CHAPTER ONE

Heat shock and thermotolerance in *Caenorhabditis elegans*: An overview of laboratory techniques

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Abstract

The soil nematode worm *Caenorhabditis elegans* is a simple and well-established model for the study of many biological processes. Heat shock and thermotolerance assays have been developed for this nematode, and have been used to decipher the molecular relationships between thermal stress and aging, among others. Nevertheless, a systematic and methodological comparison of the different approaches and tools utilized is lacking in the literature. Here, we aim to provide a comprehensive summary of the most

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commonly used strategies for carrying out heat shock and thermotolerance assays that have been reported, highlighting specific readouts and scientific questions that can be addressed. Furthermore, we offer examples of thermotolerance assays performed with wild type nematodes, that can serve as a gauge of the animal survival under diverse conditions of stress.

1. Introduction

Caenorhabditis elegans is a wild free-living nematode. It has a small size (adults are 1 mm long), which makes animals easy to handle and allows researchers to cultivate them without the need of elaborate or expensive animal facilities. Their body is covered by a transparent cuticle, so one can easily observe individual cells and monitor developmental and physiological processes. Moreover, tagging different subcellular compartments with fluorescent proteins can be used to discern cellular or subcellular characteristics with enhanced detail (Eisenmann, 2005). *C. elegans* has a simple nervous system of just 302 neurons. Moreover, the genome of the nematode has been fully analyzed and annotated. Notably, 38% of the protein-coding genes of *C. elegans* have orthologues in the human genome and 60–80% of human genes have orthologues in the *C. elegans* genome (Markaki & Tavernarakis, 2020). In addition, 40% of human disease-associated genes have orthologues in *C. elegans*.

C. elegans can survive and reproduce across a range of temperatures from 12 to 26 °C (Schulenburg & Félix, 2017). As an ectotherm, *C. elegans* body temperature is determined by the environment. These animals suffer changes of temperatures along the day and night, and they have to seek temperatures conducive to survival and avoid dangerous temperature ranges. Thermosensation is critical for nematodes (Hoffstaetter, Bagriantsev, & Gracheva, 2018), which are able to detect and respond to temperatures changes as small as 0.01° in the laboratory (Ramot, MacInnis, & Goodman, 2008). The median lifespan of nematodes decreases with temperature, with the populations cultured at 25 °C (high temperature) to display the shortest median lifespan and those cultured at 15 °C (low temperature) to show the highest median lifespan (Xiao et al., 2013). Moreover, short term increases in environmental temperature cause heat stress and affect several aspects of nematodes' physiology from tissue and cellular integrity to life expectancy. High exposure to HS often causes tissue damage and shortens life expectancy, whilst short or mild stress induces a hormetic effect and promotes stress resistance and longevity (Prahlad, Cornelius, & Morimoto, 2008).

Nematodes have evolved diverse responses to exogenous and endogenous stresses in order to maintain homeostasis (Kumsta et al., 2017). Heat Shock Factor 1 (HSF-1 in nematodes) is the master regulator of the Heat Stress Response (HSR). Specifically, in response to increased temperature, the transcription factor HSF-1 binds to heat shock elements (HSEs) in the promoters of heat-inducible genes and induces expression of heat shock proteins (HSPs) and molecular chaperones (Guhathakurta et al., 2002). The target genes of HSR aim to eliminate toxicity caused by HS, which mainly involves accumulation of misfolded proteins. Interestingly, proteotoxicity deriving from unfolded protein accumulation often accompanies aging and contributes to the onset of neurodegenerative diseases (Richter et al., 2010). Therefore, overexpression of HSF-1 has been found to promote longevity, improve stress response and protect against age-related disease development and/or aggravation (Rattan, 2005; Zeng et al., 2021).

