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Stress Responses and Energy Storage in *Drosophila melanogaster* Selected for Resistance to a Gram-Positive *Bacillus cereus* Spores

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by

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A survival response study was carried out by using *D. melanogaster* and the opportunistic pathogen *B. cereus* as the agent of selection. The spores of *B. cereus*, a gram-positive bacteria that can cause the human pathogen disease, were applied in our artificial laboratory selection. Selected lines were treated with *B. cereus* spores. Wound control lines were punctured with a needle dipped into sterile H_2O. Control lines did not apply any treatment. Three different environmental treatments were used within each line type (autoclaved spores of *B.cereus*, sterile H_2O and no treatment). The autoclaved spores were used as an inducer of the immune response in our study, with the purpose of boosting their innate immunity. Our hypothesis was that selected lines will live longer as they are immune to "*B. cereus*" spores. By comparing the average mortality rate of different line types, flies in selected lines were observed to die slowest, which was correspondent to our hypothesis. Selected and control lines were studied in different stress environment (starvation, desiccation, chill coma, oxidation) to investigate their survival response to stress. Those flies resistant to *B. cereus* spores in our selection were expected to survive longer than the flies in other line type under different stresses. To study the trade-off for these survival responses, energy components were measured. The

result showed that there was a change in lipid and glycogen concentration in selected flies, which confirmed our trade-off hypothesis between survival responses and energy component.

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Chapter 1.

Introduction

Introduction

Drosophila melangaster as the model for immunity research

Drosophila melangaster (D. melanogaster) has been used for basic and applied research for a long time. There are several reasons to choose *D. melangaster* as the genetic research model. First, it is easy to build a population in the lab environment. It only takes 7 to 10 days to breed a new generation under the room temperature. Besides, it has high fecundity. Females could lay up to 100 eggs per day and around 2000 in a lifetime (James Sang 2001). It has only four pairs of chromosomes, three autosomes and one sex chromosome, which are easy to study. Its complete genome structures have been sequenced (Adams *et al*. 2000). According to their gene sequences, *D. melanogaster* has been found to share highly similar genetic pathways with human beings. About 75% of known human disease genes have a recognizable match in the genome of *D. melanogaster* (Reiter 2001) and 50% of fly protein sequences have mammalian homologs. This is why *D. melanogaster* is widely used as an important eukaryote genetic model. In addition, *D. melanogaster* share the similar innate immune system with human beings. The innate immune responses provide immediate defense against infection. It triggers ancient defense strategy. Different from adaptive immunity, innate immune responses can be activated rapidly after infection occurs. This response plays a crucial role in the early mortality control. The Toll receptor in *Drosophila* is known to be a homologue of Toll-like receptors in mammals (Lemaitre and Hoffman 2007). The highly conserved innate immunity (Figure 1) is one of the reasons that *Drosophila* is a good model system with relevance to human health.

Laboratory Artificial Selection

Artificial selection was conducted in our laboratory for this study. Different from laboratory natural selection, where populations evolves under some environments without human intervention, artificial selection is a way of intentional breeding for certain traits or combination of different traits from breeders over generations (Catherine Linnen 2001). The purpose of artificial selection is to increase the genetic difference between different lines (selected and control lines). The result can be allele frequency changes in many genes of *D. melanogaster*. Through artificial selection, two types of responses will occur: direct responses and indirect responses. Direct responses to the selection are changes in the phenotype that was selected, while indirect (correlated) responses are defined as changes in characters, that occur as incidental consequences of selection. Direct responses have been studied previously in our laboratory (Ma *et al*. 2012). In this study, indirect responses, such as stress responses and energy storage of lipid and glycogen were studied.

For artificial selections, there are advantages and disadvantages for studying evolution of physiological system. The most important advantage is that we can simulate selection for a specific direction in the laboratory environment according to their experimental design. Enough replications in the experiment can especially provide a greater statistical power than in a similar natural environment (Garland and Adolph 1994). The artificial selection can be repeated in different laboratory environment with different populations, as the overall process is still similar. Moreover it can be modified according to the experimental design to include not only selected and control lines. In this study,

other than control and selected lines, we designed to include another control line as the wound control of selected lines.

However, there are still many disadvantages in artificial selection studies. First, indirect responses (correlated responses) are not always consistent among different experiments. Through artificial selection, many different factors can affect the experiment and cause the various results, e.g. inadvertent selection on controls or random errors caused by researchers. According to the population genetics theory, small population size can also cause inconsistenies. To avoid this, large population sizes are applied in our study. Different conditions between selection experiments and related assays might contribute to inconsistencies in the outcomes. For instance, while flies selected for starvation were maintained under the condition being lack of food source, correlated assays were performed under a condition with abundant of nutrition and food source (Zera and Harshman 2001). Genetic correlation were highly context-dependent. Thus the correlated responses observed in one laboratory might not be the same as in another laboratory (Clark 1987).

Artificial selection is usually conducted inside the laboratory environment, which is not equivalent to natural environments. One major difference is that selection in the laboratory occurs in an environment with a super-abundant food source. Consequently, the results of such selection experiments can be "biased" through the accumulation of energy due to the unlimited amount of food during the response to the selection. This could explain why in some cases, storage of energy sources is common for stress resistance and longevity selection experiments (Chippindate *et al*. 1996, Harshman *et al*. 1999, Djawdan *et al*. 1997, Van Herrewege and David 1997). Furthermore, artificial

selection in the laboratory is considered as the result by increasing intensity of the same pattern of selection. However, it cannot represent the fluctuating environments and selection in natural environment.

Stress Response and Energy Storage

This section will first cover the four stress responses conducted in this study, which is starvation, desiccation, cold response, and oxidation. Then it will explain the relationship between these stress responses and energy storage components, lipid and glycogen.

Starvation Stress Response

Under the laboratory artificial selection, *D. melanogaster* has presented resistance to environmental stress in many studies. Stress resistance characters, such as starvation and desiccation were proven to be importance in evolutionary physiology (Hoffmann and Parsons 1991). Selection for postponed senescence increased the resistance of *Drosophila* adults to desiccation and starvation (Rose 1984, Service *et al*. 1985). Starvation resistance study and longevity were also associated by phenotypic manipulation experiments in *D. melanogaster* (Zwaan *et al*. 1991, Chippindale *et al*. 1993). Levels of lipid storage were used to explain the relationship between starvation and longevity (Service 1987). However reduced metabolic rate could also simultaneously increase longevity and starvation resistance.

During the starvation response (Figure 2), Adipokinetic hormone(AKH) activates cAMP-dependent protein kinase first, then this will stimulates lysine-specific

demethylase 1(Lsd1) and Brummer lipase, which is located at the surface of the intercellular lipid droplets, to release diacylglycerols to transport through the haemolymph by lipophorins (Leopold and Perrimon, 2007). When liberated diacylglycerols reach the oenocytes, they are converted to free fatty acids by the actions of lipases. During this process, a series of enzymatic reactions convert the fatty acids to acetyl-CoA, which can be used in the Krebs cycle for ATP synthesis in the later process (Hong and Park, 2010). In the later phases, the liberated free fatty acids are converted into ketone bodies to be used in the *Drosophila* brain for energy (Baker and Thummel 2007, Hong and Park 2010).

Desiccation Stress Response

Desiccation stresses also contribute to the energy storage and metabolism in *D. melanogaster*. *Drosophila* living desert environment were reported to be more resistance to high temperature and desiccation than species living other habits (Stratman and Markow 1998, Krebs 1999, Gibbs and Matzkin 2001, Patton and Krebs 2001). The mechanisms responsible for these differences include expression of heat-shock proteins (Krebs 1999) and reduction of water-loss rates (Gibbs and Matzkin 2001).

Cold Stress Response

In the evolutionary study, genetics and mechanisms of cold resistance can be productively investigated in *Drosophila* (Stanley *et al*. 1980, Kimura 1988, Gibert *et al*. 2001), including cold resistance selection (Tucic 1979, Chen and Walker 1993, Watson and Hoffmann, 1996). But this selection study has proved to be difficult to conduct as its

carryover effects were brought on by stressful conditions. Cold exposed parents, especially mothers, often had the issue of their offspring with a low fitness and lack of replication (Hoffmann *et al*. 2003). This selection was replaced by chill coma recovery experiment. Chill coma is a narcosis-like state induced in many species under cool temperatures (Schenker 1984, Leather *et al*. 1993). This state is reversible in *Drosophila* upon returning to warmer temperature. The amount of time taken for flies to recover can be used as a reliable measure of cold tolerance (Gibert *et al*. 2001). As less severe stress levels are used, this method is likely to be more amenable to genetic analysis than methods based on mortality.

Oxidation Stress Response

Oxidation has been tested as a form of stresses, which is less obviously associated with endogenous energetics. Oxidative stress, or the overabundance of reactive oxygen species (ROS) as an unavoidable consequence of aerobic respiration, has been implicated in aging (Harman 1957, Finkel and Holbrook 2000), neurodegenerative and cardiovascular disease (Barnham *et al*. 2004, Finkel 2005), and the disruption of cell signaling processes that control cell growth and death (Giorgio *et al*. 2007) .

Energy Storage

In this study, we simulate four different stresses in the laboratory environment, which is starvation, desiccation, oxidation, and chill coma recovery. Energy storage components, such as lipid and glycogen concentrations, are measured for these stress responses. A previous study has reported that starvation and desiccation resistance were

positively correlated with lipid levels (van Herrewege and David, 1997). Lipids are considered as important fuels to store the energy for starvation resistance. It provides more than twice energy per gram as carbohydrates produced (Withers, 1992). However , Glycogen is less energy-dense but provides slightly more metabolic water per gram than lipid. So inspecting the lipid and glycogen concentration of *D. melanogaster* might provide us a good interpretation of the stress response result.

Bacillus cereus* (*B. cereus*) Selection for Resistance of *D. melanogaster

In this study, we choose *Bacillus cereus* to conduct the artificial selection in the laboratory environment. *Bacillus cereus* is a gram-positive human pathogen bacterium. It can be easily found in human food products. Previous studies have proved that *B. cereus* can be detected in the faecal samples of both adults and children (Ghosh 1978, Turnbull 1985). It can also cause the food-borne diseases, like diarrhoeal syndrome and emetic syndrome (Kotiranta *et al*. 2000, Granum and Lund 1997) and local and systemic infection as a human opportunistic pathogen.

The reason we choose *B. cereus* in this study is because it is closely related to *Bacillus anthracis (B. anthracis),* which has been used as a bioterrorism weapon against humans with a long history. However, previous study has proved that *B. anthracis* cannot infect *D. melanogaster* (Guillemin 1999). Thus we have to find another bacteria species, which is closely related to *B. anthracis* for our study. According to the phylogenetic tree, *B. cereus* shares the highest similarity to *B. anthracis* among other species (Figure 3). There are five other species in the *B. anthracis* cluster, which is *B. thuringiensis*, *B.s mycoides, B. pseudomycoides, B. weihenstephanensis* and *B. cereus* (Fratamico *et al*.

