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Methodological Considerations for Heat Shock of the Nematode *Caenorhabditis elegans*

Shannin C. Zevian and **Judith L. Yanowitz***

1. Introduction

1.1.The stress response

Heat shock is the effect caused by exposing a cell or organism to a temperature that is higher than ideal body temperature. Exposure to heat shock/heat stress causes a response that is similar in bacteria, plants, and mammals ([1] & reviewed by [2]). Resistance to heat stress is capacitated by heat shock proteins (HSPs) [3], molecular chaperones that maintain proteostasis by destabilizing protein aggregates and promoting protein folding and sorting [4–6]. During heat shock, transcription and translation of most proteins is inhibited. The HSPs, however, are defined by their enhanced expression after heat shock (reviewed in [7]). Some HSPs, predominantly the large, ATP-dependent chaperones of the hsp70 and hsp90 families, show constitutive expression and have roles in development as well as stress response [8,9]. Others HSPs, mostly the small, monomeric hsp-16 family, are induced by and function during stress [7,10–13]. HSP-reporter proteins now serve as rheostats for stress induction [14–17]

Increased lifespan and an organism's ability to withstand stress tend to be positively correlated [18–20, 21] as many long-lived strains of animals are more resistant than wild-type to a variety of stresses, including heat (reviewed in [13], radiation [22], or oxidative damage [23–25]. The reverse is also true as stress resistance genes have been used to identify novel longevity mutants [26]. In fact, in *C. elegans*, ~80% of genes that promoted thermotolerance also increased lifespan by >15% [27]. This has led many in the field to view aging as an organismal stress.

Because heat shock responses are conserved across species and because there is a growing interest in how stress responses are integrated to affect lifespan, healthspan, and disease pathology, use of this technique is widespread. Since most laboratories have access to the tools to induce heat stress, scores of papers are published each year that employ this technique. Nevertheless, during our efforts to initiate such studies in *C. elegans*, we found the available literature sorely lacking in consistent protocols. This, combined with running

***Corresponding author** Magee-Womens Research Institute University of Pittsburgh School of Medicine 204 Craft Avenue Pittsburgh, PA 15217 412.641.7843 fax: 412.641.8519 jly@alum.mit.edu.

into serious barriers to reproducibility, led us to examine every aspect of our heat shock procedure and to devise detailed protocols that can be adapted by any lab.

1.2. Heat shock and hormesis

Hormesis is a phenomenon by which a mild stress predisposes an organism with resistance to a subsequent and more extreme stress [28]. The mild stress can bestow increased resistance both to the stressor to which the organism was originally exposed and to a variety of different stressors and toxins (reviewed in [29,30]). To cite just two examples: *Drosophila* exposed to radiation [31], and *C elegans* exposed to the metabolic inhibitor, sodium azide [32] increased survival following a subsequent acute heat shocks.

Mild exposure to stress has been shown to impinge on—and indeed, slow down—the aging process [18,33–35]. The observations that HSPs accumulate in response to various stresses [16] and during aging [36–38], that mutations that alter HSP expression influence lifespan [39], and that HSP expression can be predictive of lifespan [17] have led to the model that the perdurance of HSPs (and other proteins) from an initial stress exposure will be protective when an organism is exposed to subsequent insults [40–43].

Nevertheless, our understanding of hormesis and the inter-relationship between different stresses is still quite limited. It is precisely the breadth of stresses capable of causing a hormetic effect that underscores the importance for precise protocols in heat shock experiments. The technical difficulties that plague heat stress experiments are potentially even more problematic when considering hormesis due to the added complication of determining the appropriate timing between exposures to the mild and acute stresses. A recent review of the hormetic effect of heat stress on longevity across multiple species found that both the temperatures and the timing of the mild and acute heat stresses impacted the magnitude of the response [44].

1.3 Heat shock in C. elegans

C. elegans can grow and reproduce at temperatures from 12°-26°C. Interestingly, growth rates differ more than 2-fold between 16°C and 25°C and whereas animals are viable at 26°C, they are not sustainable at 27°C and are most fecund at temperatures near 16°C [45]. Similar to other organisms, even slight increases from maintenance temperature can elicit the response of HSPs. By using microwaves to increase the temperature of a worm from 26.0°C to 26.2°C, one group showed that this relatively small change was enough to increase the expression of an *hsp-16* reporter [46]. As worms have great utility as a simple model system to study aging as well as other complex human diseases (reviewed by [27]) and, as thermotolerance and aging have been linked in many studies (described above), heat shock experiments are a great tool for the worm researcher. Unfortunately, choosing a protocol is fraught with complications.

Scanning the literature, one will find many variations in heat shock protocols: with different labs preferring different exposure temperatures and times, different media for exposure, and differently aged worms. In part, these differences may be explained by a desire to study different outcomes, e.g. survival versus lethargy versus gene expression. However, even for

the same outcome, different protocols are frequently employed. For instance, if one wants to know how heat tolerance differs in a new genetic background compared to wild type worms, early adult worms are often used (see [19,47,48] for examples). However, use of larval stages is not uncommon; for example, one group used L2 worms to study the involvement of *skn-1* in the stress response [48](see [37] for another example).

Some of the differences in protocols are attributable to inherent problems in incubator design that prevent precise control of the heat stress temperature. Although the published protocols are a useful starting point, the lack of standardization makes it difficult to compare results across laboratories and presents a quandary when initiating these studies in one's own laboratory. In the text that follows, we discuss the wide range of variables that can influence heat shock responses and how protocol standardization can address these issues.

2. Considerations for HS experiments

2.1. Choosing the right temperature

C. elegans is exquisitely sensitive to temperature. Biological functions including growth, fecundity, behavior, and longevity are affected by changes in temperature: even a 0.2°C change in temperature can be sensed by the worm and alter its behavior [46]; less than a 2°C change in growth temperature can alter protein homeostasis as demonstrated when worms shifted from 25° to 26.5°C accumulated aggregates of the meiotic synaptonemal complex proteins [49]; and allowing worms to develop at 25.5°C instead of 25°C will result in sterility [42]. With regard to acute heat exposure, *C. elegans* manifest differential responses depending on the duration and timing of exposure. This extreme sensitivity to temperature underscores the need to carefully consider the temperature used in heat shock experiments.

Throughout the literature, the parameters used for heat shock differ between labs. Although, 35°C is most often used for acute heat stress [16,19], examples of other temperatures crop up frequently (e.g. 32°C [50,51]; 33°C [52]; 37°C [53]; 38°C [1,54]; 40°C [53]). We chose 37°C for our heat stress experiments for several reasons. First, historically 37°C was the temperature used to analyze the induction of HSPs in different organisms e.g. [53,55]. Second, acute exposure at this temperature is short enough that the worms do not have time to develop to a new stage during the course of the treatment. Finally and conveniently, at 37°C the lethal dose occurs within 3 hours for all growth stages, which allows a heat shock regimen to be easily conducted in a day.

2.2. Liquid versus solid medium

Heat shock experiments can be done in pre-warmed liquid medium, on pre-warmed agar plates (with or without food), or by moving growth plates into an incubator at a higher temperature. Each method has its advantages, however, we have found that moving the worms as little as possible reduces unintended stresses on the worms.