Apart from transcriptional reprogramming upon HSR, several other factors have been shown to impact the ability of animals to respond to HS. Epigenetic regulators (cbp-1 and swsn-1) and a stress-activated protein kinase (pmk-1) enhance resistance to pathogens, promoting longevity after early heat stress (Bokszczanin, Solanaceae Pollen Thermotolerance Initial Training Network (SPOT-ITN) Consortium, & Fragkostefanakis, 2013; Zhou, He, Deng, Pang, & Tang, 2019). Protein phosphorylation is essential in the regulation of nematode activities at high temperatures (Huang, Wu, & Zhang, 2020). In addition, phosphorylation is a key regulator that enables signal conversion in response to external environmental stimuli for a short period of time, indicating that it may also mediate mild responses to temperature stress in early life stages. Finally, besides the cell autonomous benefits conferred by the HSR, its cell non-autonomous effects are starting to emerge. Specifically, HSF-1 activation in thermosensory neurons triggers a HSR in distal tissues, like the intestine, the body wall muscles and reproductive organs, conferring protection against proteotoxicity (Fig. 1) (Douglas et al., 2015; Prahlad & Morimoto, 2009; Tatum et al., 2015).

Aging is the time-dependent functional decline that affects most living organisms. A better understanding of the molecular mechanisms during age-associated health decline is essential in the search for a therapy of age-related diseases. Hallmarks that represent common denominators of aging in different organisms like chronic inflammation (Gomez, 2021) proteostasis decline (Fernández-Fernández, Gragera, Ochoa-Ibarrola, Quintana-Gallardo, & Valpuesta, 2017; Ferreira, da Rosa Soares, & Pereira, 2022), mitochondrial dysfunction (Li, Berliocchi, Li, & Rasmussen, 2024) and resistance to insulin (Karpe & Tikoo, 2014; Kuppuswami & Senthilkumar, 2023), are

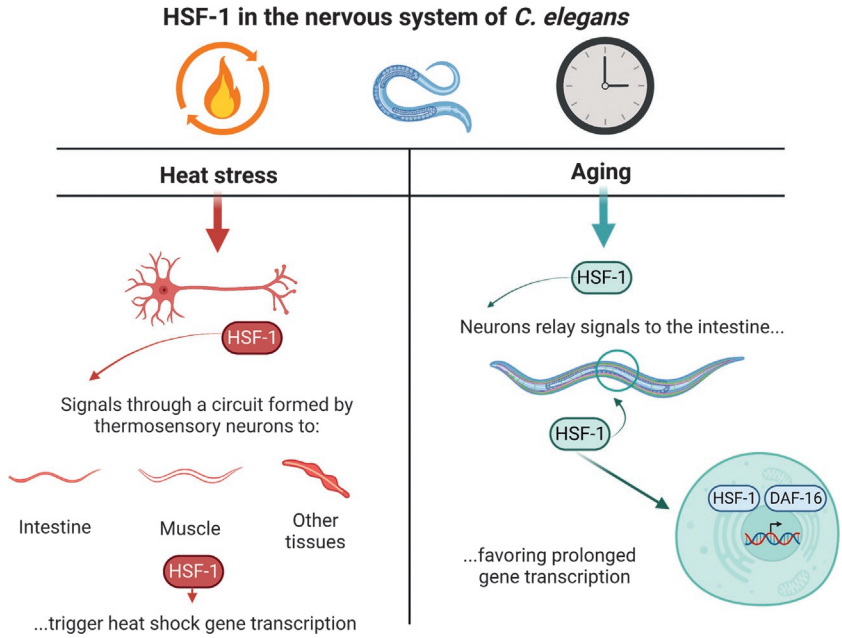

Fig. 1 HSF-1 in the nervous system of *C. elegans*. HSF-1 signals through thermosensory neurons and favors transcription of heat shock genes. It is also related with aging (Douglas, 2015).

associated with a decline in HSF1 function (López-Otín, Blasco, Partridge, Serrano, & Kroemer, 2023). Therefore, it becomes apparent that the identification of novel factors that regulate HSF-1 and HSR may provide new tools to combat age-related decline.

Here we describe published methods to assess nematodes thermotolerance highlighting the read outs and the scientific question that can be answered each time. Such assays can be used to screen for conditions that affect HSR and HSF-1 activation with critical impact on the animals' stress response in diverse genetic backgrounds. Furthermore, we offer an example of thermotolerance assays performed in our laboratory with wild type nematodes strain (N2) that can serve as a guide of the animal survival/mobility upon thermal stress.