2005). *B. cereus* is further divided into seven phylogenetic groups (I to VII) based on thermotolerance ecological differences (Guinebretiere *et al*. 2008). *B. cereus* strain ATCC 10987 is grouped together with *B. anthracis* in group II based on fluorescent amplified fragment length polymorephism (fAFLP) (Økstad *et al*. 1999). It contains genes similar to those on virulence plasmids pXO1 (Read *et al*. 2003). *B. cereus* ATCC 10987 became the strain of *B. cereus* we used to infect *D. melanogaster*. In our study, we used the live spores of *B. cereus* ATCC10987, as it is most similar to the *B. anthracis* spores, which has been used as bioterrorism weapon. The *B. cereus* strain used in our study was ATCC 10987. It was isolated from a survey of cheese spoilage in Canada in 1930 (Smith 1952, Herron 1930).

Experimental Design and Treatments

The artificial selection process of this study has described in Ma *et al*. (2012). Our study has three different lines. The selected lines are flies punctured with *B. cereus* spores by a fine tungsten needle. This acute infection triggers their innate immune responses and simulates the pathogenic mechanism. The concentration of *B. cereus* spores in each generation is adjusted to reach 50% mortality rate, which is the goal for this laboratory selection. During this selection, another injury factor has to be considered. In this case, a wound-healing control groups are involved. Flies in these wound control lines are punctured with sterile water, which will cause the injury only but no bacteria infection is included. This is the "real" control line we are interested in, compared to the selected lines. The third line is regular control lines, without causing any perturbation to the flies.

In this research, we used flies from generation 26 and relaxed two more

generations for stress responses and energy storage study. The purpose of relaxing two generation was to make sure they were not too stressed for the following experiments. Two generations later, three treatments are applied in each single line. The first treatment is to puncture flies with autoclaved (dead) spores. This is to induce their immune response. Another treatment is to puncture flies with sterile water, which aims to induce the response of wounding. The last treatment is the control, where nothing was done.

Specific Aims

The goal of this study is to study the stress responses and energy storage in *D. melanogaster* selected for resistance to gram-positive human pathogen *B. cereus*. After the artificial selection in the laboratory, autoclaved spores of *B. cereus* ATCC 10987 are induced to boost the immunity of *D. melanogaster*. Then stress survival studies, like starvation, desiccation, oxidation, and chill coma recovery were considered in the context of life history traits (life span, development time, and egg production). Energy storage compounds, glycogen and lipid concentration in *D. melanogaster* lines and treatments were also investigated. It was anticipated to illustrate a trade-off between stress responses and energy storage in the body of *D. melanogaster*. This could help to study the immunity response of *D. melanogaster* and pathogen related mechanism behind.

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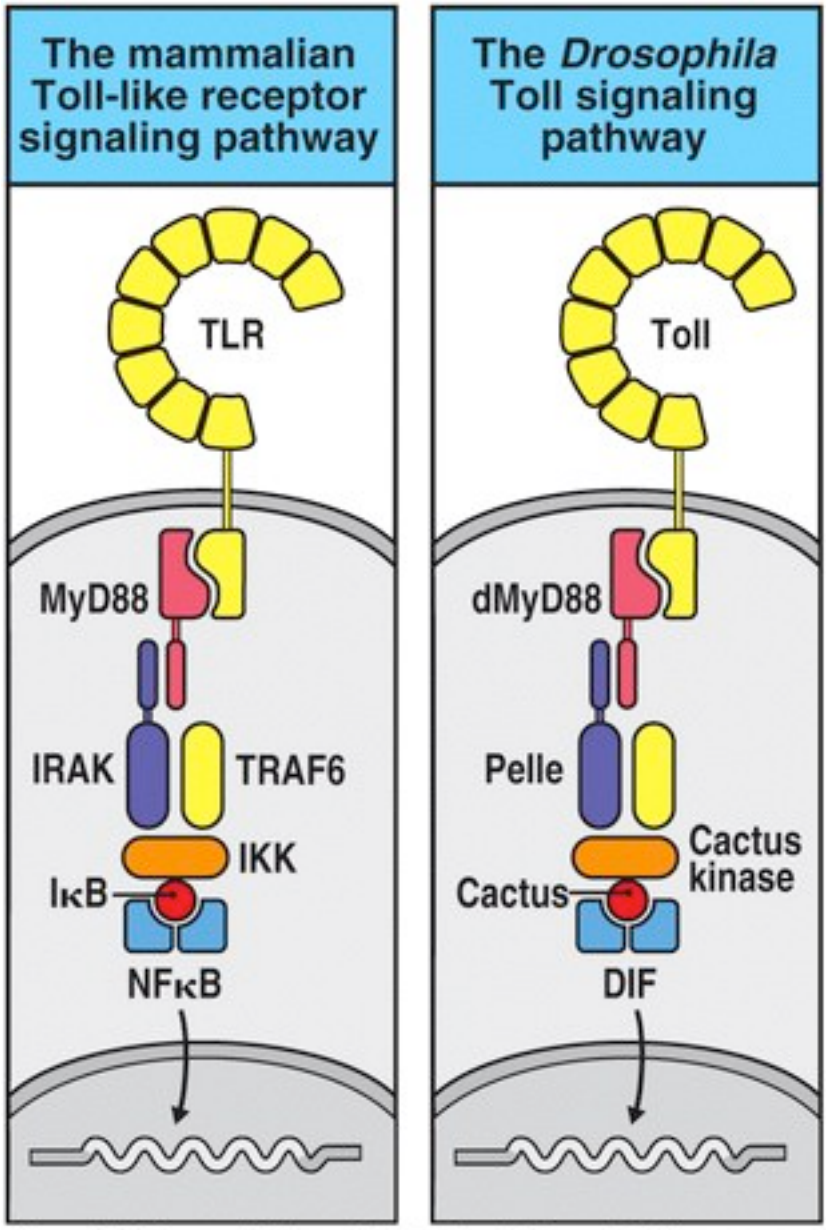

Figure 1. Homologous proteins described in the *Drosophila* Toll signaling pathway and the mammalian TLR signaling pathway.

Source : Immunobiology. Garland Science (Janeway et al., 2005.)

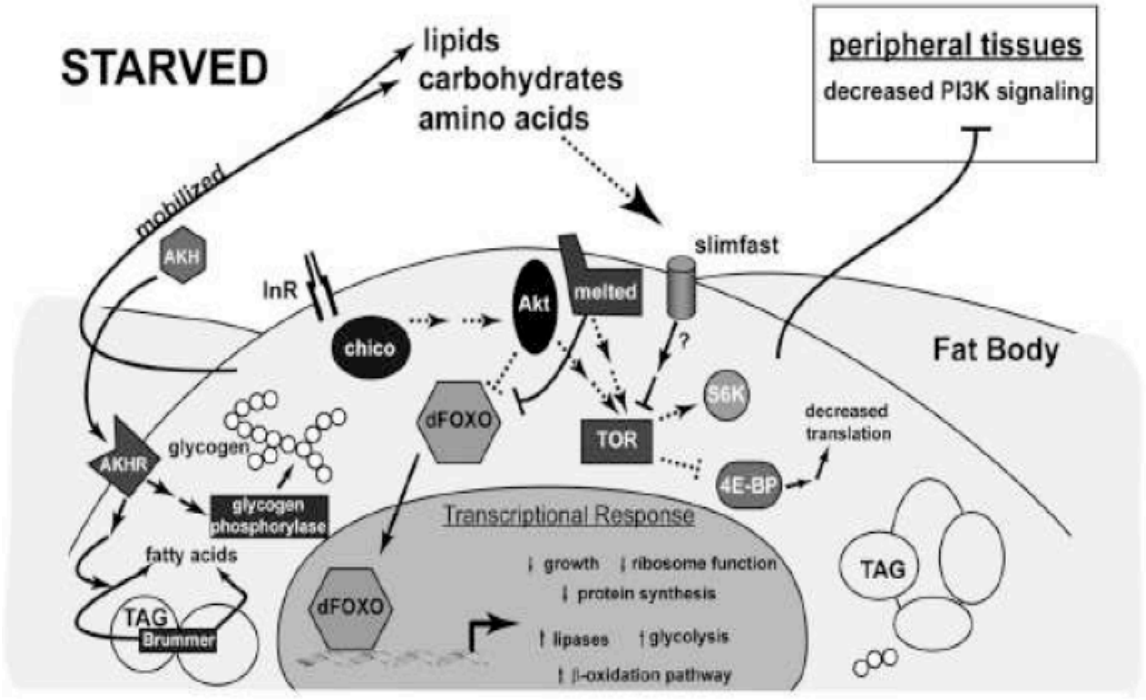

Figure 2. Metabolic signaling in *Drosophila melanogaster* during the starved state.

Source: Baker, 2007.

