Comparing the effect of heat shock on locomotion, posture and head oscillation of wild-type (N2 strain) and mutant (*VC854* strain) *Caenorhabditis elegans*

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ABSTRACT

In response to extreme temperatures, *Caenorhabditis elegans* combat cellular stress through activation of a heat shock transcription factor (HSF-1). The downstream effects of HSF-1 can lead to a sleep-like state in nematodes, characterized by decreased feeding and locomotion. Our objective was to compare sleep-state responses exhibited by the wild-type (N2) and mutant (*VC854*) strains during heat shock. Our experiment implemented an air incubation technique, exposing the nematodes to 35ºC for 30 minutes in the heat shock treatment and a control treatment at 15ºC. The mean number of head oscillations in a 30 second interval, posture and locomotion were measured and recorded for each replicate. Following heat shock treatment, the average number of head oscillations by the mutants was 4 ± 2 (SD: 3.32), while the average number of head oscillations by the wild-type was 9 ± 3 (SD: 5.88). Our results suggest that the effect of heat shock at 35˚C on the activity of mutants (*VC854*) was not greater than the effects seen on wild-type *C. elegans*. A possible explanation for this phenomenon is the restricted locomotive potential conferred by the *unc-2* gene deletion mutation in the *VC854* strain.

INTRODUCTION

Caenorhabditis elegans nematode worms have a nervous system comprised of only 302 neurons and move by generating an oscillatory neuromuscular wave through alternation of dorsal and ventral muscles (Kaletta and Hengartner 2006). A meta-analysis compiled by Zevian and Yanowitz (2014) noted the optimal temperature for growth of *C. elegans* to be between 16°C to 26°C, and the optimal temperature for experimental heat shock to be around 35ºC. At extreme temperatures, *C. elegans* exhibit a behavioural pattern of thermotaxis, evidenced by the migration of nematodes away from areas of elevated or decreased temperatures and toward their cultivation temperature (Hedgecock and Russell 1975). Under prolonged noxious heat exposure (30 minutes heat shock at 35˚C), suspension of the thermotactic behaviour typically occurs, at which point *C. elegans* demonstrate decreased evasive ability and are rendered immobile after

just two minutes (Hedgecock and Russell 1975). To explain this immobilization, Hill *et al.* (2014) found that *C. elegans* enter a protective sleep-like state when exposed to cellular stresses such as heat shock. The model of heat shock response in *C. elegans* is dependent on the thermosensory neuron, AFD, which senses ambient temperature and regulates temperature-dependent behaviour (Prahlad *et al.* 2008). The heat shock response in *C. elegans* is coordinated by the ubiquitously expressed heat shock transcription factor-1 (HSF-1), which acts to induce transcription of specific genes to combat the stressful stimulus (Morton and Lamitina 2013). The downstream effects of the pathway include a sleep-state response, acting to reduce locomotion, feeding activity, and anatomical curvature (Nelson and Raizen 2013).

Our study focuses on two specific strains of *C. elegans*; the wild-type (N2) and mutant (*VC854*) strain. Organisms of the mutant (*VC854*) strain are characterized by the deletion of the *unc-2* (gk366) gene, which is required for normal locomotion and stress induced modulation in neurons through the *unc-2*/TGF beta pathway (Schafer and Kenyon 1995). The *unc-2* gene encodes a voltage-activated calcium channel, and is expressed primarily in motor neurons and several sensory neurons (Bargmann 1998). The observed phenotype of the mutant (*VC854*) strain displays a slower and more uncoordinated movement pattern in comparison to the wild-type strain.

Building on the preliminary studies discussed above, our objective was to compare the physiological and behavioural responses of wild-type and mutant (*VC854*) strains of *C. elegans* under induced temperature stress. In particular, we were interested in testing how heat shock could affect the activity (reflected in the rate of head oscillation) of both wild-type and mutant *C. elegans.* This investigation is applicable to human models, as the product of *unc-2* gene expression in *C. elegans* has been found to function similar to a human P/Q-type voltage gated

calcium channel (Bargmann 1998). Thus, investigation of *C. elegans* under temperature stress can provide insight into similar body structures and responses of the human body (Mignot 2008). Moreover, a sleep-like state similar to *C. elegans*' has been exhibited in other animal species; therefore our research may help in understanding heat shock behavioural responses that are observed throughout the animal kingdom (Nelson and Raizen 2013).