2.2.1. Considerations for heat shock in liquid medium—With pre-warmed liquid medium, worms are washed off plates with room temperature buffer, allowed to settle, and the medium replaced with pre-warmed buffer at the desired temperature. The worms then remain in liquid for the duration of the exposure. Unfortunately, this method has two

disadvantages: 1) Maintaining nematodes in liquid culture changes the lifespan, metabolic rate, and growth rate of worms compared to worms maintained on agar [56]. As even brief daily periods of swimming in liquid in animals otherwise reared on plates can extend lifespan (M. Driscoll, unpublished results), it is very likely that being kept in liquid medium during the course of an experiment will have a complex relationship with heat stress; 2) Liquid culturing requires significant manipulation of the worms as they are washed from their original medium, rinsed, and moved again at the end of the experiment. Each transfer can physically damage worms and/or cause significant population attrition due to handling errors (e.g. sticking to plastic tubes and pipettes). The damage incurred can alter results either by inducing a hormetic response or by pre-stressing the animals and enhancing response to the subsequent heat shock.

Liquid culture is however the ideal option for severe heat stress where the time of exposure is less than 1 hour. A good example was recently published by the Tavernarakis lab [57], where they describe a hormetic 34°/39°C protocol that mimics heat stroke. We note that protocols for liquid exposure differ between labs but that many labs are now using PCR machines to maintain tube/liquid temperature during the course of exposure. Since worms tend to stick to plastic, we recommend a vast excess of animals be used in these studies.

Liquid culturing is an option for large-scale screening [58], despite the concerns related to altered physiology on plates versus in liquid. Using a Copas biosorter (Union Biometrica Inc., Holliston, MA), individual worms can be distributed into wells containing different compounds or RNAi clones and the liquid temperature precisely controlled in a thermocycler. SYTOX Green (ThermoFisher Scientific, Waltham, MA) can be included in the medium and fluorometric readings taken every half hour to assess mortality. Because thermotolerance curves vary between experiments [58], controls must be included on each 384 well plate as absolute values cannot be compared between assays.

2.2.2. Considerations for heat shock on pre-warmed agar plates—Experimental protocols using pre-warmed plates have the advantage of rapidly getting the animals to the desired temperature. However, these protocols also require significant manipulation, usually washing large populations of animals off plates and into liquid before plating onto pre-warmed plates Thus, these protocols are fraught with the problems described above for liquid exposure (Section 2.2.1). For smaller populations of experimental animals, they can be rapidly “picked” onto pre-warmed plates. However, this limits the scope of any experiment to the number of worms and plates that can be picked in a reasonable amount of time and also creates significant variability resulting from the time required to passage the worms. Testing pre-warmed plates immediately out of the incubator, we observed a drop of 1°C in temperature within one minute and of 5°C within 5 minutes (data not shown). Maintaining a uniform temperature across the plates therefore is extremely challenging due to differences in ambient lab temperature.

2.2.3. Heat shock on seeded agar plates—In order to circumvent the problems created by moving worms more than necessary, we prefer keeping the worms on the same plate throughout an experiment. When plates are moved from room temperature, generally between 21° and 23°C, into an incubator or water bath with a higher temperature (in our

case 37°C), the agar increases in temperature gradually. The temperature increases quickly at first, then slows and ultimately requires about three hours to fully equilibrate (Figure 1). This procedure is scalable as up to several hundred animals can be grown on each plate and numerous plates can be placed in the incubator at one time. Because of time required for the plates to get to temperature, this method cannot be used for heat stroke models which require shorter amounts of time at higher temperatures [57].

2.3. Duration of Heat Shock

Heat shock is most often used to investigate the resistance to heat stress wherein percent survival is the measurable output. Figure 2A provides a time course of survival data for different larval stages following exposure to 37°C (see also Section 2.5 below). These results were the basis for our decision to perform the vast majority of our experiments on late L4 worms with a 2.0-hour exposure at 37°C. The expected 15-20% survival of wild-type worms allows enough plasticity to see significant differences when comparing populations that may experience either increased or decreased survival.

When setting up hormetic heat shock experiments choosing a temperature for the mild heat stress requires a bit more nuance. The timing should not be so long that any of the worms are killed by the treatment, but must be long enough to induce a stress response. If worms are being maintained on seeded plates, the timing of the heat stress must also be long enough for the plates to come up to temperature in the incubator. In our experiments, a 60-minute mild heat stress produced no detectable hormetic response while 90 minutes elicited a good response, but killed 5-10% of the exposed worms. For our purposes we have achieved reproducible results when worms are exposed for 75 minutes to 34°C: this temperature is strong enough to induce a hormetic response, yet allows all of the treated worms survive.

2.4. Incubator Setup

2.4.1. Temperature Gradients—Because incubators rely either on built-in fans or passive heat transfer, we find that almost all incubators have temperature gradients from top to bottom and/or front to back. Depending on the design of the incubator and the severity of the temperature differential, these gradients can be overcome. Front-to-back gradients can be caused by either low airflow or poor insulation in and around the door of the incubator. The airflow problem is common in many incubators and can be overcome by additional fan(s). However, if the gradient is too extreme, generally more than three or four degrees, the problem may be insurmountable, as too much airflow has the potential to dry out plates during the course of an experiment. Further, if the problem is the door, such as we encountered with a TriTech DigiTherm incubator, correcting the gradient may be even more difficult. If cooler air is constantly finding its way into the incubator, it becomes nearly impossible to sustain a constant temperature. By retrofitting our incubator with “thermal packaging” and a small fan, we maximize air exchange from pre-warmed objects and eliminate temperature gradients inside the incubator Figure 3. Although we show our Forma Scientific model 3327 water-jacketed incubator, the same approach works for many makes, models, and sizes.

Heat shock can also be accomplished by placing parafilmed plates directly into a water bath (the plates will float). Although water loses temperature much more slowly than air, similar to frozen pierogies added to boiling water, the transfer of plates to a pre-heated water bath will cause the water to cool down. Obviously, the effect is only significant when multiple plates are used. Though our experience using water baths for heat shock is admittedly limited, our trials with heat exchange in incubators (and trial and error when designing our own reliably reproducible protocols) leads us to suggest using a large, circulating water bath. This type of equipment should limit temperature gradients in the water and at the air-water interface and increase reproducibility. The chief issue with the water bath method is differences in ambient air temperature that will complicate maintaining temperature at the water/air interface: This may lead to substantially different heating times from one experiment to the next. Water baths are also limited by available space and are not ideal for multi-well plates, such as used in large-scale screens. For these reasons, we favor the use of dedicated incubators for heat shock studies.

2.4.2. Air Exchange—It is important to know how the temperature in your incubator changes every time the door is opened and how the incubator recovers from this change. Incubators with both heating and cooling capabilities will sometimes overshoot the temperature and compensate by blowing cold air into the unit. If this cold air is coming into direct contact with the plated worms, it will be difficult to know if the plates are coming up to temperature. We recommend using incubators without the cooling feature or, if possible, turning off the cooling compressor during heat shock experiments.