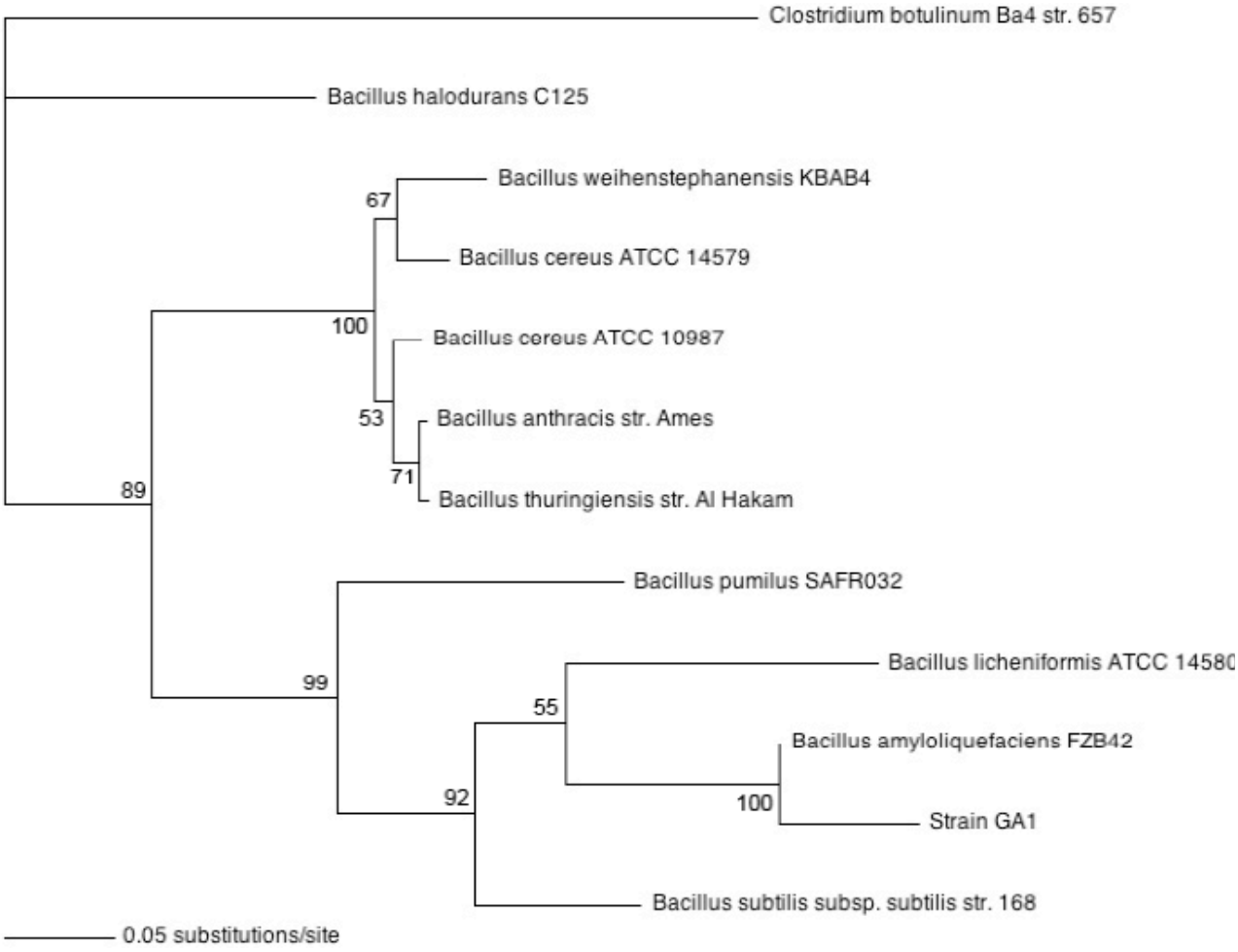

Figure 3. The maximum likelihood phylogenetic tree of *Bacillus* genera. All sequences were aligned on *Clostridium botulinum* Ba4 str. 657 (NC_012658). Values for frequencies less than 50% are not given. The scale bars represent the number of substitutions per base position.

Source: Anthony Arguelles-Arias et al. 2009

Chapter 2.

Stress Responses and Energy Storage in *Drosophila melanogaster* Selected for Resistance to a Gram-Positive *Bacillus cereus* Spores

Abstract

D. melanogaster was developed as a model for resistance to *Bacillus* infection using *B. cereus* spores as an agent of artificial selection. The direct response to selection in *D. melanogaster* survival after *B. cereus* infection was already investigated in our laboratory. In this study, the indirect responses to our laboratory selection, such as stress responses and energy storage of lipid and glycogen, were investigated. Stress survival studies against starvation, desiccation, oxidation, and chill coma recovery were performed in the context of life history traits (life span, development time, and egg production). Energy storage compounds, glycogen and lipid concentration in *Drosophila melanogaster* lines and treatments were investigated to explain the trade-offs of these survival responses under stress environment. Our expectation was to detect the resistance in selected line type by comparing the average mortality rate among different line types. In the starvation stress experiment, females in selected and wound control groups died more slowly than those in control group under all treatment conditions, while selected males died slower than control males under non-treatment conditions. In the desiccation experiment, selected males always died more slowly than flies in any other line type. In oxidation experiment, males in selected and wound control groups died more slowly than males in control group under all treatments conditions. In chill coma recovery experiment, selected females had a higher recovery time compared to flies in other line types. These results were correspondent to our hypothesis, which illustrated a higher survival rate of selected flies. By investigating the energy component, males in selected lines had already presented a higher body lipid concentration, while females in control lines showed a higher glycogen concentration. This difference detected might be considered as one

explanation for our survival responses study. By studying the trade off between different traits, our goal is to understand the evolved resistance and/or tolerance mechanism behind.

Introduction

Stress responses may be caused by a trade-off between different traits. A phenotypic trade-off was observed between reproduction and two other traits, longevity and starvation resistance (Chippindale *et al*. 1993). According to Chippindale *et al*. 1993, dietary stimulation of reproduction might lead to a net depletion of lipids reserves, resulting in decreasing starvation resistance. Selection for starvation and desiccation resistance have increased mean longevity and reduced early fecundity, which represented a positive genetic correlation between these stress-resistance traits and longevity. Besides, a negative genetic correlation was detected between such survival-related characters and early fecundity (Luckinbill *et al*. 1984, Rose 1984, Serivce and Rose 1985, Rose *et al*. 1990, 1992, Leroi *et al*. 1994 a, 1994 b). Starvation-selected populations of *D. melanogaster* were detected to accumulate high lipid and carbohydrate levels (Guillemin J., 1999, Chippindale *et al*. 1998), while desiccation-selected populations were detected to store less lipids but much more glycogen than control populations (Djawdan *et al*., 1998).

A trade-off between oxidation stress responses and lifespan has been observed (Harman 1956, Wallace 1992, Dudas and Arking 1995, Martin *et al*. 1996). A cost of female reproduction increasing and oxidative stress resistance was studied by using Methoprene, a juvenile hormone III analog, to stimulate vitelogenesis and egg production (Salmon *et al*. 2001). Temperature stress, like cold and heat environment, also played a crucial role for detecting the trade-off between phenotypic responses and their related proteins. For example, heat shock proteins (Hsps) were important in environmental stress tolerance and in thermal adaptation (Feder and Hoffman 1999, Hoffmann AA *et al*. 2003,

Frydenberg *et al*. 2003, Sørensen *et al*. 2003). The report of a cold-induced response in insects was provided by Burton *et al*. (1988) who noticed the induction of a 70 kDa protein after a cold treatment.

In this study, we will investigate the stress responses and energy storage in *D. melanogaster* selected for resistance to gram-positive human pathogen *B. cereus*. Stress survival responses, including starvation, desiccation, chill coma recovery, and oxidation stresses, will be studied as the indirect responses and energy storage compounds, glycogen and lipid body concentration will be considered as the trade-offs. By comparing the mean mortality rate of *D. melanogaster* in different line types, we expected to see a resistance tendency of those flies in selected lines and their associated energy components concentration changes as the trade offs for their survival responses.

Materials and Methods

Fly Population

The base population and subpopulation used for the selected and control lines had been described in Ma. *et al*. (2012). More than 10,000 individuals were maintained in a laboratory environment, and then divided into 9 subpopulations for laboratory artificial selection. In our laboratory artificial selection, there were nine lines from outbred populations which evolved independently; selected lines, wound-control lines and no perturbation lines. Each line had three replicated lines and within each replication, 1000 males and 1000 females were used as breeders in each generation.

Selected lines (S) are flies injected with *B. cereus* spores, a species that is closely

related to *B. anthracis*. Spores of *B. anthracis* have been used as a bioterrorism agents and an ultimate goal of the selection experiment is to identify spore resistance genes in *D. melanogaster*. The spores were introduced by puncturing them into the thorax with a fine needle. The concentration of *B. cereus* spores was adjusted to result in 50% mortality in each generation. During this selection, another factor injury had to be considered. In this case, a wound-healing control groups were involved. Flies in these wound control lines (CI) were punctured with sterile water, which might cause the injury only, but no bacteria infections. This was the "real" control line we were interested in, compared to the selected lines. The third line was no perturbation line (Cn), without any injury in each generation.

Treatments

In the present study, we used flies from the second generation without selection after 26 generations of selection. After two generations, 200 males and 200 females virgin flies were collected for treatments. Three different conditions were used for each line (autoclaved spores, sterile water and no treatment). Autoclaved spores (As) are dead *B. cereus* spores. Live *B. cereus* spores were left into autoclaved machine to generate dead *B. cereus* spores. For the autoclaved spores, they were not supposed to kill the flies as they had lost their pathogenic toxicity. Instead they were just to work as an inducer to boost flies innate immunity. For our study, the dose concentration for autoclaved *B. cereus* spores was 5×10^8 g/ml. Another treatment was the sterile water (H_2O), which was used as the wound control for the *B. cereus* spores treatment. No treatment (Non) was also used in the study. The detailed experiment design was illustrated in Figure 1.

Stress Response

For the following stress response study (starvation, desiccation, chill coma recovery and oxidation), we used flies from generation 26 and relaxed for two generations. When the flies were hatched, virgin flies were collected in both sexes. After 3 to 5 days later, three different treatments mentioned above were applied for each line. For each treatment, 50 males and 50 females were tested among 9 lines. Then mortality rate were recorded up to 72 hours as the result of these treatments. Those survival flies were used for the following stress responses experiments.

Starvation Assay

For the starvation experiment, the survival flies after the treatments were kept in the environment without adequate food. Within each cage, 50 males or females were fed with agar. Then put all the cages into a huge bag with wet paper towel inside. The purpose of this is to keep the humidity of overall environment. Vials with agar food and wet paper towel were changed every 48 hours. Then the mortality rate was monitored every 8 hours and the number of dead flies was recorded until all flies in the cage were dead.

Desiccation Assay

For the desiccation experiment, we put each single fly in one empty vial and taped the vial along the side of a glass tank. The bottom of tank was covered with desiccant, which was used to dry the atmosphere in the tank. An IButton (Maxim Integrated Products, Inc) was left inside the tank to monitor the humidity and temperature of the

overall tank environment. Then top of the tank was sealed with glass and vaseline. Every 60 minutes, the mortality rate of each fly was monitored until all flies were dead.

Chill Coma Recovery

For the chill coma recovery experiment, we used the similar methods described in Colinet *et al* (2010). Flies were kept in the empty vials and plug tightly. Then these vials were embedded in ice for 5 hours. Ice should cover upto the bottom of the plugs. Five hours later, flies were removed from ice box and kept on a 40 cm × 27 cm white tray. Then flies were monitored for 60 -75 minutes and the time to standing was recorded as each fly. After 75 minutes later, the remaining flies were considered as non-recovering flies and recorded as dead. Average recovery time for each line was calculated at the same time.

Oxidation Assay

For the oxidation experiment, the flies were fed with food full of nutrients, which had yeast and cornmeal nutrients. 50 males and 50 females were kept in vials with cotton plugs and flies were transferred to new food every 48 hours. All the vials were kept inside a huge sealed bag, with one side connected to oxygen tank. The oxygen tank had the 95 % pure oxygen inside and continuously releasing 5 psi to the bag. One bottle of water was connected between the oxygen tank and experiment bag, which was used to maintain humidity. Then flies were monitored every 8 hours and the number of dead flies was recorded until all flies were dead.

Lipid Assay

The lipid and glycogen experiments used flies from generation 37 after relaxing selection for two generations. Lipid concentration was measured by the method from Van Handel (1985). Ten males and ten females were homogenized in 100ul chloroform: methnanol (2:1) using plastic pestles and 1.5 ml Eppendorf. Then 0.88% KCl was added into the homogenized solution and centrifuged them with low speed (5000 rpm). After centrifugation, solvent was added to a glass tube. Triolein was prepared into different concentration (0.1 g/ml. 0.2 g/ml, 0.6 g/ml, 0.8 g/ml, 2 g/ml, 3 g/ml, 4 g/ml) as standard to determine the standard curve and add into 2:1 chloroform : mathnanol to generate a total solution volume of 1ml . The glass tubes with samples and standards were incubated in water bath at 90 degree Celsius for 10 minutes. Ten minutes later, sulfuric acid (30 ul) was added into the glass tube, then left them into the water bath with same temperature for another 10 minutes. After this period, the tubes were removed from water bath and allowed to cool to room temperature. Thereafter, vanillin-phosphoric acid reagent (1 ml) was added and mixed gently. 250 ul of each sample and standard were transferred to each cell in 96 well plates and read in a spectrophotometer at 525 nm. Concentrations of lipid present in different treatment for each line were determined by comparison to the standard curve.