With consideration of the above, our proposed hypotheses are:

H_{o1}: Heat shock at 35˚C increases or has no effect on the activity (head oscillations per thirty seconds) of *C. elegans.*

H_{a1}: Heat shock at 35˚C decreases the activity (head oscillations per thirty seconds) of *C. elegans.*

H_{o2}: Presence of the *unc-2* mutation increases or has no effect on the activity (head oscillations per thirty seconds) of mutant (*VC854*) *C. elegans.*

H_{a2}: Presence of the *unc-2* mutation decreases the activity (head oscillations per thirty seconds) of mutant (*VC854*) *C. elegans.*

H_{o3}: The effect of heat shock at 35˚C on the activity (head oscillations per thirty seconds) is the same or less in the mutant (*VC854*) than wild-type (N2) *C. elegans.*

H_{a3}: The effect of heat shock at 35˚C on the activity (head oscillations per thirty seconds) is greater in the mutant strain (*VC854*) than wild-type (N2) *C. elegans.*

METHODS

In order to reduce variability, our experimental design included two trials: one specifically for wild-type (N2) *C. elegans*, and one for mutant (*VC854*) *C. elegans.* Populations of the wild-type and mutant *C. elegans* were initially cultured at 15˚C in *Escherichia coli* medium. We then analyzed the nematodes and manually removed organisms that were in the distinctive L4 life-stage from the population in order to control for age effects. Nematodes at the L4 stage were recognized by having a distinct vulva, a half-moon shaped structure on the medial portions of their bodies. We subsequently placed three L4 replicates from the population dish into separate agar plates containing *E. coli*, thus ensuring each treatment plate consisted of the same number of replicates, and consistency among the life cycle stage of all replicates. In total, we prepared 48 plates; 24 plates for each strain. The 24 plates in each trial were placed in a 15°C air incubator for approximately two hours to limit further growth of the L4 nematodes into full-grown adults.

We split the 24 plates of each strain into two divisions: control plates and treatment plates. As only 12 plates could be measured at one time so we kept the other half of the plates in the 15°C incubator. Administration of heat shock occurred by placing the 12 designated heat shock plates into an air incubator at 35˚C for 30 minutes, as these conditions have been proposed to induce heat shock environments that are non-lethal to *C. elegans* (Zevian and Yanowitz 2014). Our technique of administering heat shock followed a staggered pattern. We placed two plates into the 35˚C air incubator at the conclusion of every 3-minute interval, for a total duration of 6 rounds. After 18 minutes, we removed the first pair of plates that had been exposed for a period of 30 minutes for analysis. Subsequent pairs of plates were removed from the incubator at the conclusion of 3-minute intervals and analyzed.

We analyzed and recorded measures for the control and heat shock replicates in the same manner. Following treatment, plates were placed under a Kyowa optical dissecting microscope and the first of the three nematodes in the field of observation was selected for analysis of its sleep-state characteristics and movement. We recorded the initial posture of the replicates based on two parameters: sinusoidal, as seen in Figure 1A or curved/straight, as seen in Figure 1B. Immediately following the initial observation, we stimulated the replicates with a heated platinum worm pick and recorded their movement with a DinoXcope digital microscope in a 30 second clip, which was later analyzed to record the number of observable head oscillations in the 30 second interval. In addition, we classified the initial locomotion of the nematodes qualitatively in terms of exhibiting either forward, backward, or no movement; all directions relative to the head. All replicates that were classified as having no movement were confirmed to be alive, as they continued to show minor head oscillations after a short recovery period of a few minutes. Following the completion of experimental analysis, the nematode was removed from the plate and terminated, in order to ensure that no replicate was observed twice. The entirety of our methodological procedure of our experiment can be visualized in Figure 2.

For quantitative analysis, we performed a two-way ANOVA test on the mean head oscillation data from both the wild-type and mutant (*VC854*) *C. elegans*.