When the door of the incubator is opened, the majority of the air inside the incubator is rapidly (<5 seconds) exchanged with the room temperature air outside. Thus, placing the incubator in a room maintained at or near the desired temperature would be ideal. Unfortunately, many laboratories do not have the luxury of warm rooms. Therefore, great care must be taken to ensure rapid heating of the air within the incubator itself in order to restore the desired (heat stress) temperature.

The equation, $Q = ha(\Delta T)$, where Q is the rate of heat transfer, h is the heat transfer coefficient for your medium, a is the surface area, and ΔT is the difference in temperature between desired temperature and initial temperature, reveals that the time required to increase the air temperature in the incubator following closing the door is proportional to the surface area of the objects in the incubator that are at the higher temperature, how many degrees the temperature must change, and the rate of heat exchange between the air and the objects. Thus, methods that call for using insulated boxes or lidded plastic containers within incubators increase the time for equilibration and are not recommended. Placing plates into containers within the incubator should only be considered for long-term heat exposures (>12 hours).

What the equation above clearly demonstrates is that increasing the warm surface area inside the incubator would significantly decrease the time it takes to reach the desired temperature. We found that using solid freezer bricks to fill as much of the unused space in the incubator as possible increases the amount of surface area at the desired temperature. The surface area is only increased significantly if the freezer bricks lean against each other much like a house

of cards (Figure 3). This way, even though all the air may be exchanged each time the door is opened, a battery-operated fan at the bottom of the incubator is able to move air sufficiently to bring the temperature back up to 37°C in less than three minutes. For us, using the incubator shown in Figure 3, this was a huge improvement over the 3-5 hours required to return to temperature when it was mostly empty.

Before heat shock experiments are commenced, it may be advantageous to find absolute values for the timing of plate dynamics experimentally with a calibrated thermocouple, such as can be purchased from Omega Engineering (model HH506RA). We accomplished this by inserting the thermocouple probes through a hole in the lid of a seeded, agar plate and placing one probe at the surface of the agar while embedding the other in the agar. Everything was held in place with tape and the plate was placed onto a 37°C gel pad in the incubator. The thermocouple probes will not allow the plate to be completely covered, but smaller gel pads can be placed carefully around the probes to cover the plate as well as possible. The probes should be long enough to allow the digital thermometer to be left outside of the incubator for recording temperature manually or for connection with a computer for automatic readings.

To facilitate heat transfer to the agar plates, we keep a gelatinous, 2 cm thick pad on the bottom of the shelf designated for heat shock experiments (See section 3.1). Four thermometers surround the gelatinous pad, one at each corner, to verify before each experiment that there are no temperature gradients on the shelf. Experimental plates are placed directly onto the gel pad and covered with a second gel pad for the duration of the experiment. As previously mentioned, it is rarely appropriate to put worm plates into any type of container before going into the incubator, as this limits airflow. Stacking plates before putting them into the incubator should also be avoided as this affects heat transfer. The pockets of room-temperature air trapped within the plates will be warmed at inconsistent rates if some plates are contacted on either side by other room-temperature plates while others directly contact the warm air in the incubator.

2.5. Age/Developmental Stage

The effect of heat shock differs markedly throughout the life cycle of *C. elegans*. Figure 2A and 2B shows survival rates of N2 worms throughout the life cycle at 37°C in the incubator set-up described above. Resistance to heat shock is highest in L1/L2 larvae and lowest in L4. However, heat stress also causes a developmental delay that may confound experiments, especially in younger worms. We have observed L1 worms that did not reach adulthood for up to 4 days following heat shock. In addition to the profound differences by stage of the worm, we have found dramatic differences in survival within stages, e.g. early versus late L4 can have up to 3-fold difference in survival.

The L4 stage in the life cycle of *C elegans* can be broken down into 6 stages based largely on development of the gonad [59]. When resistance to heat stress is measured at each sub-stage of L4, later L4 worms survive better than earlier L4 worms. Although very late L4 worms performed best in our hands, we use “late” rather than “very late” L4s, in our standard protocol due to concern that with very late L4 we were more likely to accidentally heat shock worms that were close to, or in the process of, molting.

Adult worms are often used for heat shock experiments, with 1-4 day-old adults showing up most often in our literature searches. However, we observe significant differences in thermotolerance at different adult ages (Figure 2B). Although the nature of these differences is unknown, one could hypothesize that they may be due to differences in fecundity or to age-related deterioration of proteostasis which may start as early as day 2 of adulthood [60]. In addition, since stress resistance may vary based on circadian rhythms in *C. elegans* [61], the time of day that experiments are performed could influence the variance between experiments. These observations underscore the importance of carefully controlling the staging of heat shocked and control animals whether an experiment involves larval or adult worms. We have found that a relatively short egg-lay (1.5-2 hours) allows us to attain well-synchronized populations with limited exposure to other stressors.

2.6. When to score survival

A search of the literature reveals protocols where worms are scored immediately [19], 6-8 hours [39], and 16-18 hours post-heat shock [57]. Unfortunately, more often than not, there is no clear indication of when worms were scored for survival. Because heat shock makes animals lethargic and they will appear lighter in color, visual inspection of worms immediately after heat shock is not a reliable determinant of their ultimate survival. In addition, we have found that these worms to be more fragile than non-stressed animals as they can be easily damaged in touch assays. In addition, worms that respond to touch immediately after heat shock often do not survive much longer and rarely lay viable eggs before dying. Thus, during this early time period, worms that for all physiologically relevant purposes did not survive the heat stress may be scored as alive.

We have found that 12-14 hours post-heat shock, worms (maintained in a 20°C incubator) can reliably be assayed for viability. By this time point, many dead worms appear swollen and are often oddly oriented. The remaining worms can be readily tested for tap response and pharyngeal pumping. Twelve hours post heat shock is sufficient time to be confident that the surviving worms will continue to grow and survive and, depending on the extent of heat shock, produce progeny.

2.7. Food source

Changes in the growth [62], behavior [63], fecundity [64] and longevity [65] of *C. elegans* have been reported on different food sources. Likewise, we have noticed that changes in food source can affect the survival to heat shock (unpublished data). Although we have noticed only slight effects when using the same strain of bacteria, for best results, we recommend standardizing the timing of seeding plates prior to use and being diligent regarding contamination.

Standardization of plate thickness and age, as well as the size and age of the bacterial lawn, should be done to ensure reproducibility between experiments. We have found that 14 ml of nematode growth medium (NGM) agar in standard, vented 6 cm plate works best. Plates are poured and allowed two days to dry at room temperature before seeding >65% of the plate with OP50. The bacterial lawn is dried overnight and seeded plates are stored at 4°C for up to 5 days prior to use. Although freshly seeded plates are ideal, we have found no

differences in heat stress responses between new plates and those stored for up to 1 month. Thus far, when plates are stored unseeded at 4°C for many months we have not found appreciable differences in heat shock response.