2. Materials and methods

2.1 Materials

1. Pipette tips 2–200 μL Eppendorf® epT.I.P.S. (Eppendorf, catalogue number: 022492039)

2. Pipette tips 50–1000 µL Eppendorf® epT.I.P.S. (Eppendorf, catalogue number: 022492055)
3. Petri dishes 60 × 15 mm 500/cs (Fisher Scientific, catalogue number: FB0875713A) or Petri dishes 35 × 10 mm 500/cs (Fisher Scientific, catalogue number: FB0875711YZ)
4. Eppendorf® Safe-Lock 1.5 mL microcentrifuge tubes (Eppendorf, catalogue number: 022363204)
5. Toothpick
6. 99.95% Platinum, 0.05% Iridium wire (3 ft./pk) (Tritech Research, catalogue number: PT-9901)
7. OP50 *Escherichia coli* bacteria (University of Minnesota, *C. elegans* Genetics Center, MN)
8. HT115 *E. coli* strain (Source BioScience)
9. Experimental and control *C. elegans* strains (University of Minnesota, *C. elegans* Genetics Center (CGC), MN)
10. Sodium chloride (NaCl) (Sigma-Aldrich, catalogue number: S7653)
11. BactoPeptone (BD, BactoTM, catalogue number: 211677)
12. Agar (Sigma-Aldrich, catalogue number: A1296)
13. Commercial Bleach 60 g/L
14. Potassium phosphate monobasic (KH_2PO_4) (Sigma-Aldrich, catalogue number: P5655)
15. Cholesterol (Sigma-Aldrich, catalogue number: C8667)
16. 100% ethanol (Sigma-Aldrich, catalogue number: E7023)
17. Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) (Sigma-Aldrich, catalogue number: M1880
18. Calcium chloride dihydrate ($CaCl_2.2H_2O$) (Sigma-Aldrich, catalogue number: C3881)
19. Potassium phosphate dibasic (K_2HPO_4) (Sigma-Aldrich, catalogue number: P2222)
20. Sodium hydroxide (NaOH) (Sigma-Aldrich, catalogue number: S8045)
21. Streptomycin sulfate salt powder (Sigma-Aldrich, catalogue number S6501)
22. Nematode growth medium (NGM) (see Recipes)
23. M9 buffer (see Recipes)
24. 5 mg/mL cholesterol (see Recipes)
25. 1 M $MgSO_4$ (see Recipes)
26. 1 M $CaCl_2$ (see Recipes)
27. Phosphate buffer (see Recipes)
28. 1 N NaOH (see Recipes)
29. S buffer (0.1 M NaCl and 0.05 M potassium phosphate buffer pH 6.0)

2.2 Equipment

1. Erlenmeyer flask (Fisher Scientific, catalogue number: FB5006000)
2. Pipetor (Gilson, catalogue number: F167300)
3. Platinum worm pick. Worm picks can either be purchased (Genesee Scientific, catalogue number: 59-AWP) or made in-house as described in Wollenberg, Visvikis, Alves, and Irazoqui (2013)
4. Pasteur glass pipette (Fisher Scientific, catalogue number: 22-378893)
5. Autoclave
6. Stirring hotplate (Corning, catalogue number: 6795-620)
7. Centrifuge (Eppendorf, model: 5430)
8. Tabletop centrifuge (Eppendorf, model: 5424)
9. Pellet pestle (Kimble Chase Life Science and Research Products, catalogue number: 7495211590)
10. Refrigerated incubator (Thermo Fisher Scientific, Thermo ScientificTM, model: HerathermTM General Protocol Microbiological Incubators, catalogue number: 51028064)
11. Bunsen burner (Humbolt, catalogue number: H-5870)
12. Dissecting stereomicroscope (Olympus, model: SMZ645)
13. Incubators for stable temperature (AQUA® LYTIC incubator 20 °C)
14. Freezers (−20 °C; So-Low Environmental Equipment) (Siemens, model: C85-22)
15. Water bath
16. 96-Well plate transparent bottom (Greiner Bio-One International GmbH, Kremsmünster, Austria)
17. Tecan Infinite NANO+ plate reader (Tecan Group Ltd., Männedorf, Switzerland).
18. Gas perfused chamber (Echotherm IN35 incubator)
19. Wheaton Unispense peristaltic pump liquid dispenser with digital display (American Laboratory Trading, model: 374301)