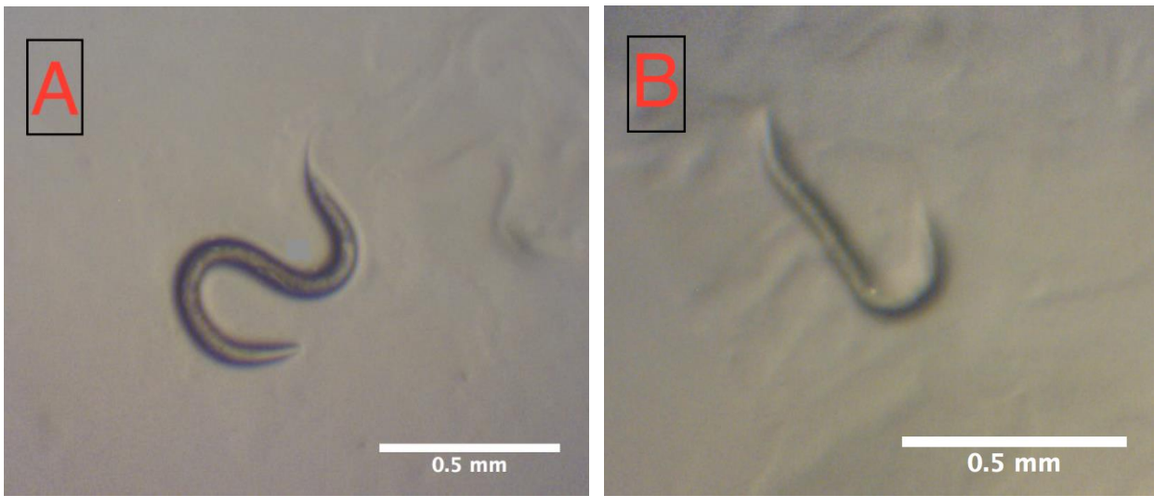

Figure 1. Image captured with DinoXcope microscope eyepiece camera that shows a **(A)** "sinusoidal" heat shock wild-type (N2) and **(B)** "curved" heat shock wild-type *C. elegans* at 45x magnification.

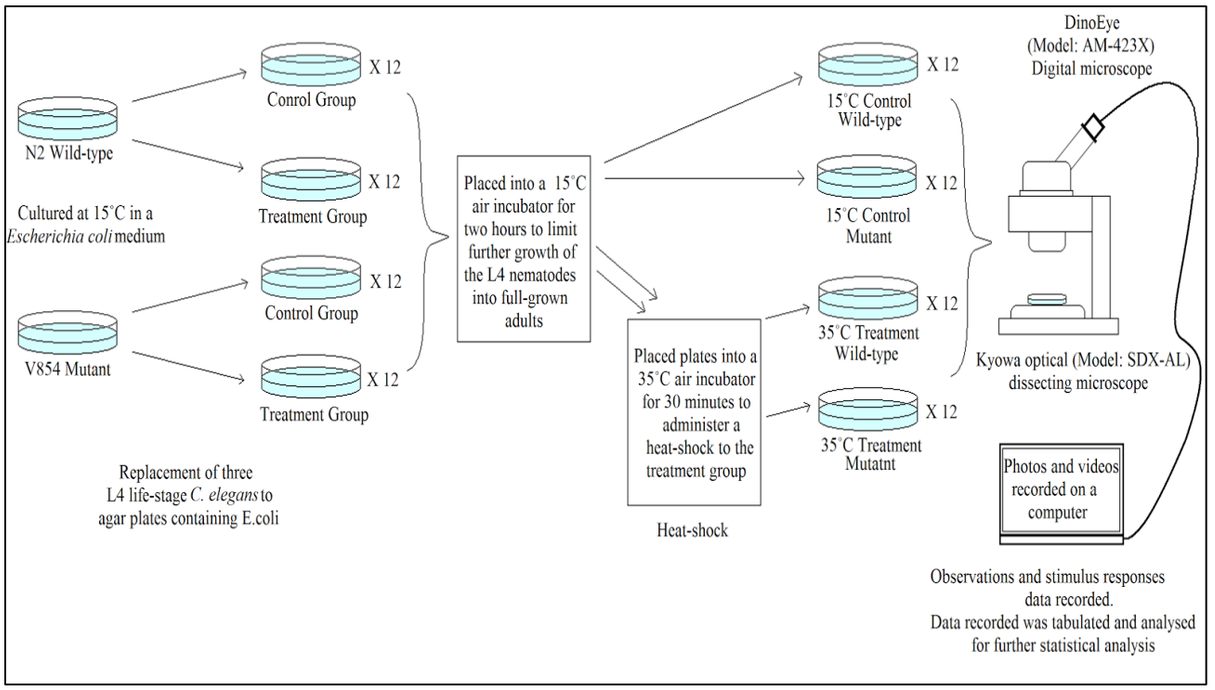

Figure 2. Pictorial representation of the methodology and procedure for the control and heat shock treatments.

RESULTS

Figure 3 shows the comparison between the mean number of head oscillations over 30 second intervals between wild-type and mutant *C. elegans*. The mean number of head oscillations by wild type decreased under heat shock conditions as opposed to control, specifically 9 ± 3 (SD: 5.88) at 35ºC, and 34 ± 4 (SD: 9.25) at 15ºC. The difference between the means of wild-type head oscillation at heat shock and control temperatures is significant because no overlap of confidence intervals is evident (Figure 3). The mean number of head oscillations for mutants (*VC854*) at control and heat shock conditions was 7 ± 2 (SD: 4.68), and 4 ± 2 (SD: 3.32) respectively, indicating a decrease in activity among mutant replicates at higher temperatures. The difference between the means of mutant head oscillation at heat shock and control temperatures is not significant because confidence intervals overlap (Figure 3). At control temperatures, the wild type had a significantly higher mean number of head oscillations in comparison to the mutant. During heat shock conditions, an overlap between the confidence intervals of mean head oscillations by the wild-type and mutant strains is noted (Figure 3). Therefore, the difference between mean head oscillations by wild-type and mutant *C. elegans* is not significant at heat shock temperatures.