2.8. Synchronizing populations

While it is convenient to synchronize worms using bleach preparations; this method can be quite stressful on the embryos, as evidenced by hatching rates below 99%. This is likely due to the time required to remove the bleach after "popping", which also leads to variability between trials. The stress of a bleach preparation has the potential to cause a hormetic response in the worms that will increase their resistance to heat stress (Figure 4). We have found that animals hatched from bleach-prepped embryos may exhibit a 20-30% increase in survival following heat shock compared to worms from laid eggs (Figure 4). We have also observed that the resultant bleach prepped worms are less synchronous than a 1.5-2 hour egg-lay plate and that variation in L4 age can affect heat sensitivity (data not shown). For these reasons, we favor allowing adults to lay eggs directly onto seeded plates.

2.9. Population Density

Finally, one must consider the number of worms on each plate during any given experiment. Failure to take note of the number of worms on a plate can destroy any hope of reproducibility. In our hands, 6 cm plates with 800-1,000 L4 larvae survived at 37°C better than similarly staged worms where only 150-200 worms were plated. Fortuitously, 150-200 worms/plate has given us reproducible results and is a reasonable number of worms to expect on a plate using our egg-lay protocol (described in Section 3.2.1).

3. Heat Shock Protocols

3.1 Preparing your incubator

The gelatinous pads used both to maintain the temperature of the incubator after opening and closing the door and to give the agar plates a pre-warmed place to sit are easy to assemble. We opted for a low-tech option, as heated pads were often equipped with power cords that would have needed an external outlet, potentially affecting the seal and insulation around the door. To assemble our gelatinous pads, we emptied the contents of room temperature, non-toxic freezer packs into resealable plastic bags until the bag was 2 cm thick when flattened. The pads pictured in Figure 3 are 27×27 cm bags, but we have used other sizes to fit the space available in other incubators. Alternatively, one could use gelatin or agar flakes (purchased from supermarket) or re-melted old agarose gels (~0.7% agarose would be ideal). We do not recommend heat-sealed bags as air bubbles coalesce over time and would be difficult to remove.

When setting up an incubator as described in Section 2.4.2 the incubator will take 1-2 days to equilibrate due to the time required to bring all the solid material to the desired temperature. Small battery-powered fans, such as Travelon Personal Fan (Style#12267), are an inexpensive and low profile option for increasing airflow in the incubators. These small fans do not have power cords; consequently, they can be used in all types of incubators without affecting seals and they allow the user to control airflow in different regions of the

incubator. We strongly recommend extra sets of rechargeable batteries to eliminate downtime of the incubator. We cannot emphasize enough the necessity of monitoring the incubators with multiple thermometers. We recommend placing four liquid-embedded thermometers at each corner of the desired shelf to control for temperature gradients across the incubator. We have used H-B instrument's FRIO-Temp liquid in glass thermometers (catalog #20403C-RMD) with good results. This style of thermometer has a relatively small footprint, is easy to read at a distance, and the liquid provided changes quickly with ambient air temperature.

3.2. Protocols on seeded plates

3.2.1. Basic 37°C Heat Shock Protocol—Move 20 adult, day 1-2 gravid worms onto uniformly seeded NGM plates with equal amounts of agar in each.

Allow the worms to lay eggs for 1.5 hours.

Pick off all the adults (count as you pick them off to ensure all have been removed). Take care to not disturb the eggs. 30-60 minutes later, confirm that all adults have been removed. Because worms may crawl onto the side of the plate, we recommend starting several additional plates per experiment: Plates with even one adult left behind overnight will lbe useless for your experiment.

Maintain plates at 20°C.

At 20°C, the late L4 stage should be 56-58 hours post egg-lay. However, just as significant effort was made to get the heat shock incubators to have uniform temperature, most common incubators (Percival, ThermoFisher, Thomas) suffer from temperature gradients. Therefore, one needs to determine experimentally the exact timing of the L4-adult molt at a given spot within your incubator and choose a time point approximately 2 hours prior to enrich for late L4s.

Note: if you are growing multiple plates, be aware that stack effects occur: plates at the top and bottom of the stack may be at different temperatures compared to plates in between. We recommend “buffer” plates at top and bottom to ensure even growth.

Prior to heat shock, confirm the stage of the animals using a stereomicroscope. Large, dark L4s are a good read-out of the late L4 stage. A reasonable effort should be made to remove/pick off worms that are not at the desired developmental stage.

At least 30 minutes prior to the start of the heat shock, the fan should be turned on in the incubator. This eliminates pre-existing temperature gradients within the incubator.

When moving plates into the incubator, open the door of the incubator as little as possible and remove the fan to reduce the flux of cold air over our warmed incubator surfaces.

Place experimental plates onto a pre-warmed gel pad and place a second pre-warmed gel pad on top of them.

Replace the fan and close the door immediately.

At least one control plate should be left at 20°C.

Remove plates after 2 hours. Two hours at 37°C will kill most, but not all, wild-type N2 worms and should allow for a significant surviving population of experimental worms. If you prefer to compare your experimental worms to near 100% death in wild-type worms, leave the plates in the incubator for an additional 30-60 minutes.

Following heat shock, allow the plates to sit in a single layer on the bench top for at least 20 minutes to recover. Although we have not gone to great pains to define this step, plates should not be left outside the 20°C incubator for longer than necessary.

Return plates to the 20°C incubator overnight.

Score worms for survival 12-14 hours post-heat shock as described in Section 2.6.

When scoring live worms it is helpful to divide the plate into horizontal sections by drawing six lines on the bottom of the plate using an ultra fine-tipped, red marker. If possible, score the worms with the lid of the plate on, as this will allow you to tap the plate from above, if necessary, to stimulate movement in a worm that may look alive but is not moving. Tapping from the top rather than tapping the whole plate against the surface of the stereomicroscope will make it easier to keep track of your place on the plate while counting. If you are still not sure about the status of a worm, use touch to verify.

Training yourself to recognize dead worms can be done by shocking plates of worms at 37°C for 30 minutes or three hours. 99-100% of N2 wild-type worms will survive 30 minutes on a plate at 37°C, while most will not survive the three-hour heat shock.

3.2.2. Hormetic 34°C Mild Heat Shock Followed by 37°C Acute Heat Shock Protocol—Before beginning this experiment it is important to note that you may need two incubators that are appropriately prepared for heat shock experiments. This is because the large volume of warm material in each incubator takes a long time to equilibrate to a new temperature.

Prepare the mild heat shock plates as described above for a basic 37°C heat shock. Briefly, move 20 gravid, adult worms onto as many uniform, seeded plates as necessary.

Allow adults to lay eggs for 1.5-2 hours and pick off.

Maintain the plates at 20°C to allow eggs to develop.

56-58 hours later, check that your worms are late L4 stage.

Move experimental plates onto a pre-warmed gel pad in the 34°C incubator with a pre-warmed gel pad on top of them. At least one control plate should be left at 20°C.

Remove plates after 75 minutes. Allow the plates to sit in a single layer on the bench top, at least 20 minutes, to recover.

Move the plates back to the 20°C incubator.

Twelve hours later, perform the 37°C heat shock as described above.

Score the worms for survival 12-14 hours post acute heat shock. 12 hours of recovery time between the mild and acute heat treatments mimics a previously published protocol [66] and is long enough for the L4 worms that we are interested in to reach adulthood.