Glycogen Assay

Quantify of glycogen was measured as reported in Tiffany (2012). Ten males and ten females were homogenized in 200 ul of a 2% sulfate solution in water by using plastic pestles and 1.5 ml Eppendorf. Then 1ml of methanol was added to the homogenized

samples prior to a 1-minute centrifugation at 2000 rpm. After centrifugation, glycogen within the sample was present in the precipitated sodium sulfate with fly tissue. An anthrone reagent was added to the glycogen solution to generate a total reaction volume of 5 ml. This solution was mixed and left into water bath at 90 degree Celsius for 10 minutes. Ten minutes later, the solution was removed from water bath and waited to cool to room temperature. Optical density readings were obtained for each sample using the Versa Max microplate reader (Molecular Devices) reading at 625 nm. Glycogen was used at different concentrations (1.0 g/ml, 2.0 g/ml, 2.5 g/ml, 5 g/ml, 7.5 g/ml) as the standard to establish a standard curve at 625 nm at the same time. The concentrations of glycogen was determined by comparison to the standard curve.

Statistical Analysis

Our statistical analysis was conducted using SAS 9.3 (SAS 2009). The data are analyzed using a mixed model. Fix effects include line types and treatments. Random effects included linear nested with line types in addition to the residual. All the data were created as continuous data. A mixed model analysis of variance was used with line types and treatments as fixed effects. Random effects consisted of variation among the three lines of each type. Variation among lines of the same type was nested within fixed effects for the analysis.

For the starvation, desiccation, and oxidation assays, the comparison of average death time for reaching 50% mortality rate among different lines and treatments were analyzed. For the chill coma recovery assay, using statistical analysis also compared a total recovery time of flies among different lines and treatments. The lipid and glycogen

assays were determined for all lines, by comparing different treatments. Males and females were also analyzed separately to compare the sex difference between treatment and line types for each experiment.

Results

Starvation Assay

The average mortality rate among different lines types for males treated with autoclaved spores treatments was presented in Figure 2. The selected males had a lower mortality compared to the other two groups. The same situation occurred in control injected and non-perturbation males (Figure 3 and Figure 4), especially for non-perturbation males (Figure 4). There was an interaction detected between treatments and line types for male flies ($P = 0.02$) (Table 1). We thus inspected the simple effect of the treatment and line type interaction. Based on the statistical analysis, males in control lines with treatment were dying significantly faster than selected males ($P = 0.0023$) (Table 2).

For the female flies, control females were dying significantly faster than CI and S females under all three treatments conditions (Figures 5, 6, and 7). The statistical analysis showed no interaction detected between line types and treatments. In this case, treatment effect did not affect the result of line types. So we only need to inspect the line type effects (main effect) for female flies. The result illustrated a significant difference between selected and non-perturbation females, also between wound control females and non-perturbation females, with P value of 0.0406 and 0.0074 separately (Table 3). In this case, the selected and control injected females survived acute starvation longer than those

in non-perturbation lines.

Desiccation Assay

When compared the average mortality rate of males under H_2O injected treatment of different line types (Figure 9), selected males died significantly slower than other two lines with P value of 0.0005 (Table 4). There was no significant difference between injections with autoclaved spores compared to non-perturbation treatment (Figures 8 and10). But there was an interaction between line types and treatments (P = 0.0142) (Table 1). Besides, there was a significant difference detected between wound control and non-perturbation lines (P = 0.0213) (Table 4). Without any treatment, wound control lines survived longer than non-perturbation lines (P = 0.0344) (Table 4).

Female flies did not present any difference between line types under all treatments conditions (Figures 11, 12, and 13). Comparing the survival time when they reached 50% mortality rate, there was no interaction detected between line types and treatments for females, and also no significantly difference detected between line types (Table 5).

Chill Coma Recovery

For chill coma recovery assay, there was no significantly difference of total recovery time detected between line types for male flies (Table 6) and the overall mean of males for total recovery time under among different line types were very close under each treatment (Figures 14, 15, and 16). However for female flies, selected group had a quicker recovery time than the other two groups under autoclaved spore treatment (Figure 17). There was a significant interaction between line types and treatments among females

($P < 0.0001$) (Table 1). Selected females recovered significantly slower under autoclaved spores treatment than females in non-perturbation lines ($P = 0.0014$) (Table 7). Under H_2O injected treatment, a significant difference of recovery time among selected females and H_2O injected females were detected ($P = 0.004$) (Figure 18). Without any treatment, there was also a significantly difference of recovery time among different line types ($P = 0.0022$ and $P = 0.0062$) (Figure 19).

Oxidation Assay

For oxidation assay, the no-perturbation control males (Cn) died faster than other two line types under all three treatments (Figures 20, 21, and 22). Based on the statistic analysis, when flies reach 50% mortality rate, control males had a significantly lower survival time compared to the other two groups under all treatments ($P = 0.0069$ and $P = 0.0002$) (Table 8). However, for female flies, selected groups died slower than other two groups for some specific time point, especially under H_2O treatment (Figure 24), but there was no interaction detected between line types and treatment effect for females at 50% mortality and no significantly difference of survival time among female line types (Table 9).

Lipid Assay

For lipid assay, the average lipid concentration in selected males was higher than other two groups under all treatment conditions, followed by H_2O injected groups (Figures 26, 27, and 28). There was a significantly difference of male lipid concentration detected among lines types ($P = 0.0071$) (Table 11). Selected males presented a

significantly higher lipid concentration than non-perturbation lines (P = 0.0019) (Table 12). However for female flies, the average lipid concentration between different line types for under each treatment condition (Figures 30, 31, and 32) did not significantly among line types (Table 13).

Glycogen Assay

The average glycogen concentration of selected males was higher than other two line types, especially under autoclaved spores treatment (Figure 32). There was no interaction detected among line types and treatments, and no significant difference detected among line types (Table 14). However, for females, control groups presented a significantly higher glycogen concentration than any other two groups, especially under autoclaved spores treatment (Figure 35) and no treatment condition (Figure 37). Significant differences in glycogen concentration were detected under all treatments among selected females and non-perturbation females (P = 0.0407) (Table 15).

Discussion

Evolution of resistance to bacterial spores has been observed in laboratory and field population of *Aedes aegypti* populations when *Bacillus thuringiensis* subspecies *israelensis* was the pathogen (Goldman *et al*. 1985). In our study, the level of resistance to *B. cereus* spores was many-fold greater than previously observed for selection on *A. aegypti* using *B. thuringiensis* spores. After 26 generations, the dose concentration of *B. cereus* for artificial selection have increased to 1.47×10^{10} g/ml from 2×10^{6} g/ml in the first generation of selection. The selected lines are considered as resistant to *B. cereus*

spores. Moreover, autoclaved spores are used as an inducer of the immune response in our study. “Deployment” is the cost of maintaining the immune system in an activated state under conditions where its function is unnecessary and indeed detrimental, and this can also exert a cost (McKean and Lazzaro 2011). This is in many ways analogous to the detrimental effects of inflammation on multiple systems in humans (e.g. metabolic disorders, arthritis) as illustrated by the inflammatory bowel diseases (Anderson *et al*. 2011). Life history traits, like life span, development time and egg production, have also been investigated in the present study (Ma *et al*. 2012). Survival flies in selected lines might present indirect responses to the stress conditions as the effect of selection resistance to *B. cereus* spores. In this stress survival studies, starvation, desiccation, oxidation and chill coma recovery will be consider as different stress conditions for the experiment flies and stress responses are studied as the trade-off behavior for comparison among different line types and treatments.

For starvation, desiccation and oxidation experiments, we compared the overall mortality rate among different line types. For the chill coma recovery experiment, we compared the overall recovery time among different line types. In our hypothesis, selected lines were supposed to survive longer and recover slower compared to the other two line types, as they were resistant to *B. cereus* spores.

Our results support our hypothesis. For starvation assay, control females died significantly faster than selected and control injected females under all treatment conditions (Table 3), while selected males died significantly slower than control males under non- perturbation situations (Table 2). For desiccation assay, selected males always died slower than other two line types (Table 10). For chill coma recovery assay, selected

females presented significantly higher recovery time for cold environment than other two line types, under autoclaved spores treatment and non-perturbation treatment (Table 7). For oxidation assay, both selected and control injected males died significantly slower than non-perturbation males under all treatments (Table 8). All the stress survival studies above proof our hypothesis that selected flies resistant to *B. cereus* spores might a stronger immune system, which could help them resist to the other stress environment.

To investigate the physiological mechanism for these indirect responses, energy storage components were studied. In our experiment, we inspected the lipid and glycogen concentration as their energy component. Previous studies have reported that starvation and desiccation resistance were positively correlated with lipid levels (van Herrewege and David, 1997). Desiccation-selected populations were reported to store fewer lipids but much more glycogen than control populations (Djawdan *et al*., 1998). In our result, selected males presented a significantly higher body lipid concentration than males in control lines (Table 12), while control females presented a significantly higher glycogen concentration than selected females (Table 15). This could explain the survival responses results of starvation and desiccation experiment.

A future plan of this study could work on the insight into the evolution of *B. cereus* spore infection resistance and/or tolerance in *D. melanogaster*. According to Schneider (2008), when challenged with a pathogen, a host would evolve two types of defense mechanisms to increase its fitness, which is tolerance and resistance mechanism. Antimicrobial peptides and phenol oxidases activity of *B. cereus* selected flies would provide insight into mechanisms of resistance or tolerance of our "*D.melangaster*" lines selected using "*B. cereus*" spores.