In quantifying the effect of temperature on mean head oscillations of wild-type *C. elegans*, the p-value was calculated to be 2.44E-13. After conducting a subsequent t-test to test the effect of mutation on the mean number head oscillations of *C. elegans* we found the p-value to be 3.03E-15. Comparing the difference in effect of heat shock between the wild-type and mutant strains by two-way ANOVA we found that the p-value was 2.02E-10.

The posture preference of 17 replicates of wild-type *C. elegans* are shown in Figure 4, and indicate a preference for the sinusoidal posture at 15ºC control conditions (88% sinusoidal)

and curved posture at 35ºC heat shock conditions (59% curved). In contrast, no trend is observed for mutant posture at either control or heat shock temperatures (Figure 5).

Both strains exhibit the same directional locomotive response at control conditions, as 76% of the wild-type replicates and 59% of mutant *C. elegans* favor forward movement at 15ºC (Figure 6, Figure 7). A difference in locomotion between strains is observed under heat shock conditions. During heat shock, the wild-type replicates displayed no observable pattern of locomotion (Figure 6). In contrast, 76% of the mutant heat shock replicates demonstrated no movement as their favored initial response (Figure 7).

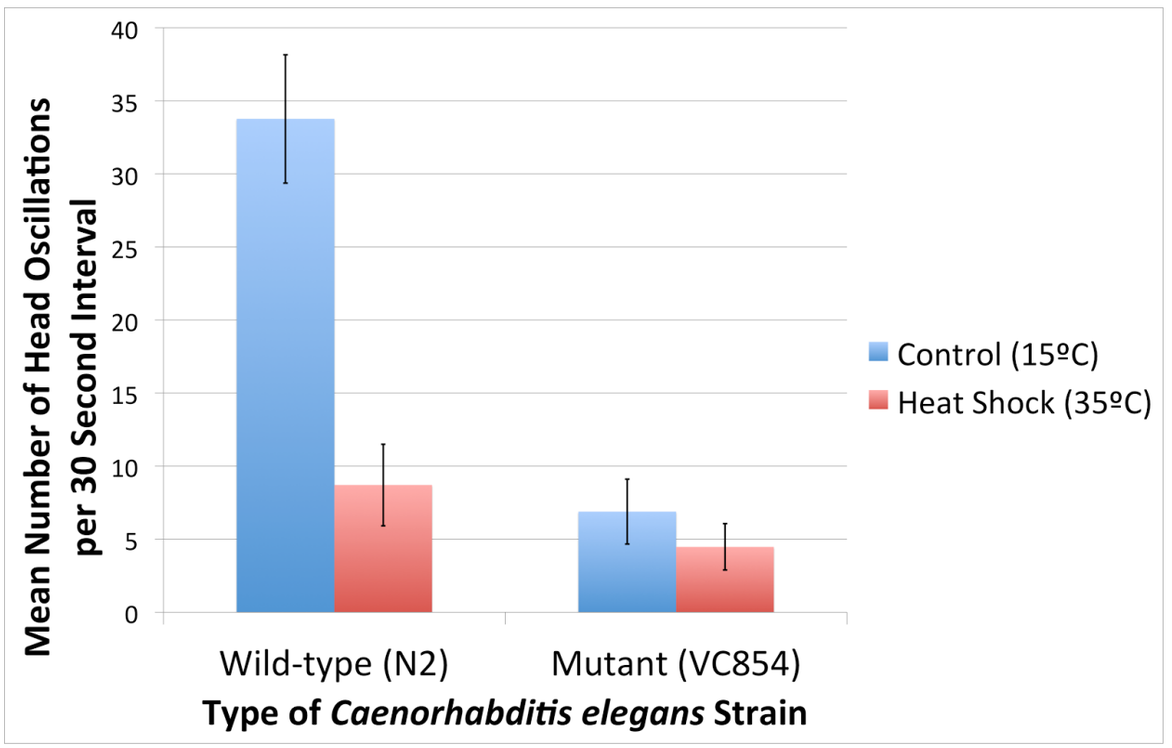

Figure 3. Graphical representation of mean number of head oscillations per 30 second interval of wild-type (N2) and mutant (*VC854*) strains of *C. elegans* under 15ºC control (blue) conditions and 35ºC heat shock (red). The errors bars on the graph represent the 95% confidence intervals, n =17 for each control and heat shock trial.