4. Conclusion

Heat shock is a widely used method within the *C. elegans* community to study the impact of stress on physiology, behavior, fecundity and survival. Because heat shock is inexpensive and requires little additional equipment, it is frequently used to study the interrelationships between different types of stresses and their impact on lifespan. Nevertheless, getting reproducible results with heat shock can be tricky as numerous parameters impact the results. We show differential sensitivity to heat stress during larval development and into adulthood. We also show that different methods of worm preparation can cause mild stress that attenuates the heat shock response. These results underscore the importance of controlling for the age of the worm, the extent of synchronization, the worms' prior life histories (bleach prepped or laid), and the number of worms. Here we emphasize numerous other details of worm growth that can impinge on these parameters from the thickness and age of the bacterial lawn they are grown on to the type of food they are eating. Importantly, we also provide a detailed discussion of major issues in incubator design and an inexpensive method for overcoming problems in heating found in most incubators. We have found that these efforts allow us to attain reliable and reproducible results for heat shock of nematodes.

References

1. Yokoyama K, Fukumoto K, Murakami T, Harada S, Hosono R, Wadhwa R, et al. Extended longevity of *Caenorhabditis elegans* by knocking in extra copies of hsp70F, a homolog of mot-2 (mortalin)/mthsp70/Grp75. FEBS Lett. Apr 10; 2002 516(1–3):53–7. [PubMed: 11959102]
2. Åkerfelt M, Morimoto RI, Sistonen L. Heat shock factors: integrators of cell stress, development and lifespan. Nat Rev Mol Cell Biol. Aug; 2010 11(8):545–55. [PubMed: 20628411]
3. Parsell DA, Taulien J, Lindquist S, Viitanen P, Jaenicke R, Horwich A, et al. The Role of Heat-Shock Proteins in Thermotolerance [and Discussion]. Philos Trans Biol Sci. Mar 29; 1993 339(1289):279–86.
4. Hartl FU. Heat shock proteins in protein folding and membrane translocation. Semin Immunol. Jan; 1991 3(1):5–16. [PubMed: 1680013]
5. Pechan PM. Heat shock proteins and cell proliferation. FEBS Lett. Mar; 1991 280(1):1–4. [PubMed: 2009952]
6. Jäättelä M. Heat shock proteins as cellular lifeguards. Ann Med. Aug; 1999 31(4):261–71. [PubMed: 10480757]
7. Schlesinger MJ. Heat shock proteins. J Biol Chem. Jul 25; 1990 265(21):12111–4. [PubMed: 2197269]
8. Snutch TP, Heschl MF, Baillie DL. The *Caenorhabditis elegans* hsp70 gene family: a molecular genetic characterization. Gene. Apr; 1988 64(2):241–55. [PubMed: 2841196]
9. Heschl MFP, Baillie DL. Characterization of the hsp70 Multigene Family of *Caenorhabditis elegans*. DNA. May; 1989 8(4):233–43. [PubMed: 2766926]
10. Russnak RH, Jones D, Candido EP. Cloning and analysis of cDNA sequences coding for two 16 kilodalton heat shock proteins (hsps) in *Caenorhabditis elegans*: homology with the small hsps of *Drosophila*. Nucleic Acids Res. May 25; 1983 11(10):3187–205. [PubMed: 6190129]

11. Russnak RH, Candido EP. Locus encoding a family of small heat shock genes in *Caenorhabditis elegans*: two genes duplicated to form a 3.8-kilobase inverted repeat. Mol Cell Biol. Jun 1; 1985 5(6):1268–78. [PubMed: 4033652]
12. Jones D, Russnak RH, Kay RJ, Candido EP. Structure, expression, and evolution of a heat shock gene locus in *Caenorhabditis elegans* that is flanked by repetitive elements. J Biol Chem. Sep 15; 1986 261(26):12006–15. [PubMed: 3017958]
13. Muñoz MJ. Longevity and heat stress regulation in *Caenorhabditis elegans*. Mech Ageing Dev. Jan; 2003 124(1):43–8. [PubMed: 12618005]
14. Fire A. Integrative transformation of *Caenorhabditis elegans*. EMBO J. Oct; 1986 5(10):2673–80. [PubMed: 16453714]
15. Stringham EG, Dixon DK, Jones D, Candido EP. Temporal and spatial expression patterns of the small heat shock (hsp16) genes in transgenic *Caenorhabditis elegans*. Mol Biol Cell. Feb 1; 1992 3(2):221–33. [PubMed: 1550963]
16. Link CD, Cypser JR, Johnson CJ, Johnson TE. Direct observation of stress response in *Caenorhabditis elegans* using a reporter transgene. Cell Stress Chaperones. Dec; 1999 4(4):235–42. [PubMed: 10590837]
17. Mendenhall AR, Tedesco PM, Taylor LD, Lowe A, Cypser JR, Johnson TE. Expression of a Single-Copy hsp-16.2 Reporter Predicts Life span. J Gerontol A Biol Sci Med Sci. Jul; 2012 67(7): 726–33. [PubMed: 22227523]
18. Lithgow GJ, White TM, Hinerfeld DA, Johnson TE. Thermotolerance of a long-lived mutant of *Caenorhabditis elegans*. J Gerontol. Nov; 1994 49(6):B270–6. [PubMed: 7963273]
19. Lithgow GJ, White TM, Melov S, Johnson TE. Thermotolerance and extended life-span conferred by single-gene mutations and induced by thermal stress. Proc Natl Acad Sci USA. Aug; 1995 92(16):7540–4. [PubMed: 7638227]
20. Lin Y- J, Seroude L, Benzer S. Extended Life-Span and Stress Resistance in the *Drosophila* Mutant methuselah. Science. Oct 30; 1998 282(5390):943–6. [PubMed: 9794765]
21. Johnson TE, Henderson S, Murakami S, de Castro E, de Castro SH, Cypser J, et al. Longevity genes in the nematode *Caenorhabditis elegans* also mediate increased resistance to stress and prevent disease. J Inherit Metab Dis. May; 2002 25(3):197–206. [PubMed: 12137228]
22. Murakami S, Johnson TE. A Genetic Pathway Conferring Life Extension and Resistance to Uv Stress in *Caenorhabditis Elegans*. Genetics. Jul; 1996 143(3):1207–18. [PubMed: 8807294]
23. Larsen PL. Aging and resistance to oxidative damage in *Caenorhabditis elegans*. Proc Natl Acad Sci U S A. Oct 1; 1993 90(19):8905–9. [PubMed: 8415630]
24. Vanfleteren JR. Oxidative stress and ageing in *Caenorhabditis elegans*. Biochem J. Jun 1; 1993 292(Pt 2):605–8. [PubMed: 8389142]
25. Honda Y, Honda S. Oxidative Stress and Life Span Determination in the Nematode *Caenorhabditis elegans*. Ann N Y Acad Sci. Apr 1; 2002 959(1):466–74. [PubMed: 11976220]
26. Walker GA, Walker DW, Lithgow GJ. Genes That Determine Both Thermotolerance and Rate of Aging in *Caenorhabditis elegans*. Ann N Y Acad Sci. Jun 1; 1998 851(1):444–9. [PubMed: 9668638]
27. Muñoz MJ, Riddle DL. Positive Selection of *Caenorhabditis elegans* Mutants With Increased Stress Resistance and Longevity. Genetics. Jan 1; 2003 163(1):171–80. [PubMed: 12586705]
28. Calabrese EJ, Baldwin LA. Hormesis as a biological hypothesis. Environ Health Perspect. Feb; 1998 106(Suppl 1):357–62. [PubMed: 9539030]
29. Minois N. Longevity and aging: beneficial effects ofexposure to mild stress. Biogerontology. Mar 1; 2000 1(1):15–29. [PubMed: 11707916]
30. Hercus MJ, Loeschcke V, Rattan SIS. Lifespan extension of *Drosophila melanogaster* through hormesis by repeated mild heat stress. Biogerontology. May 1; 2003 4(3):149–56. [PubMed: 12815314]
31. Lamb MJ, McDonald RP. Heat tolerance changes with age in normal and irradiated *Drosophila melanogaster*. Exp Gerontol. Aug; 1973 8(4):207–17. [PubMed: 4199650]
32. Massie MR, Lapoczka EM, Boggs KD, Stine KE, White GE. Exposure to the metabolic inhibitor sodium azide induces stress protein expression and thermotolerance in the nematode *Caenorhabditis elegans*. Cell Stress Chaperones. Jan; 2003 8(1):1–7. [PubMed: 12820649]