2.3 Maintenance of *C. elegans*

Maintain worms at 20 °C on Nematode Growth Medium (NGM) plates seeded with OP50 strain of *E. coli* bacteria by transferring approximately five to seven L4 animals per plate, depending on their ability to grow, self-fertilize and give rise to progeny. If a specific strain has a rapid life cycle it is better to transfer less than five L4 animals to avoid starvation. On the contrary, up to 10–15 worms can be transferred if a mutant strain grows slowly.

Note: Take special care that they do not run out of food as starvation affects their physiology and results.

Preparation of NGM plates

- Mix 6 g NaCl, 5 g BactoPeptone, 34 g Agar, 0.4 g streptomycin and deionized H_20 up to 1.5 L in a flask.
- Autoclave for sterilization.
- Let the mixture cool down to 55 °C.
- Add 2 mL $CaCl_2$ (1 M), 2 mL $MgSO_4$ (1 M), 2 mL Cholesterol (5 mg/mL), 2 mL Nystatin (10 mg/mL), 50 mL KPO4 (1 M) and deionized H_20 to full volume (2 L). All the solutions and water added to the medium after autoclave must also be priorly sterilized through autoclave.
- Pour NGM medium in P60 Petri dishes (P60: 6 cm) with a peristaltic pump machine. Poor 7 mL NGM medium per plate.
- Let the plates dry for 1–2 days at RT.

Seeding NGM plates with OP50

- Grow an overnight OP50 culture in LB medium at 37 °C.
- Add 300 µL of the OP50 culture in every plate.
- Let plates dry and OP50 grow by consuming cholesterol available on NGM overnight at RT. After that store them at 4 °C.

2.4 Synchronization of worms

Worm synchronization can be achieved by egg laying or hypochlorite treatment (bleaching). For egg laying 10–15 gravid adults are placed on a seeded plate for 1–2 h at 20 °C and are allowed to lay eggs which hatch with 1–2 h time interval. Alternatively, eggs can be collected from gravid adults by hypochlorite treatment (bleaching) which dissolves their body and releases the embryos. Hypochlorite treatment consists of the following steps:

- Collect *C. elegans* using 1 mL/plate of M9.
- Centrifuge 2 min 8000 rpm or 1 min full speed.
- Discard 700 µL supernatant. Keep the pellet.
- Add 700 µL bleaching buffer (7 mL dH_2O + 2 mL NaOCl (bleach) + 1 mL NaOH (5 N)).
- Shake or vortex 5 min until the bodies are dissolved and the eggs are released in the bleaching solution (check periodically in the stereoscope).
- Centrifuge 2 min 8000 rpm.
- Discard supernatant and resuspend egg pellet in 1 mL of M9.
- Centrifuge 2 min 8000 rpm and repeat the wash in M9 twice.
- Discard supernatant.

- Resuspend the pellet in M9.
- Divide the egg suspension in the desired number of plates.

Note: Synchronization by hypochlorite treatment is not as precise as egg laying, since the collected embryos may hatch with an up to 12 h time interval. The common practice to precisely synchronize them is to allow them hatch in liquid medium without food and produce arrested L1 larvae. Upon refeeding, all arrested L1 larvae resume growth simultaneously and are precisely synchronized. However, one should consider that starvation in early developmental stages cause epigenetic alterations which persist and affect the life of the adults and their descendants (Markaki & Tavernarakis, 2020). Specifically, extended L1 arrest is able to increase thermotolerance of adults as well as of their F3 generation progeny. Therefore, synchronization by L1 arrest is not preferred for thermotolerance assays.

3. Technical considerations for thermotolerance assays

Heat shock can be performed either in a water bath or in an air incubator at the indicated temperature.

Note: The use of non-heat shocked control population is desirable; control *C. elegans* should be maintained at 20 °C for the duration of heat shock.