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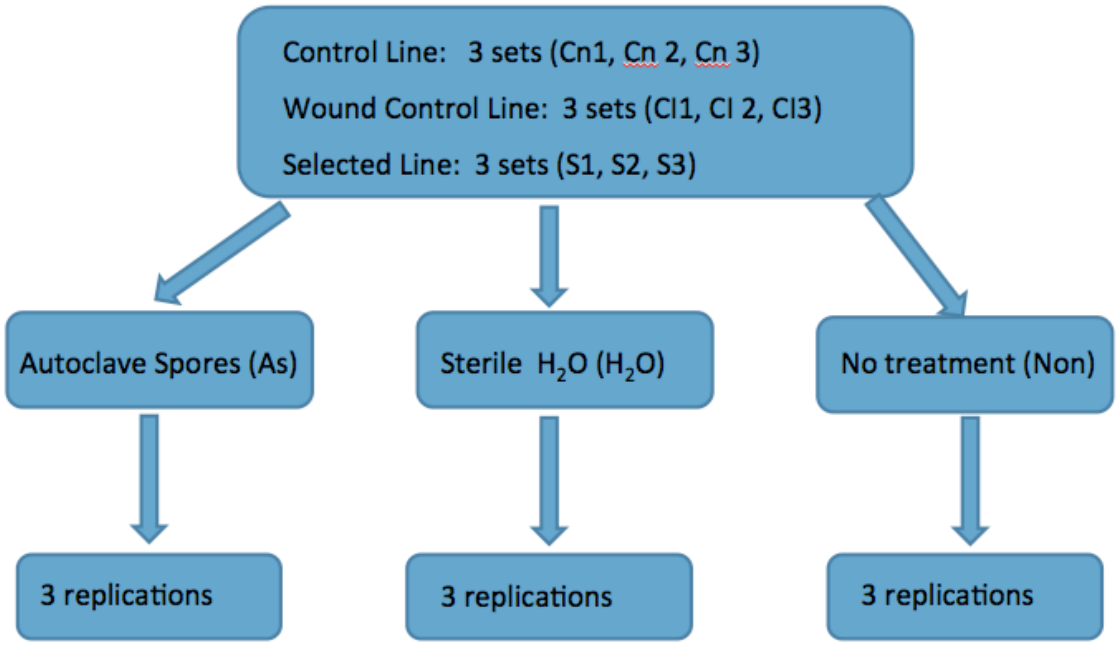

Figure 1. Experiment design of line types and treatments.
Three line types: Selected line (S), wound control line (CI) and control line (Cn). Each line type has 3 replications. Within each line, three treatments are applied: autoclaved spores (As), sterile water (H_2O) and no treatment (Non). Each treatment also has 3 replications.

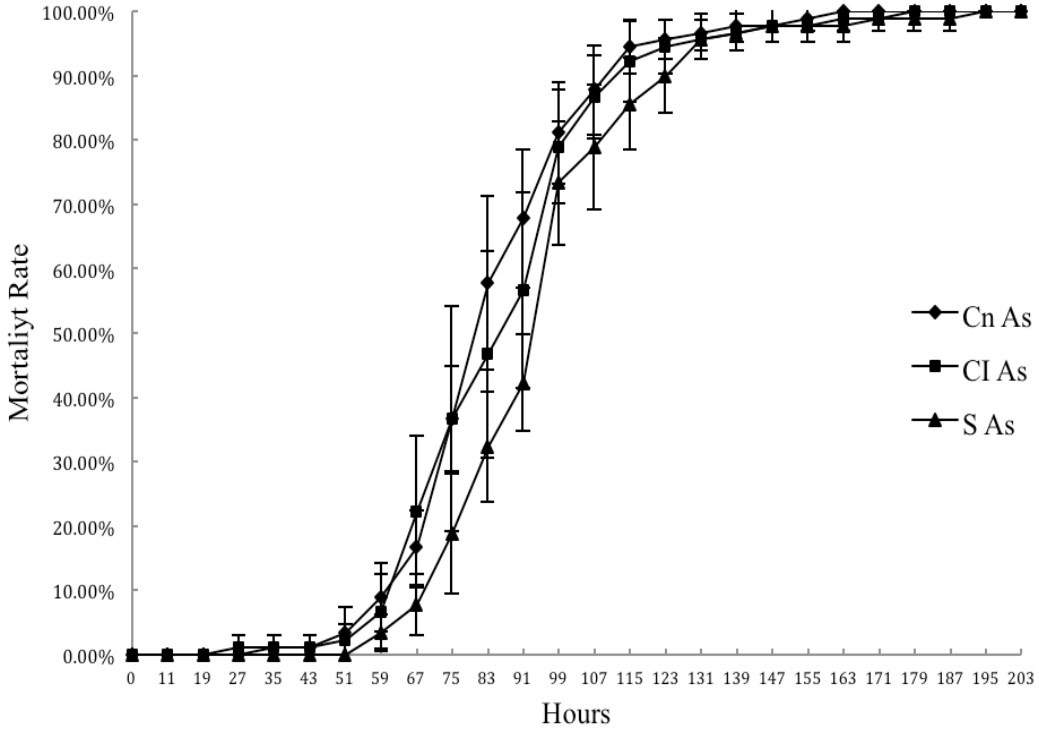

Figure 2. A comparison of average mortality rate for autoclaved spores (As) injected male flies from the selected and two control lines for starvation assay. The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

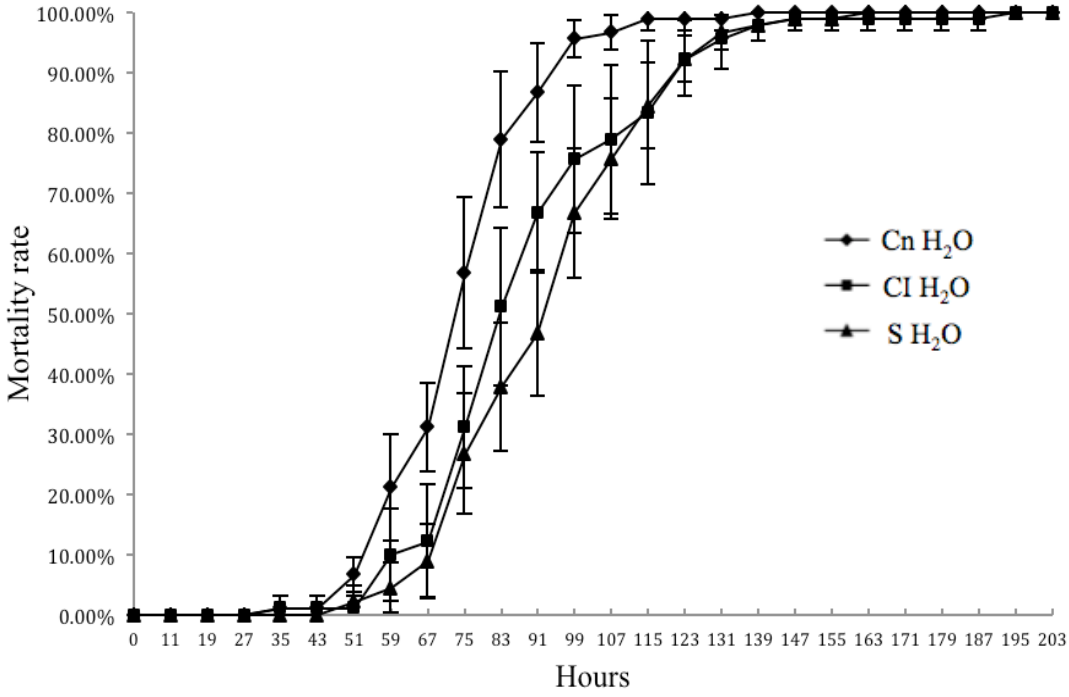

Figure 3. A comparison of average mortality rate for sterile H_2O injected male flies from the selected and two control lines for starvation assay. The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

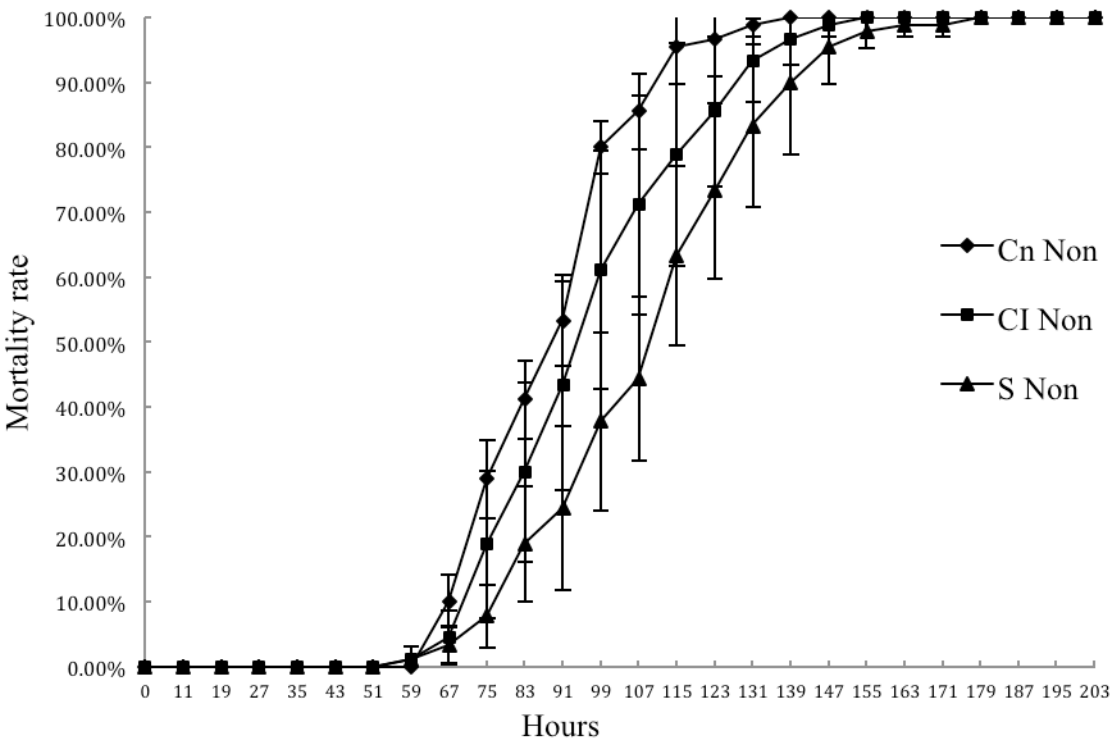

Figure 4. A comparison of average mortality rate for control male flies without any treatment (Non) from the selected and two control lines for starvation assay. The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

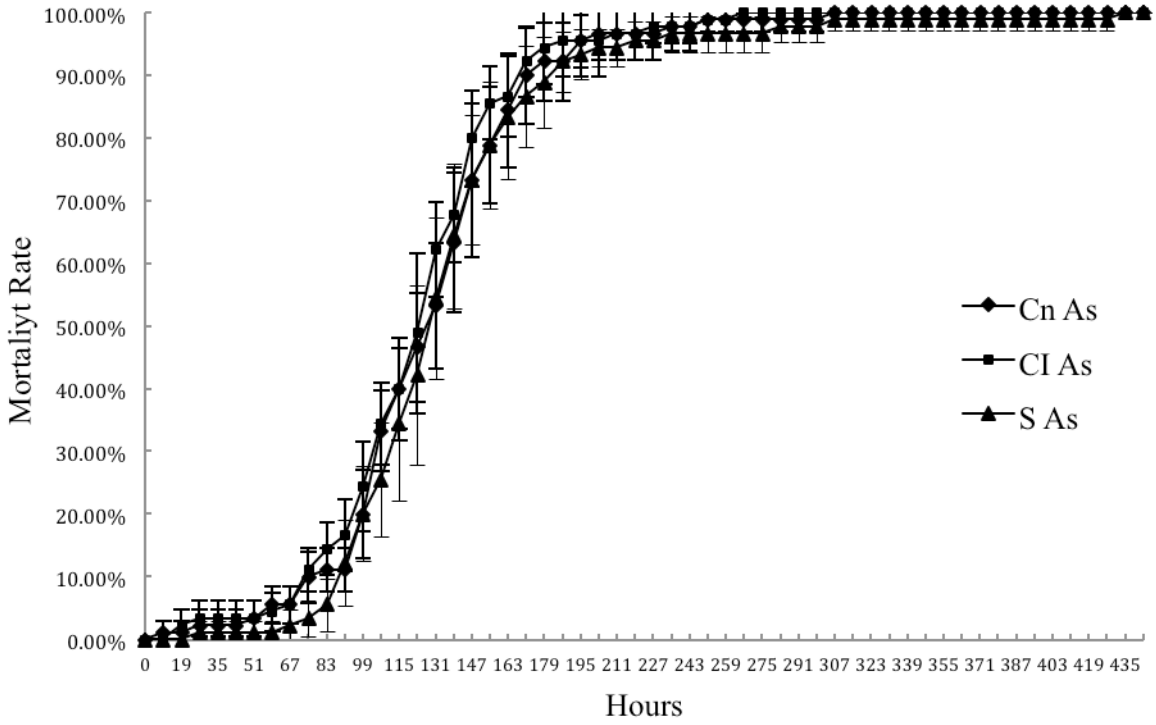

Figure 5. A comparison of average mortality rate for autoclaved spores (As) injected female flies from the selected and two control lines for starvation assay. The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