33. Khazaeli AA, Tatar M, Pletcher SD, Curtsinger JW. Heat-Induced Longevity Extension in *Drosophila*. I. Heat Treatment, Mortality, and Thermotolerance. J Gerontol A Biol Sci Med Sci. Jan 1; 1997 52A(1):B48–B52. [PubMed: 9008657]
34. Shama S, Lai C- Y, Antoniazzi JM, Jiang JC, Jazwinski SM. Heat Stress-Induced Life Span Extension in Yeast. Exp Cell Res. Dec 15; 1998 245(2):379–88. [PubMed: 9851879]
35. Johnson TE, Hartman PS. Radiation Effects on Life Span in *Caenorhabditis Elegans*. J Gerontol. Sep 1; 1988 43(5):B137–B141. [PubMed: 3418030]
36. Walker GA, White TM, McColl G, Jenkins NL, Babich S, Candido EPM, et al. Heat Shock Protein Accumulation Is Upregulated in a Long-Lived Mutant of *Caenorhabditis elegans*. J Gerontol A Biol Sci Med Sci. Jul 1; 2001 56(7):B281–B287. [PubMed: 11445592]
37. Walker GA, Lithgow GJ. Lifespan extension in *C. elegans* by a molecular chaperone dependent upon insulin-like signals. Aging Cell. Apr 1; 2003 2(2):131–9. [PubMed: 12882326]
38. Rea SL, Wu D, Cypser JR, Vaupel JW, Johnson TE. A stress-sensitive reporter predicts longevity in isogenic populations of *Caenorhabditis elegans*. Nat Genet. Aug; 2005 37(8):894–8. [PubMed: 16041374]
39. Morley JF, Morimoto RI. Regulation of longevity in *Caenorhabditis elegans* by heat shock factor and molecular chaperones. Mol Biol Cell. Feb; 2004 15(2):657–64. [PubMed: 14668486]
40. Butov A, Johnson T, Cypser J, Sannikov I, Volkov M, Sehl M, et al. Hormesis and debilitation effects in stress experiments using the nematode worm *Caenorhabditis elegans*: the model of balance between cell damage and HSP levels. Exp Gerontol. Dec; 2001 37(1):57–66. [PubMed: 11738147]
41. Michalski AI, Johnson TE, Cypser JR, Yashin AI. Heating stress patterns in *Caenorhabditis elegans* longevity and survivorship. Biogerontology. Mar 1; 2001 2(1):35–44. [PubMed: 11708615]
42. Yashin AI, Cypser JR, Johnson TE, Michalski AI, Boyko SI, Novoseltsev VN. Ageing and survival after different doses of heat shock: the results of analysis of data from stress experiments with the nematode worm *Caenorhabditis elegans*. Mech Ageing Dev. Sep 15; 2001 122(13):1477–95. [PubMed: 11470134]
43. Yashin AI, Cypser JW, Johnson TE, Michalski AI, Boyko SI, Novoseltsev VN. Heat Shock Changes the Heterogeneity Distribution in Populations of *Caenorhabditis elegans* Does It Tell Us Anything About the Biological Mechanism of Stress Response? J Gerontol A Biol Sci Med Sci. Mar 1; 2002 57(3):B83–B92. [PubMed: 11867644]
44. Lagisz M, Hector KL, Nakagawa S. Life extension after heat shock exposure: Assessing meta-analytic evidence for hormesis. Ageing Res Rev. Mar; 2013 12(2):653–60. [PubMed: 23570942]
45. Hirsh D, Oppenheim D, Klass M. Development of the reproductive system of *Caenorhabditis elegans*. Dev Biol. Mar; 1976 49(1):200–19. [PubMed: 943344]
46. Dawe AS, Smith B, Thomas DWP, Greedy S, Vasic N, Gregory A, et al. A small temperature rise may contribute towards the apparent induction by microwaves of heat-shock gene expression in the nematode *Caenorhabditis Elegans*. Bioelectromagnetics. 2006; 27(2):88–97. [PubMed: 16342196]
47. Vilchez D, Morantte I, Liu Z, Douglas PM, Merkwirth C, Rodrigues APC, et al. RPN-6 determines *C. elegans* longevity under proteotoxic stress conditions. Nature. Sep 13; 2012 489(7415):263–8. [PubMed: 22922647]
48. An JH, Blackwell TK. SKN-1 links *C. elegans* mesendodermal specification to a conserved oxidative stress response. Genes Dev. Aug; 2003 17(15):1882–93. [PubMed: 12869585]
49. Bilgir C, Dombecki CR, Chen PF, Villeneuve AM, Nabeshima K. Assembly of the Synaptonemal Complex Is a Highly Temperature-Sensitive Process That Is Supported by PGL-1 During *Caenorhabditis elegans* Meiosis. G3 GenesGenomesGenetics. Apr; 2013 3(4):585–95.
50. Beck CD, Rankin CH. Heat shock disrupts long-term memory consolidation in *Caenorhabditis elegans*. Learn Mem. Jan; 1995 2(3-4):161–77. [PubMed: 10467573]
51. Jones D, Candido EP. Feeding is inhibited by sublethal concentrations of toxicants and by heat stress in the nematode *Caenorhabditis elegans*: relationship to the cellular stress response. J Exp Zool. Jul; 1999 284(2):147–57. [PubMed: 10404644]