Water bath (adapted from Golden et al., 2020 and Smolentseva et al., 2017)

(1) Pick 20–40 worms at the specific age to fresh NGM plates. Use at least 4 plates per condition to ensure reproducibility and statistical significance.

(2) Wrap a thin trip of paraffin film twice around the plate to seal the edges.

Note: Do not cover the bottom of the plate to avoid interference with heat transfer.

Note: Ensure that the paraffin film is secure, then water will enter the plate and the plate should not be used for data collection.

(3) Submerge the plates in a circulating water bath at the indicated temperature for the indicated time.

Note: Submerge the plates upside down using a test tube rack and a lead weight.

Note: Include a negative control sample, i.e., no heat shock, but submerged in a water bath.

(4) Remove the plates from the water bath and drying with a paper towel.

(5) Remove the paraffin film to recover the worms

(6) Incubate the *C. elegans* at 20 °C for the desired time to allow recovery from heat shock.

Incubator (adapted from Kumsta et al., 2017 and used in our laboratory)

(1) For each strain, incubate 2–4 6 cm NGM plates with ~20–50 animals per plate of the desired age in a single layer in an air incubator at the indicated temperature for the indicated time.

Note: During both heat shock and recovery period plates should be placed upside down in a single layer, not stacked on top of each other. This arrangement ensures that the worm containing part of the plate does not come to close contact of with the metal parts of the incubator, and thus consistent temperature to all the plates. It is desirable to use a dedicated incubator that will not frequently open thus its temperature will not fluctuate.

4. Thermotolerance assays

4.1 Heat stress protocols for nematodes

Several protocols have been put forward to assay the capacity of nematodes to endure heat stress and elicit the HSR. These thermotolerance assays have been conducted with worms at a variety of ages and temperatures, ranging from 33 to 37 °C (Kiliçgün et al., 2011; Kiliçgün & Göksen, 2012; Rollins et al., 2017).

Well-established molecular biology techniques (qPCR, Western Blot, etc.) and behavioral assays, among other technical approaches, can be used to assess the effect of heat shock. Nonetheless, most studies evaluate survival and mobility of animals upon heat shock and recovery as a measurement of its thermotolerance capacity.

Furthermore, in order to provide a guidance for experimental design, we conducted two different thermotolerance survival assays on wild type worms (N2 strain).

1. Long-term survival after short heat shock

Animals can be stressed for a short period of time and their ability to cope with stress is assessed by their life expectancy after HS.

(a) Incubate nematodes at high temperature (e.g., 33 or 37 °C) for a few hours (e.g., 1–3 h) (Guisbert et al., 2013; Tecle et al., 2021).

Note: In all *C. elegans* assays, starved worms behave in a different way and are a source of uncontrolled variability. Hence, if the plate contains starved worms, it is necessary to chunk or transfer the animals to new plates.

Note: In all thermotolerance assays, age is an important factor that affects survival and other readouts upon heat shock response. Thus, synchronization of worms is important prior to subject the animals to stress. The same protocol albeit with older nematodes (day 5 of adulthood), different results may be observed (Smolentseva et al., 2017).

Note: An extra step of recovery period at room temperature for 30 min can be used to allow the animals to adapt gradually to their normal growing temperature (20 °C) (Tecle et al., 2021).

(b) Transfer heat stressed nematodes back to 20 °C incubator.

(c) **Assess long-term survival after heat shock**. Score for survival and transfer animals to new plates every day to avoid progeny.

Note: Importantly, at least 50 individuals should be used for each experiment (which fit on one 60 mm NGM plate) and at least 3 experimental replicates per condition are necessary to reach a robust conclusion.

Example: We measured survival after heat shock during 2 weeks. For this purpose, we distributed 100 worms (D1) among two NGM plates and incubated them at 37 °C for 2 h, 3 h and 4 h, or left untreated at 20 °C (0 h). Immediately after heat shock, worms were placed at 20 °C and their survival was assessed every 24 h (both mobile and immobile worms were count as alive). As shown in Fig. 2, the decrease in survival is dependent on the time that worms were heat shocked, with a sharp decrease in survival observed in worms that were incubated at 37 °C for 4 h. Remarkably, the highest percentage of mortality was observed during the first 24–48 h and worms that survived the first 4 days were mobile and very likely to survive for longer periods. Therefore, we suggest incubation of worms for 3 h at 37 °C as the best time-point for comparison among untreated wild type worms and treated/knock out worms.