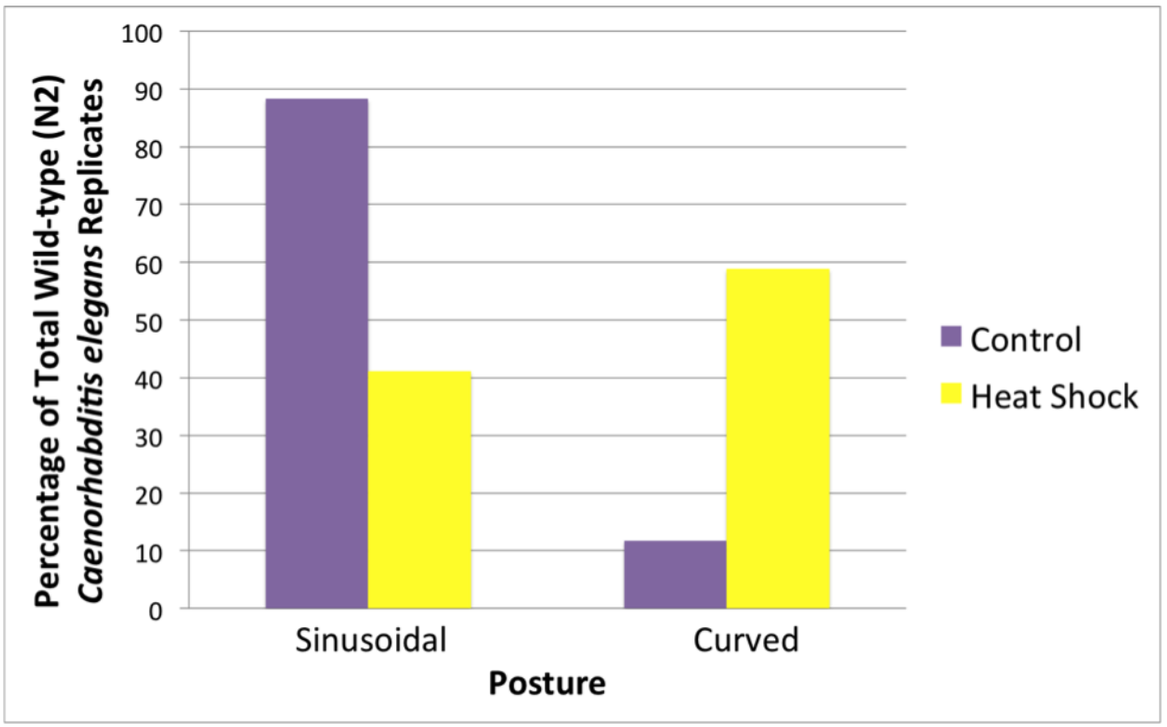

Figure 4. Graphical representation of posture preference of wild-type (N2) *C. elegans* under both heat shock (35ºC) and control (15ºC) conditions. For both the heat shock and control treatments, n=17.

