10µL RNA
   d. 5µL RNase-free water
   e. *When performing a no-reverse transcriptase control substitute water for the enzyme!*

2. Incubate complete reaction mix (iScript program on thermocycler):
   a. 5 minutes at 25°C
   b. 30 minutes at 42°C
   c. 5 minutes at 85°C
   d. Hold at 4°C (optional)

3. Store at -20°C