2. Assess survival after short heat shock automatically

Resistance to heat stress can be measured with an automated survival scoring method (LFASS) based on detecting blue fluorescence, which is considered death (excitation = 360; emission = 435).

(a) Add 200 worms/well to a black 96-well plate with a transparent bottom, which was placed in a Tecan Infinite NANO + plate reader preheated at 40 °C.

(b) Add sufficient frozen *E. coli* HT115 to prevent starvation, according to standard liquid culture protocols (Hibshman et al., 2021).

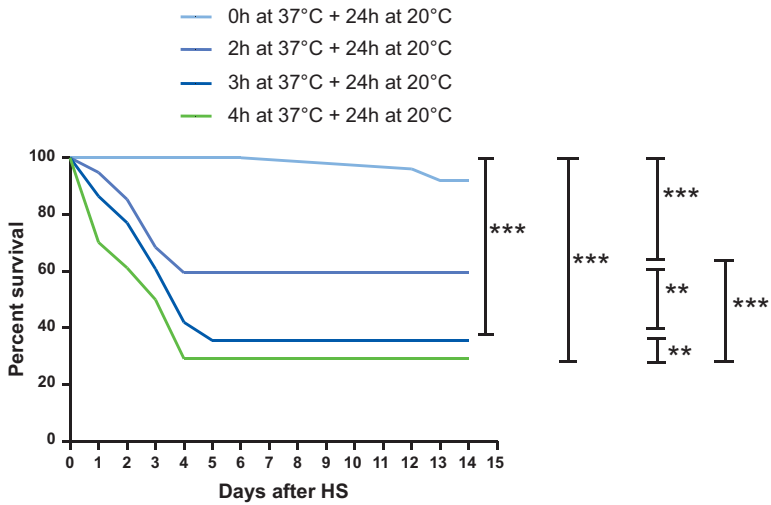

Fig. 2 Long-term survival upon heat shock. Representative survival curves of worms incubated at 37 °C for 0, 2, 3 or 4 h are depicted. Statistical significance was measured by Log-rank (Mantel-Cox) Test (**P* value ≤0.05; ***P* value ≤0.01; ****P* value ≤0.001).

To avoid the plates from drying out during heat shock, the wells with no worms are filled with water and then the plate is sealed with Parafilm.

(c) Measure fluorescence at 2 min interval time over 24 h.

Note: The median survival of the worm population is deduced by the time point where the half-maximal blue fluorescence is achieved. For the wild type strain median survival is approximately 100 min under these conditions. The average percentage survival and standard deviation can be calculated with triplicates and statistical analysis can be applied (Zečić et al., 2022).

3. HSR gene/protein expression after short HS

After short HS (e.g., incubation of nematodes at high temperature [e.g., 33 °C or 37 °C] for a least 10 min and up to a few hours [e.g., 1–3 h], similarly to protocol 1), the HSR may be assessed by expression of HSF-1 target genes and their protein products. It is imperative to allow animals to recover at 20 °C for 6–24 h after HS, prior to collecting RNA or protein extracts for these protocols. The recovery period allows for transcription and translation of these genes as well as for protein folding and fluorescence of fluorescent reporters.

4. **Chronic heat shock protocol**

In this approach we assess the survival of animals upon severe HS which lasts for longer periods and/or with higher temperatures (Jovic et al., 2019) or 16 h (Wilson et al., 2006). A survival curve at the elevated temperature could then be formed. In this type of assays one can also assess for mobility instead of death as an end point.

(a) Gather 100 synchronous animals at Day 1 of adulthood and place them in one plate. Use as many plates as the time points that need to be assessed.

(b) Heat shock nematodes at 37 for up to 8 h. Conversely, day 1 adult worms can be heat shocked at lower temperatures (e.g., 33 °C) for longer periods (e.g., more than 8 h). Worms of different ages exhibit different resistance to HS thus the time points need to be set accordingly.