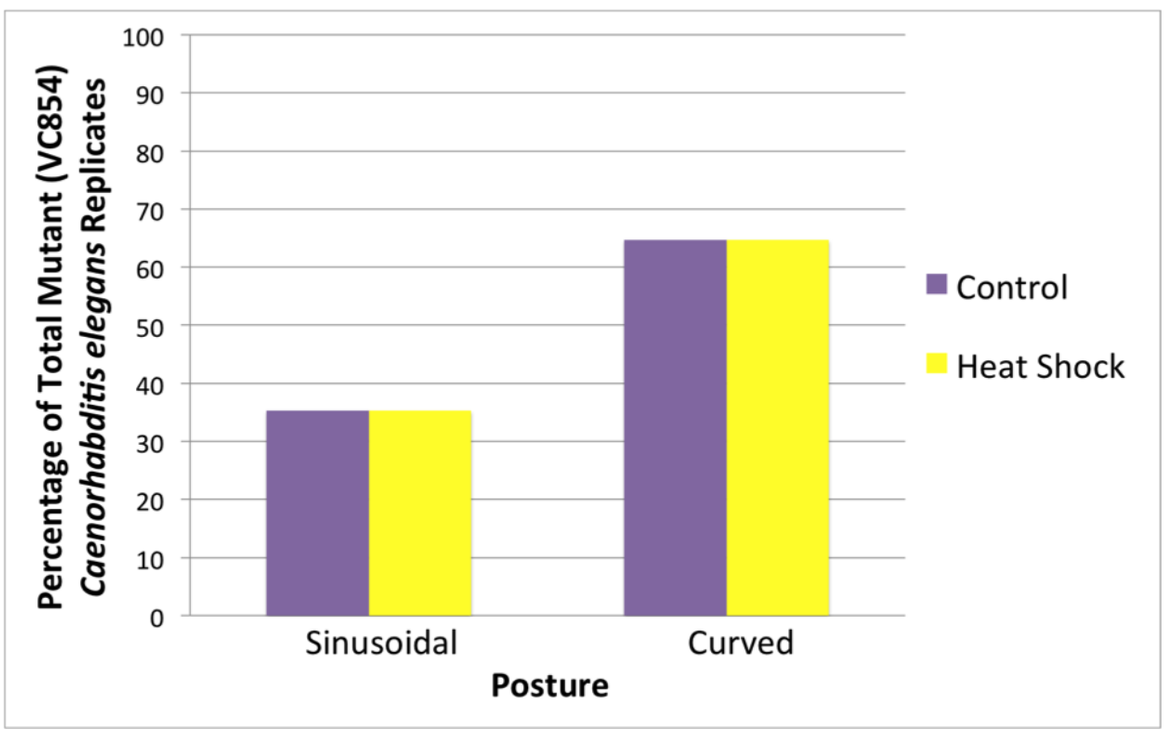

Figure 5. Graphical representation of posture preference of mutant (VC854) *C. elegans* under both heat shock (35ºC) and control (15ºC) conditions. For both the heat shock and control treatments, n=17.

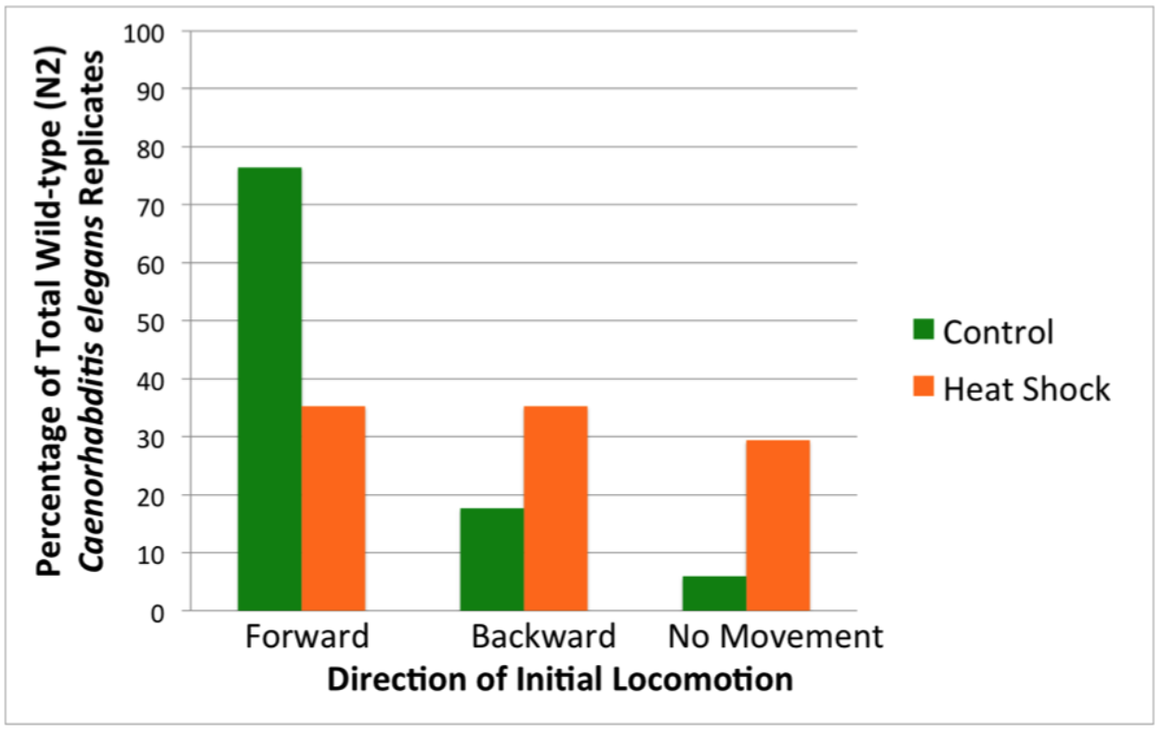

Figure 6. Graphical representation of the direction of initial locomotion of wild-type (N2) *C. elegans* under both heat shock (35ºC) and control (15ºC) conditions. For both the heat shock and control treatments, n=17.