52. Hajdu-Cronin YM, Chen WJ, Sternberg PW. The L-type cyclin CYL-1 and the heat-shock-factor HSF-1 are required for heat-shock-induced protein expression in *Caenorhabditis elegans*. Genetics. Dec; 2004 168(4):1937–49. [PubMed: 15611166]
53. Anderson GL. Responses of dauerlarvae of *Caenorhabditis elegans* (Nematoda: Rhabditidae) to thermal stress and oxygen deprivation. Can J Zool. Aug; 1978 56(8):1786–91.
54. Steinkraus KA, Smith ED, Davis C, Carr D, Pendergrass WR, Sutphin GL, et al. Dietary restriction suppresses proteotoxicity and enhances longevity by an hsf-1-dependent mechanism in *C. elegans*. Aging Cell. Jun; 2008 7(3):394–404. [PubMed: 18331616]
55. Milkman R. Temperature Effects on Day Old *Drosophila* Pupae. J Gen Physiol. Mar 1; 1962 45(4):777–99. [PubMed: 14473895]
56. Szewczyk NJ, Udranszky IA, Kozak E, Sunga J, Kim SK, Jacobson LA, et al. Delayed development and lifespan extension as features of metabolic lifestyle alteration in *C. elegans* under dietary restriction. J Exp Biol. Oct 15; 2006 209(20):4129–39. [PubMed: 17023606]
57. Kourtis N, Nikoletopoulou V, Tavernarakis N. Small heat-shock proteins protect from heat-stroke-associated neurodegeneration. Nature. Oct 11; 2012 490(7419):213–8. [PubMed: 22972192]
58. Benedetti MG, Foster AL, Vantipalli MC, White MP, Sampayo JN, Gill MS, et al. Compounds that Confer Thermal Stress Resistance and Extended Lifespan. Exp Gerontol. Oct; 2008 43(10): 882–91. [PubMed: 18755260]
59. Seidel HS, Kimble J. The Oogenic Germline Starvation Response in *C. elegans*. PLoS ONE. Dec 2.2011 6(12):e28074. [PubMed: 22164230]
60. Ben-Zvi A, Miller EA, Morimoto RI. Collapse of proteostasis represents an early molecular event in *Caenorhabditis elegans* aging. Proc Natl Acad Sci. Sep 1; 2009 106(35):14914–9. [PubMed: 19706382]
61. Simonetta SH, Romanowski A, Minniti AN, Inestrosa NC, Golombek DA. Circadian stress tolerance in adult *Caenorhabditis elegans*. J Comp Physiol. Sep 1; 2008 194(9):821–8. [PubMed: 18648821]
62. Avery L, Shtonda BB. Food transport in the *C. elegans* pharynx. J Exp Biol. Jul 15; 2003 206(14): 2441–57. [PubMed: 12796460]
63. You Y, Kim J, Raizen DM, Avery L. Insulin, cGMP, and TGF-β Signals Regulate Food Intake and Quiescence in *C. elegans*: A Model for Satiety. Cell Metab. Mar 5; 2008 7(3):249–57. [PubMed: 18316030]
64. Gracida X, Eckmann CR. Fertility and Germline Stem Cell Maintenance under Different Diets Requires nhr-114/HNF4 in *C. elegans*. Curr Biol. Apr; 2013 23(7):607–13. [PubMed: 23499532]
65. Pang S, Curran SP. Adaptive Capacity to Bacterial Diet Modulates Aging in *C. elegans*. Cell Metab. Feb 4; 2014 19(2):221–31. [PubMed: 24440036]
66. Cypser J, Johnson TE. Hormesis extends the correlation between stress resistance and life span in long-lived mutants of *Caenorhabditis elegans*. Hum Exp Toxicol. Jun; 2001 20(6):295–6. [PubMed: 11506282]
67. Stiernagle, T. Maintenance of *C. elegans*. WormBook [Internet].. 2006. [cited 2014 Apr 9]; Available from: http://www.wormbook.org/chapters/www_strainmaintain/strainmaintain.html#d0e671

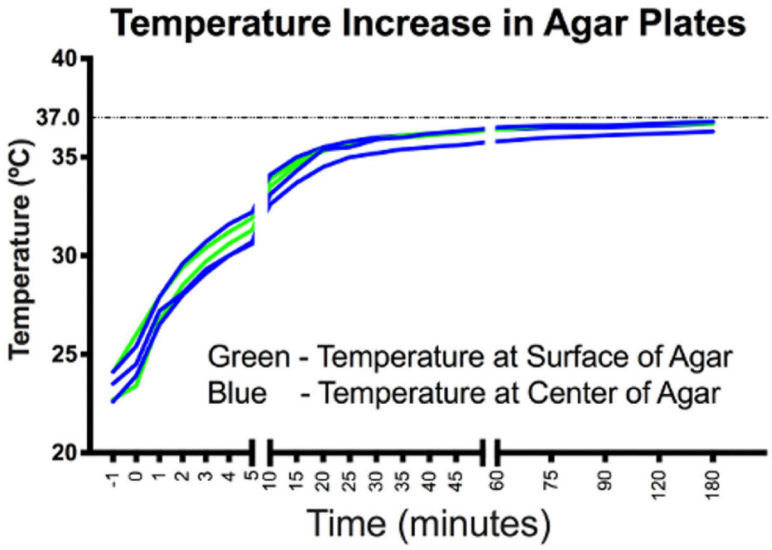

Figure 1.

Changes in agar temperature with time. Agar plates moved from a maintenance temperature (e.g. 20°C) to a 37°C incubator do not come immediately up to the temperature of the incubator. The most dramatic increase in temperature occurs within the first 5 minutes. Time -1 = room temperature immediately before being placed into the incubator, time 0 = immediately after being placed onto the pre-warmed gel pad and covered with other warm gel pads as well as possible without disturbing the thermocouple probes. Temperatures were recorded using a thermocouple attached to a digital thermometer (Omega Engineering model HH506RA).