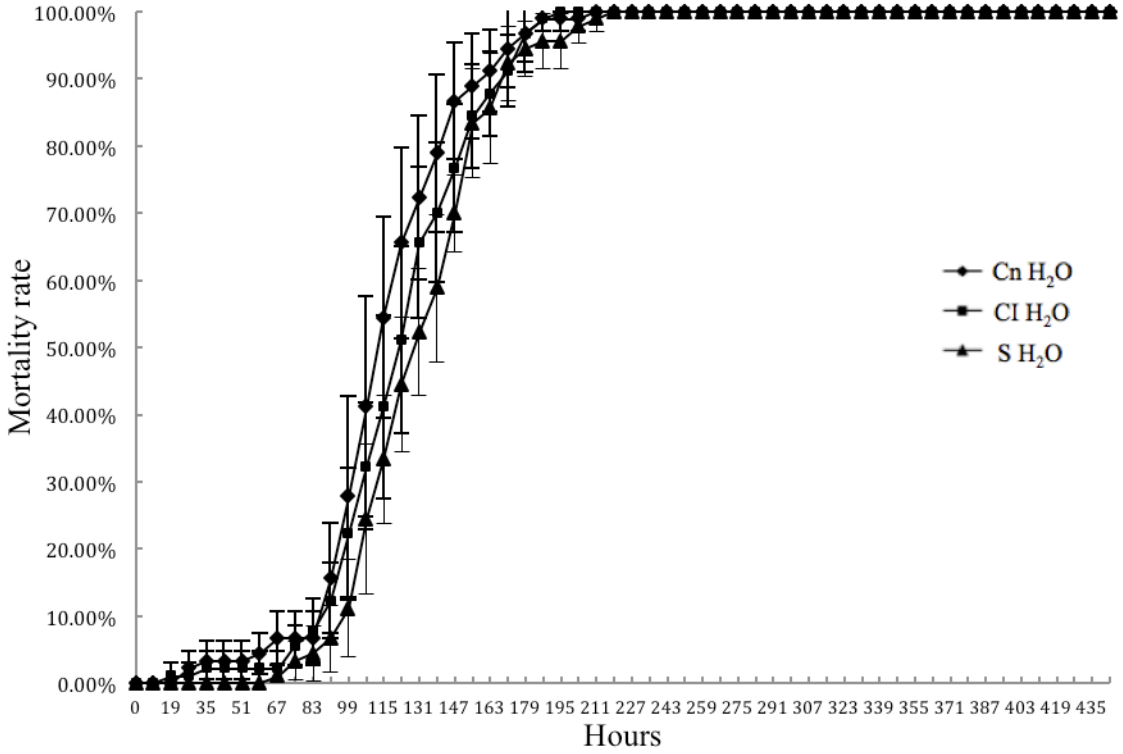

Figure 6. A comparison of average mortality rate for sterile H_2O injected (H_2O) female flies from the selected and two control lines for starvation assay. The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

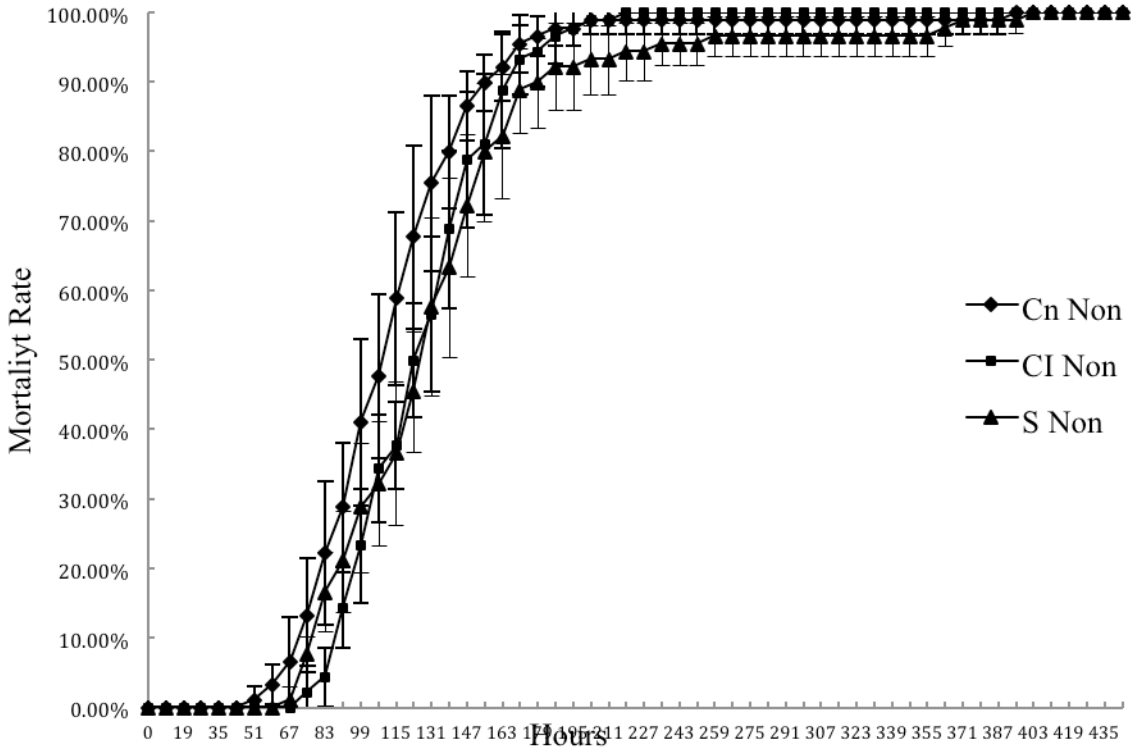

Figure 7. A comparison of average mortality rate for control female flies without any treatment (Non) from the selected and two control lines for starvation assay. The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

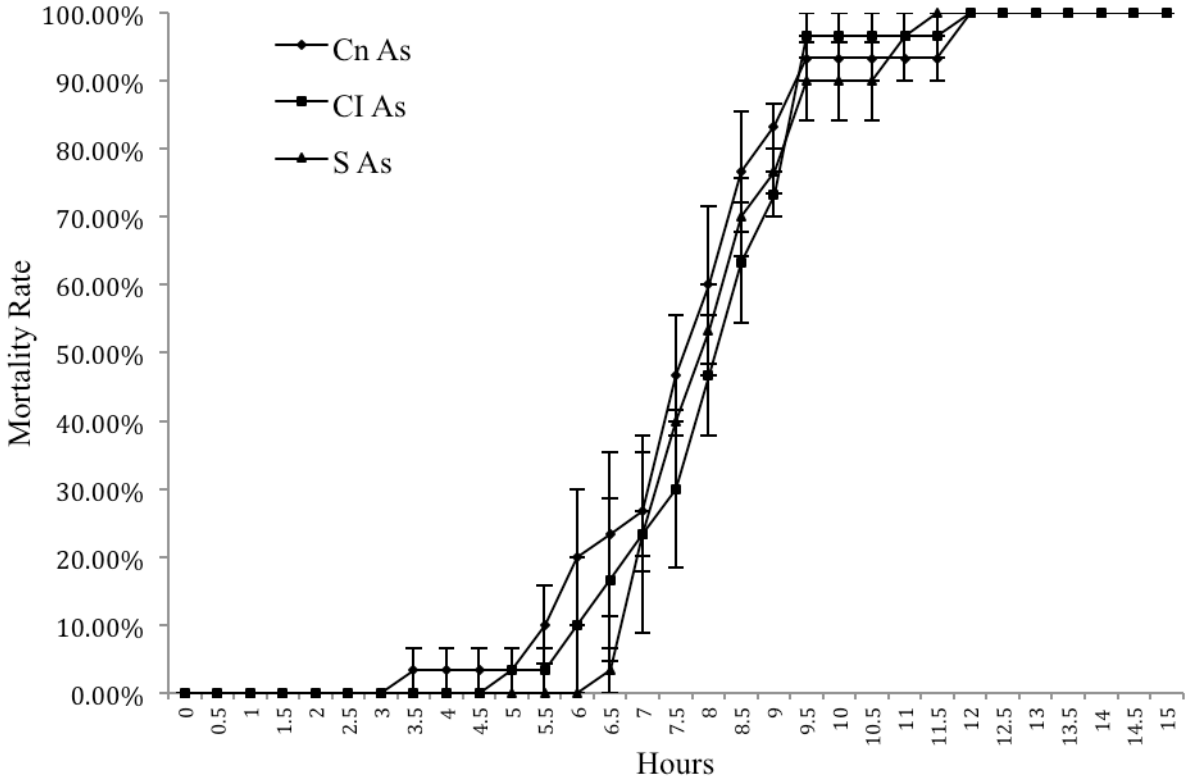

Figure 8. A comparison of average mortality rate for autoclaved spores (As) injected male flies from the selected and two control lines for desiccation assay. The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