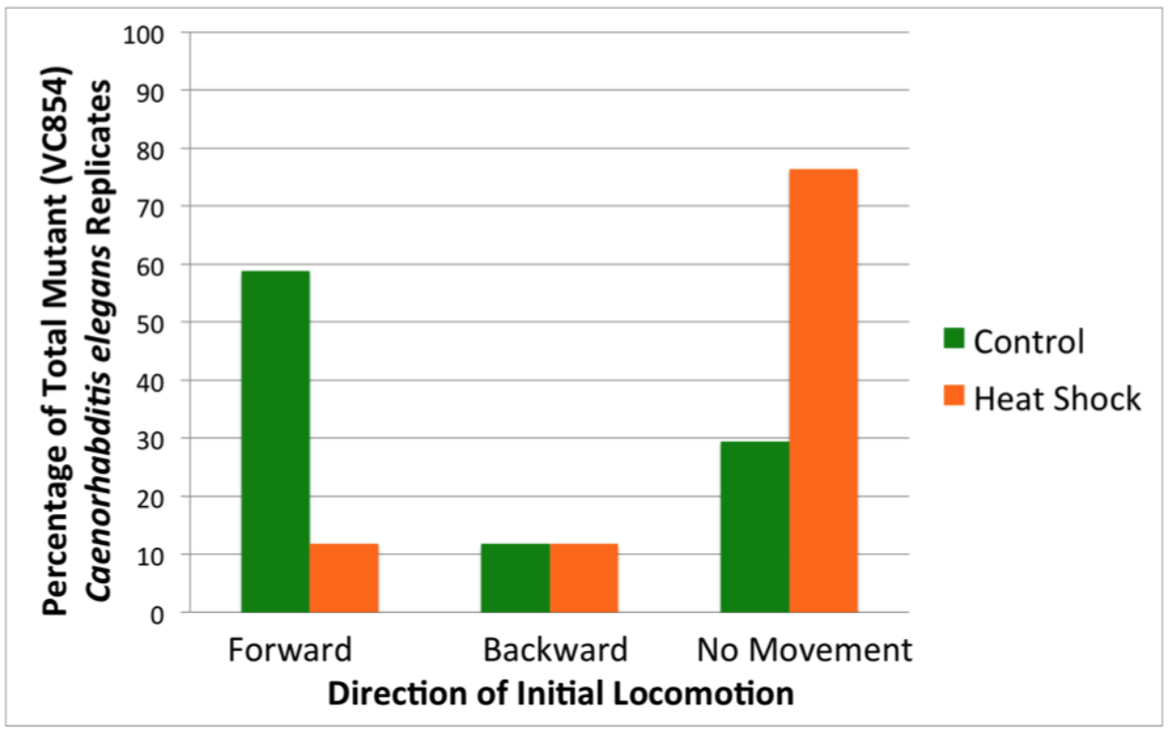

Figure 7. Graphical representation of the direction of initial locomotion of the mutant strain (*VC854*) of *C. elegans* under both heat shock (35ºC) and control (15ºC) conditions. For both the heat shock and control treatments, n=17.

DISCUSSION

Upon completion of a two-way ANOVA test, our results indicated a statistically higher average number of head oscillations in the wild-type control replicates as compared to the wild-type heat shock replicates. Analogous results were seen in the mutant trials, in which the mutant control replicates had a higher mean head oscillatory rate in comparison to the mutant heat shock nematodes. Therefore, the null hypothesis (H_{o1}) was rejected and support for the alternate hypothesis (H_{a1}) that heat shock (35˚C) decreased the average head oscillatory rate, in both the mutants and wild-type strains, was provided. This finding is supported by Prahlad *et al.* (2008), in which they reason that the AFD thermosensory neurons sense changes in ambient temperature and induce sleep-state conditioned responses. The AFD neurons are functional in both strains of *C. elegans*. Hill *et al.* (2014) agreed with this claim, and experimentally determined that the nematodes entered a sleep-like state following exposure to heat stimuli, through the downstream effects of *egl*-4 gene expression conferred by HSF-1 transcription factor within thermosensory neurons. This pathway commences as a cell-autonomous response that occurs due to unfolded proteins accumulating in the cellular domain (Prahlad *et al.* 2008). As a regulator of nematode lethargus, the *egl*-4 gene encodes a cGMP-dependent protein kinase (PKG), functioning to decrease neuronal responsiveness leading to the sleep-like state (Raizen *et al.* 2008). In addition, Schwarz *et al.* (2011) reported that reduced excitability of a sensory neuron resulted in decreased responsiveness of *C. elegans* throughout the sleep-like state. As such, our experimental results documenting decreased locomotion and excitability during heat shock conditions is supported by the literature.

Analyzing the presence of the *unc-2* gene mutation with respect to its influence on the activity of *C. elegans*, the associated p-value lead to the rejection of the null hypothesis (H_{o2}) and

support the alternative hypothesis (H_{a2}) supporting that the *unc-2* mutation does decrease activity, as measured through mean head oscillations,. In reference to our finding, the results of an electrophoresis gel run on the mutant (*VC854*) versus the wild-type strains show an extra band at 900kb, solely in the mutant organisms (Pollock 2014, BIOL342 class data, not shown). This result supports the presence of a novel gene segment that does not include the *unc-2* gene, unique to the mutant strain. The deletion mutation of the *unc-2* gene results in the lack of a calcium channel protein, which is directly correlated to proper neuronal firing in *C. elegans* (Schafer and Kenyon 1995; Schwarz *et al.* 2011). Further explanation of our results may be provided by considering the relationship between biogenic amines and the *unc*-2 gene in *C. elegans*. Both serotonin and dopamine are found in the neurons of *C. elegans*, and confer behavioural responses (Liang *et al.* 2006). At a synaptic level, serotonergic and dopaminergic responses cause inhibition of movement and subsequently paralysis of *C. elegans* (Schafer and Kenyon 1995). Schafer and Kenyon (1995) provided evidence for an *unc-2* dependent calcium influx being required for adaptation to serotonin and dopamine. Such adaptation occurs via postsynaptic response to serotonin (Shafer and Kenyon 1995). Therefore, a loss of function mutation in the *unc*-2 gene results in a failure to adapt to the serotonergic response present during stressful situations. This is in accordance with our results, as the mutant strain's failure to adapt to the inhibition of locomotion signal during heat shock response will lead to cessation of head and body movement.