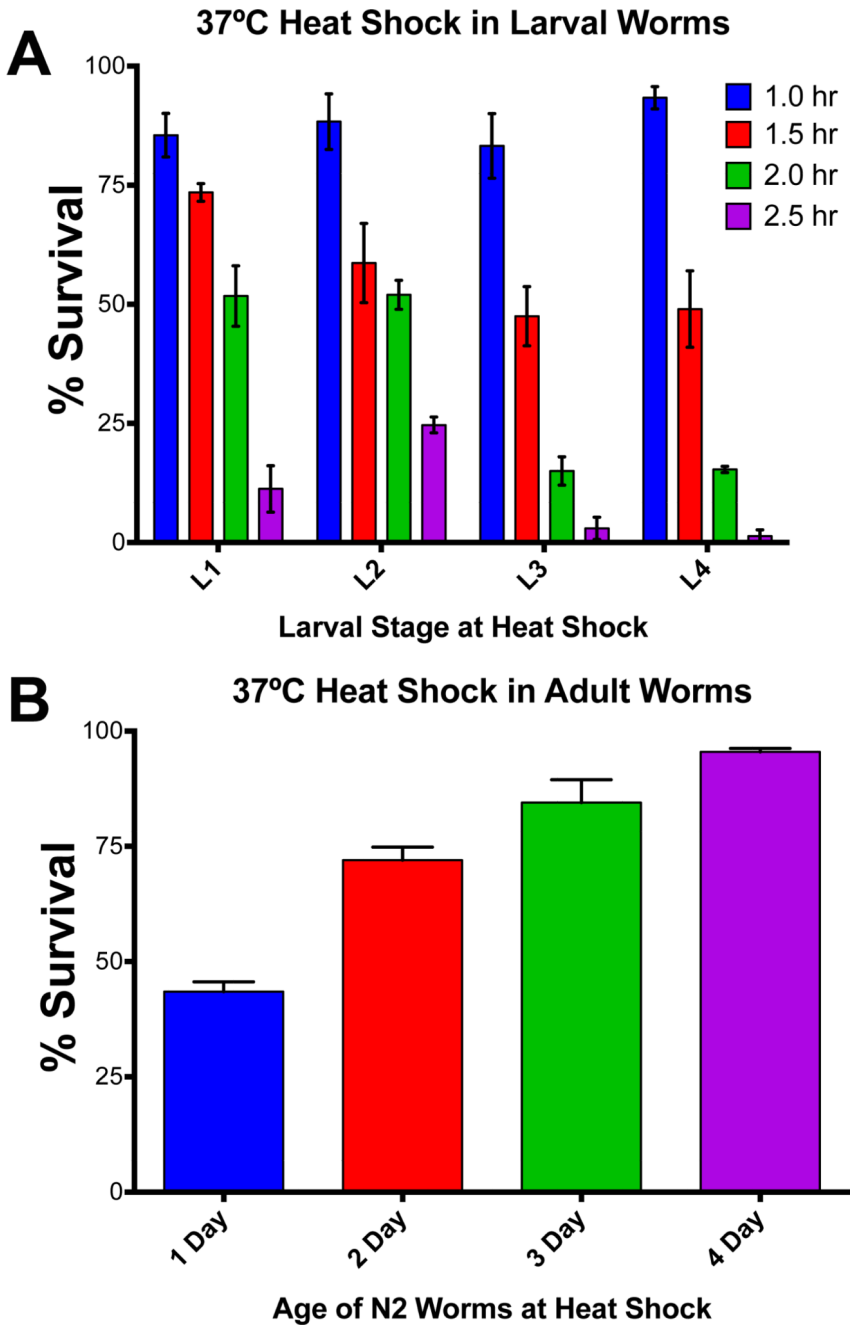

Figure 2.

Life stage differences in heat shock responsiveness. (A) Survival was compared by scoring percent (%) survival of N2 wild-type worms at each larval stage. Worm growth plates were removed from 37°C as indicated. Three or four plates/ time point were counted for each developmental stage. L1 and L2 survival was assessed 2 days post-HS (Since HS causes a developmental delay, allowing worms to fully recover increased our confidence that only worms that will ultimately survive to adulthood and produce offspring were counted). L3 and L4 survival was assessed 12 -14 hours post-HS. There are no significant differences from 1.0 to 2.5 hrs when L1 worms are exposed to 37°C. Differences in survival following

heat shock are significant beginning at 1.5 hrs of exposure to 37°C (compare L1 to L3 and L4, P=0.008 & 0.014, respectively, one-way ANOVA w/ Bonferroni correction). (B) Differences in survival of adult N2, wild-type worms when comparing worms exposed to 1.5 hours at 37°C at Day 1, 2, 3, or 4 of adulthood [71, 95, 119, 143 hours post egg-lay, respectively). Two plates were prepared as described in (A) for each age tested. Survival of day 1 worms is significantly different from day 2 and day 4 worms (P=0.02 and 0.02, respectively). Comparing day 1 to day 3 worms (P=0.054), they are not significantly different, but this is likely due to the small sample size in this experiment. Note: 1.5 hours was chosen for this experiment to ensure no plate had 0% survival.

Figure 3.

Incubator set up for heat shock experiments. The image below shows our efforts to get as much warm surface area as possible into the incubator. Shown is a Forma Scientific water-jacketed, stacked incubator, model 3327. The top shelf was chosen for the experimental space because, without the fan running, it was the shelf that most frequently stayed at the temperature set on the incubator. The middle shelf tended to be lower than desired and the bottom shelf lower still. Although this was less than a 1°C difference from top to bottom, that can be enough to affect the outcome of an experiment. The bottom shelf has a desktop

file organizer filled with gelatinous freezer packs and a small battery-operated fan. The gel pad on the second shelf is used on top of the experimental plates during heat shock.

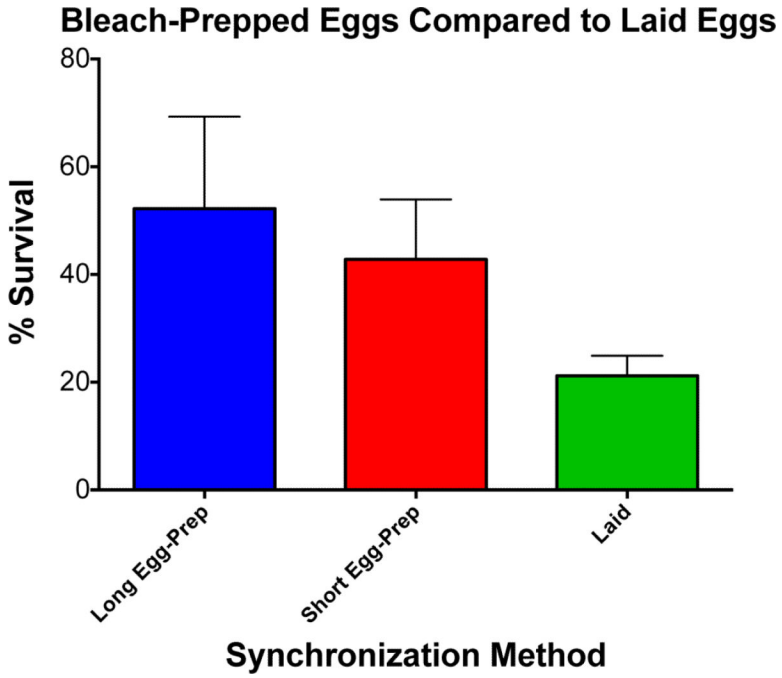

Figure 4.

Differences in survival following heat shock when eggs are laid directly onto plates compared with bleach-prepped eggs. Laid-egg plates were prepared as described in Section 3.2.1. Bleach-prepped eggs were prepared using a Wormbook protocol for decontaminating worm stocks [67]. The worms described as short bleach-prep were in lysis buffer approximately 10.5 minutes, only long enough for the worms to lyse, spin down, and be rinsed. The long bleach-prep worms were left in lysis buffer a total of approximately 14.5 minutes. Five plates were prepared for each condition and placed into a 37°C incubator for 1.5 hours as described in Section 3.2.1. 1.5 hours was chosen for this experiment instead of two to make sure no plates had 0% survival. The number of worms/plate ranged from 63 to 180 with an average of 105 worms/plate. Mean survival on the laid-egg plates was 21% and is significantly different from the short bleach-prepped plates, which had a mean survival of 45% and the long bleach-prepped plates, which had a mean survival of 52% (P=0.0359 and 0.0039, respectively, one-way ANOVA w/ Tukey's multiple comparison test).