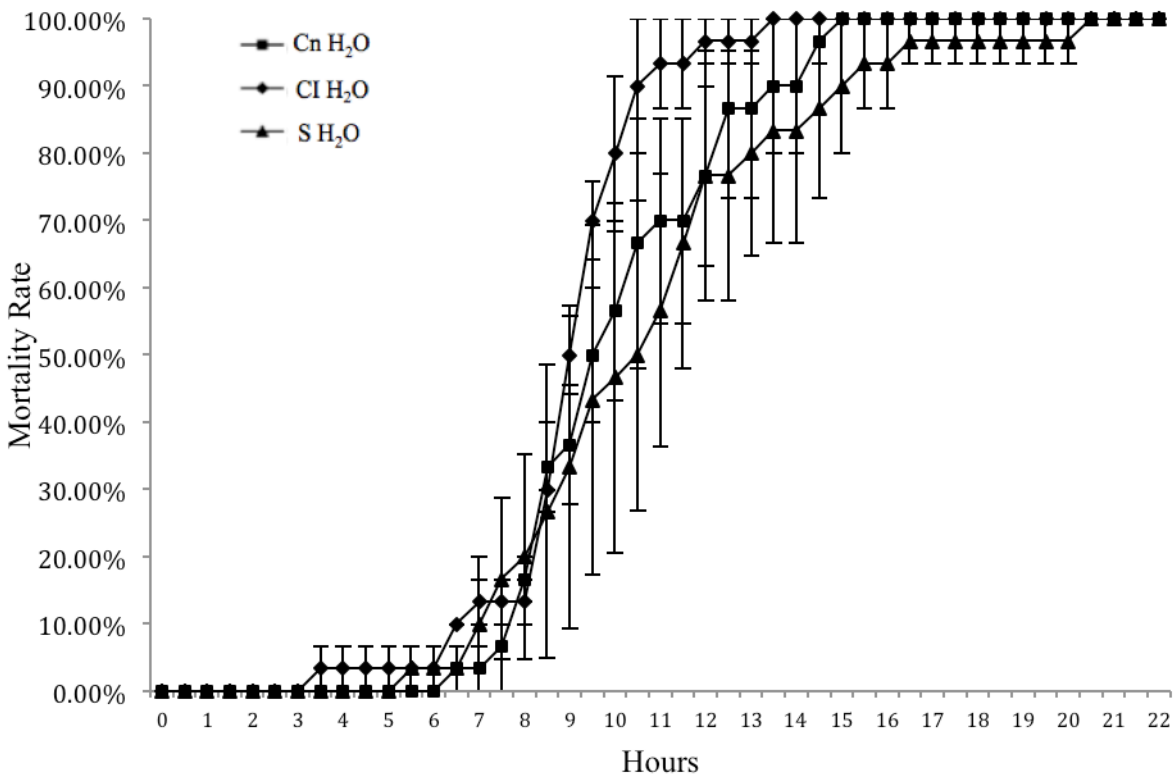

Figure 9. A comparison of average mortality rate for sterile H_2O (H_2O) injected male flies from the selected and two control lines for desiccation assay. The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

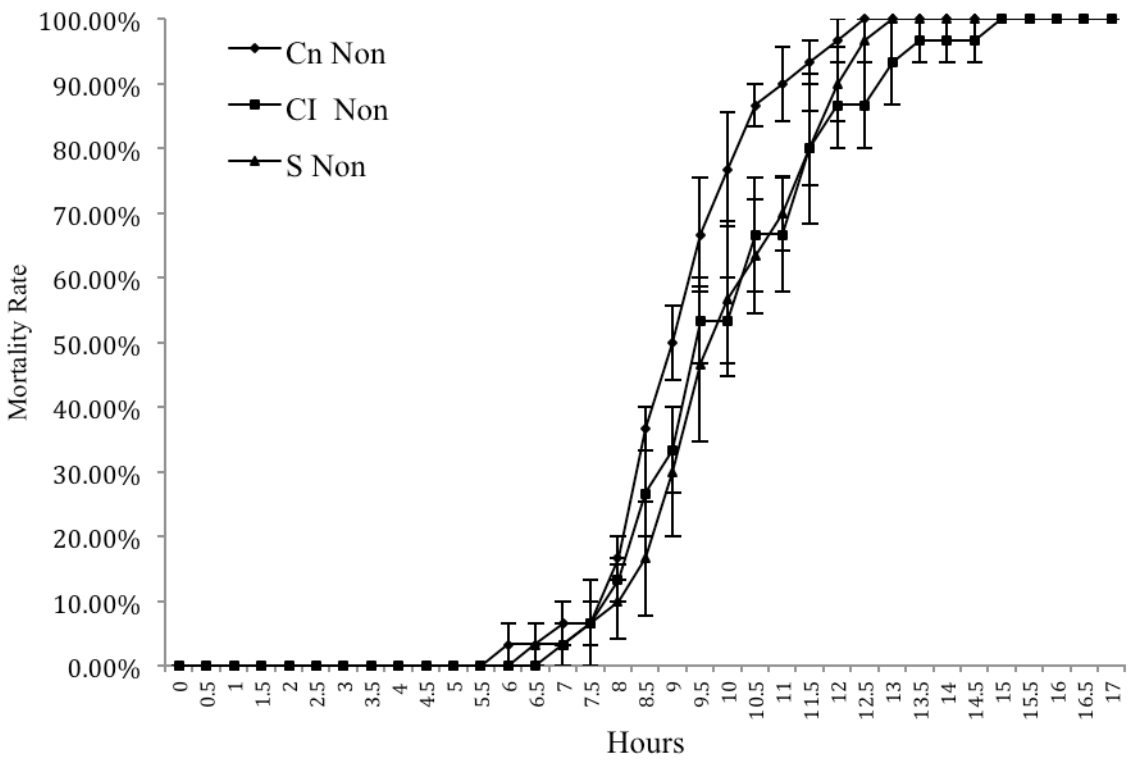

Figure 10. A comparison of average mortality rate for control male flies without any treatment from the selected and two control lines for desiccation assay. The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

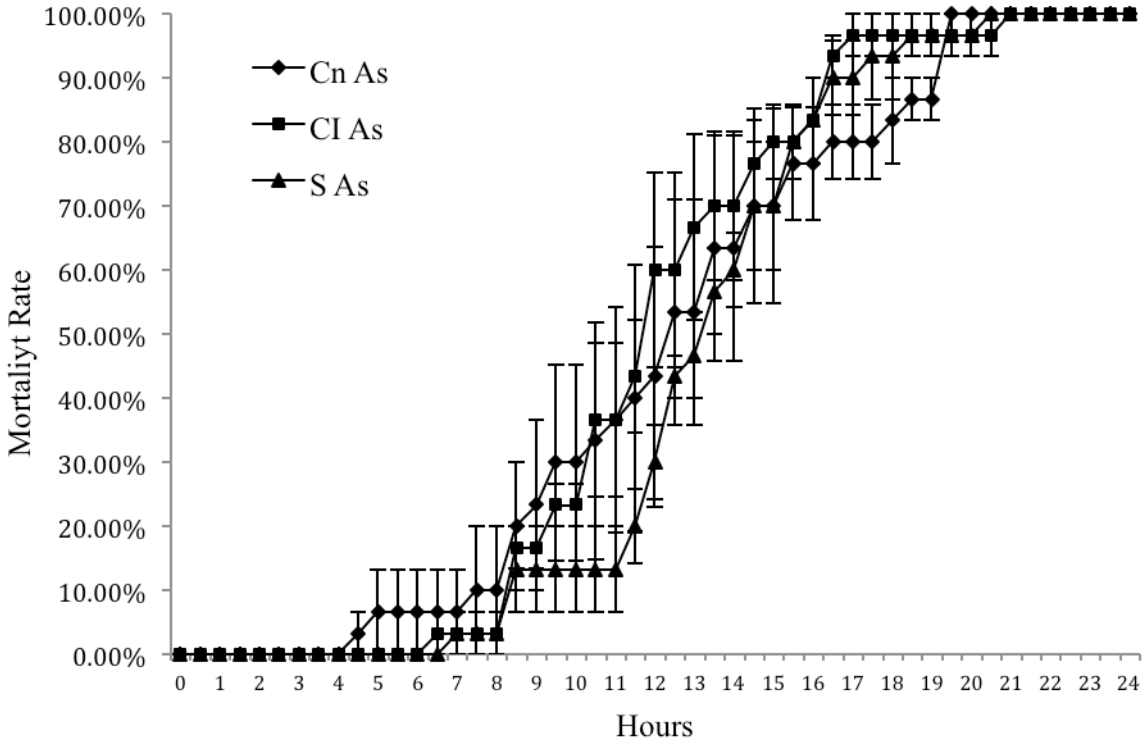

Figure 11. A comparison of average mortality rate for autoclaved spores (As) injected female flies from the selected and two control lines for desiccation assay. The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

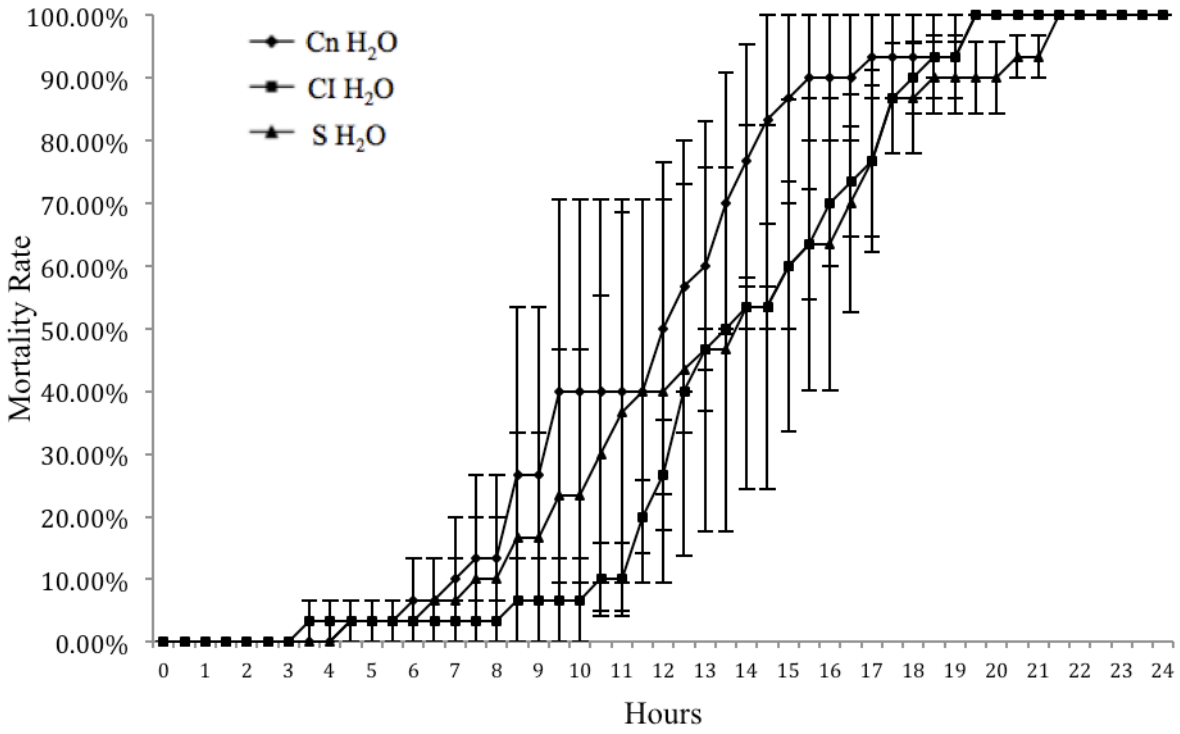

Figure 12. A comparison of average mortality rate for H_2O injected (H_2O) female flies from the selected and two control lines for desiccation assay. The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