Moreover, a statistical difference was found between the mutant and wild-type heat shock replicates with respect to mean head oscillatory rate, as wild-type heat shock replicates showed a higher mean value for head oscillations. Initially, this prompted for rejection of the null hypothesis (H_{o3}). However, further analysis of mutant and wild-type confidence intervals

associated to mean head oscillations during heat shock conditions displayed significant overlap. The notion of heat shock at 35˚C decreasing mean head oscillatory rates in the mutants to a greater extent in comparison to the wild-type replicates could not be supported. Thus, the directionality condition in our hypothesis was not met, and the null hypothesis (H_{o3}) could not be rejected. The rationale behind the greater decrease in activity seen in wild-type nematodes from control to heat shock conditions is the greater level of basal activity of the wild type in comparison to the mutant strain. The wild-type strain has no limitations on its locomotion at control conditions, whereas the mutant strain has non-maximal locomotive potential due to the absence of the *unc-2* gene product. As such, the mutant showed a low basal level of activity in the control environment, and therefore would not be able to show as great of a reduction in activity at heat shock conditions when compared to the wild type.

Analysis of the qualitative data highlight that a greater number of mutant replicates exhibited curved posture after heat shock when compared to wild-type *C. elegans*. This finding is analogous to the research of Clark *et al.* (1997), who notably used an incubation based procedural approach similar to ours. They found that *ky51* mutants showed a normal phenotype at 15°C, however less than a minute after being shifted to 25°C; the mutants exhibited sluggish locomotion and curved posture. However, this conclusion cannot be used to explain the results in our mutant, but can highlight a future area of research in order to establish a relationship between the *unc-2* and *ky51* gene products.

This experiment had certain variables that were uncontrolled and should be accounted for in attempts to understand variability in the collected results. As mentioned in the methods section, plates of L4 nematodes were placed into the 15°C air incubator to limit further growth into their adult stage. If these molts were near the end of their L4 stage, there was a chance that

they could have grown into adults during the two to three hour time frame between when they were placed in the incubator and when they were observed. Differing life cycle maturation rates for replicates may have contributed to error to our results, as not all replicates were measured from the L4 stage. Furthermore, it was observed that the temperature in the 35˚C incubator fluctuated, resulting in inconsistent temperatures over the allotted 30 minutes. The implications of such variations are discussed in a methodology study of incubating *C. elegans* done by Zevian and Yanowitz (2014), in which they mentioned that temperature fluctuations can cause inaccurate results and induce extra stress on the nematodes being studied, leading to inaccurate observations. Once removed from the incubator setups for analysis, temperature fluctuations in the agar plates also occurred. In the case of the control samples, the temperature of the plates can rise very quickly and as for the ones placed under heat shock, the temperature of the agar plates can drop very quickly. Furthermore, Zevian and Yanowitz (2014) noted out that temperatures below 35°C were not high enough to cause for *C. elegans* to enter sleep-like states. In consideration of this research, we ran preliminary trials on both strains of *C. elegans* to ensure that the heat shock temperature was sufficient enough to produce an observable response. Another reason for running preliminary trials was to ensure the heat shock temperature was not lethal to the nematodes.

It is important to note that there are actually large amounts of subjectivity within the data, attributed to the fact that the data were recorded based on human observation of a 30 second video alone. Also, the mutant worms showed to have a much different pattern of locomotion in the sense that they actually appeared to move in a longitudinal fashion rather than a sinusoidal. This has implications for the measurements of oscillations, as longitudinal motion is much harder to quantify as compared to the conventional sinusoidal measurements. Moreover, due to the high

level of activity of the wild-type nematodes, the field of view of the dissecting microscopes was sometimes insufficient for measuring the parameters, as the nematode movement may have led them in directions out of the field.

CONCLUSION

After analysing the effects of heat shock on *C. elegans,* we found that temperatures of 35˚C decreased the mean head oscillatory rate of both wild-type and mutant (*VC854*) strains. Furthermore, support for the fact that the presence of the *unc-2* gene mutation decreased the mean oscillatory rate of *C. elegans*. However, it could not be reasoned that heat shock has a greater effect on the mean oscillatory rate of the mutant as compared to the wild-type strain.

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