(c) At the end of each time point transfer the respective plate with the HS animals at 20 °C for a recovery period of 20–60 min.

(d) **Assess survival and/or mobility of the HS animals**. Animals that are freely moving or move forward/backwards upon gentle touch are considered alive and mobile. Animals that move only their head or tail region of the body either freely or upon gentle touch are considered alive but immobile. Animals that do not respond to gentle touch are scored as dead.

Example: We performed this experiment by distributing 100 worms (day 1 of adulthood [D1]) in each NGM plate and incubated them at 37 °C for the indicated time. We use a different NGM plate for every time point tested. At the end of the HS period the respective plate was moved to the 20 °C degree incubator for 20 min to allow for recovery. At the end of the recovery period the animals were scored as mobile, immobile, and dead. Fig. 3 shows the percentage of the total number of worms falling into each category at each time point.

5. **Hormetic assays**

The concept of hormesis posits that a mild stress instigates protective responses that increase fitness, stress response and survival. Similarly, nematodes that encounter a mild heat stress early in their lives display advantages later in adulthood. Induction of heat-shock proteins enhances proteostasis and lifespan and play a protective role in the pathological context of heat-stroke-associated neurodegeneration (Kumsta et al., 2017; Kourtis et al., 2012).

(a) Plate 20–40 worms at the L4 stage of development.

(b) Heat shock (preconditioning) worms for 15 min at 34 °C

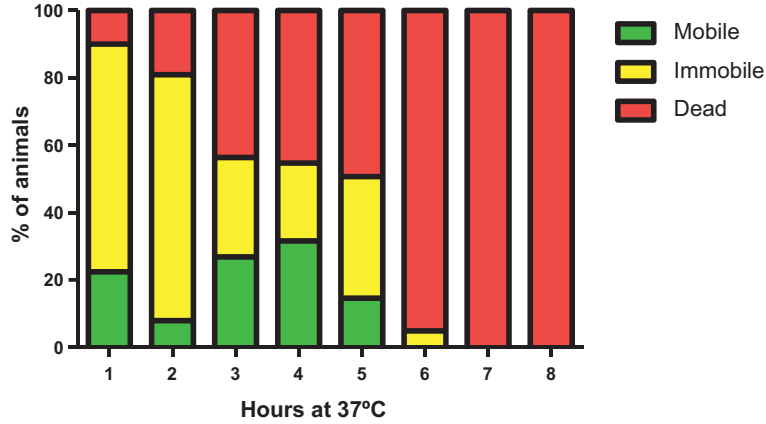

Fig. 3 Short-term survival and mobility upon heat shock. Representative graphical representation of mobile (free movement), immobile (movement upon touch) and dead nematodes were counted upon incubation at 37 °C for the indicated time and a recovery period of 20 min at 20 °C.

(c) Incubate worms at 20 °C for 10 min for recovery

(d) Heat shock (second stress) worms for 15 min at 39 °C

(e) Assess survival (see Fig. 2)

Note: As previously mentioned, age affects the effect of heat shock. Exposure on days 1, 3 and 5 of adulthood improves stress resistance and survival. The greatest effect is observed on day 1, whereas there is no significant effect on day 7, consistent with the idea that the ability of animals to respond to a hormetic treatment drops with age (Kumsta et al., 2017).

Note: Morphological signs of neurodegeneration and neuronal cell death can also be assessed by using fluorescent reporter strains and confocal microscopy. The presence of remnants of neuron cell bodies and neuron corpses, as well as axonal beading, indicate neurodegeneration.

5. Concluding remarks

Here we provide a collection of simple methods to assess thermotolerance in nematodes. Each protocol has slight variations and is suitable for different studies. Some of the presented methods could be used for high throughput screening for new factors that affect HSR. Moreover, we present examples of these methods performed with wild type animals, which can be used as a guide to choose the appropriate conditions to compare wild

type to mutants or animals subjected to specific treatments. We anticipate that the advancement of multi-omics technics will revisit the HSR and we propose the use of *C. elegans* as a simple model organism, to screen for novel regulators of this response mechanism, that could serve as therapeutic targets for age-related diseases.

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