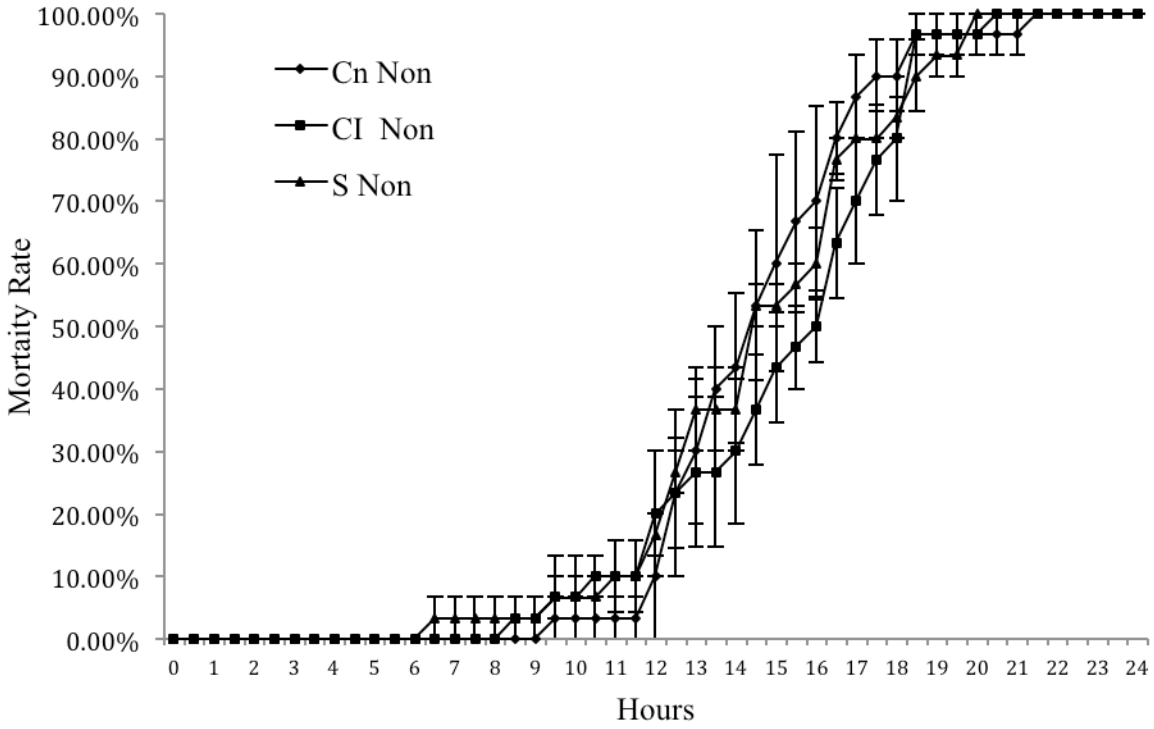

Figure 13. A comparison of average mortality rate for control female flies without any treatment (Non) from the selected and two control lines for desiccation assay. The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

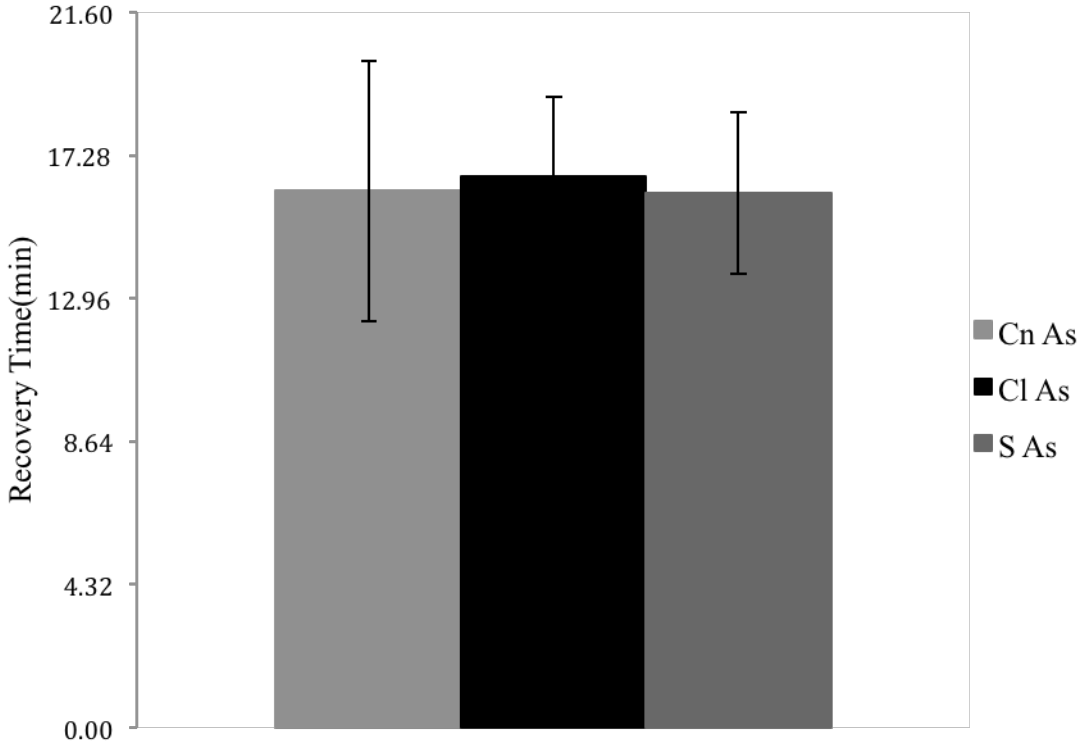

Figure 14. A comparison of total recovery time for autoclaved spores (As) injected male flies from the selected and two control lines for chill coma recovery assay. The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

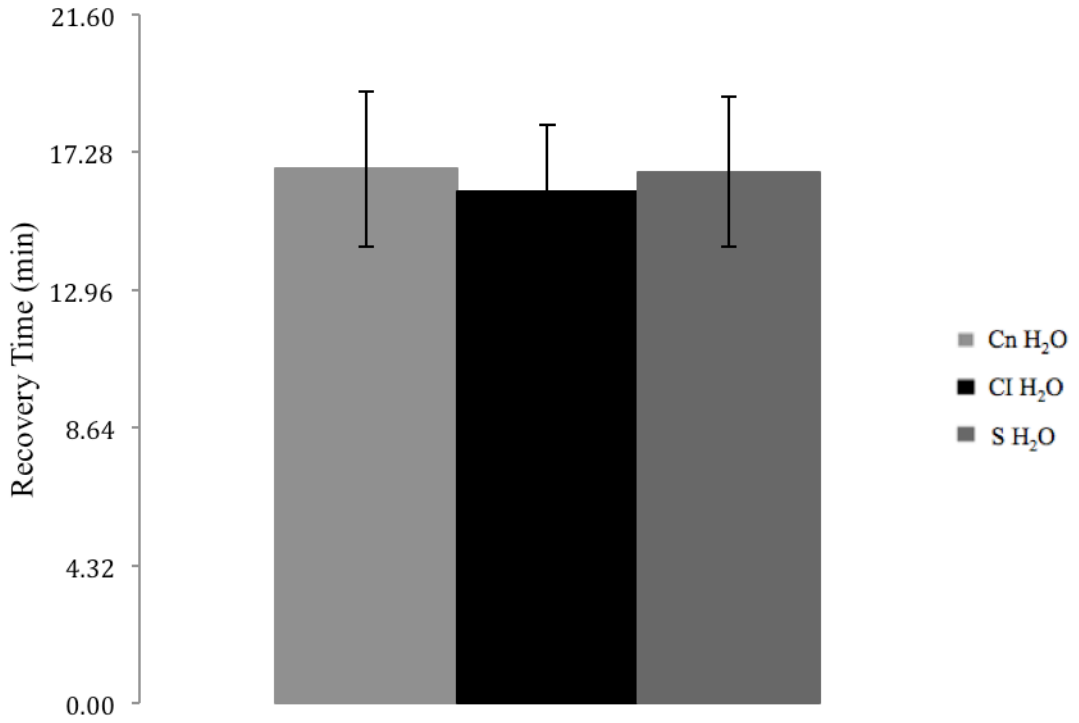

Figure 15. A comparison of total recovery time for H_2O injected (H_2O) male flies from the selected and two control lines for chill coma recovery assay. The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines

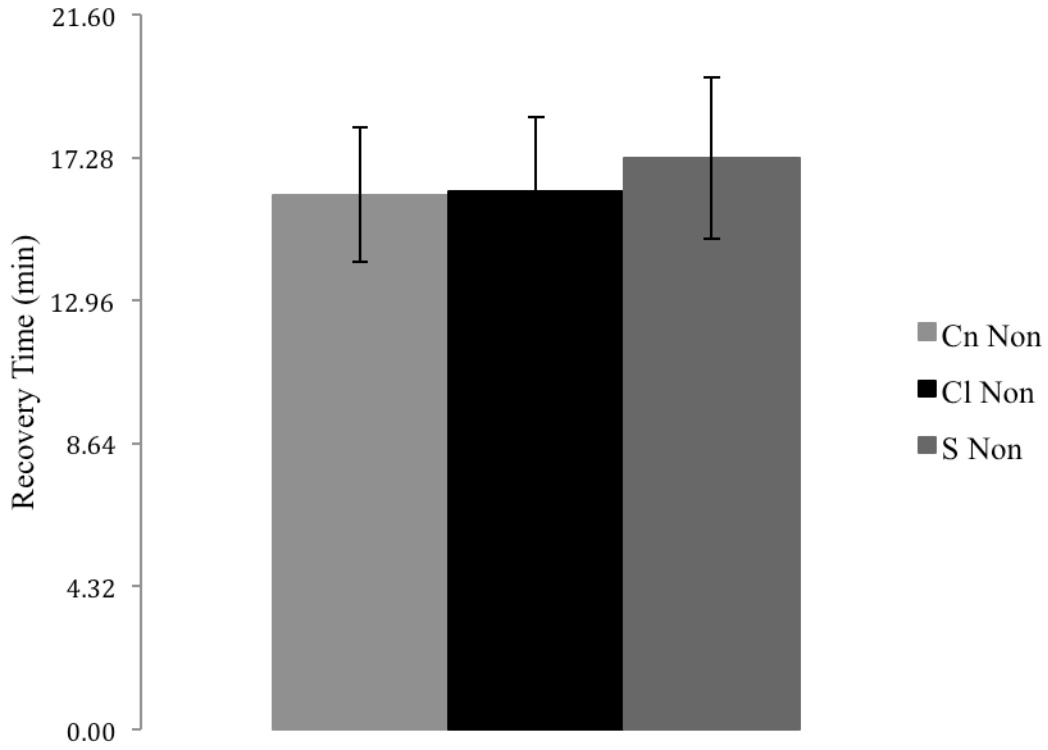

Figure 16. A comparison of total recovery time for male flies without any treatment (Non) from the selected and two control lines for chill coma recovery assay. The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines

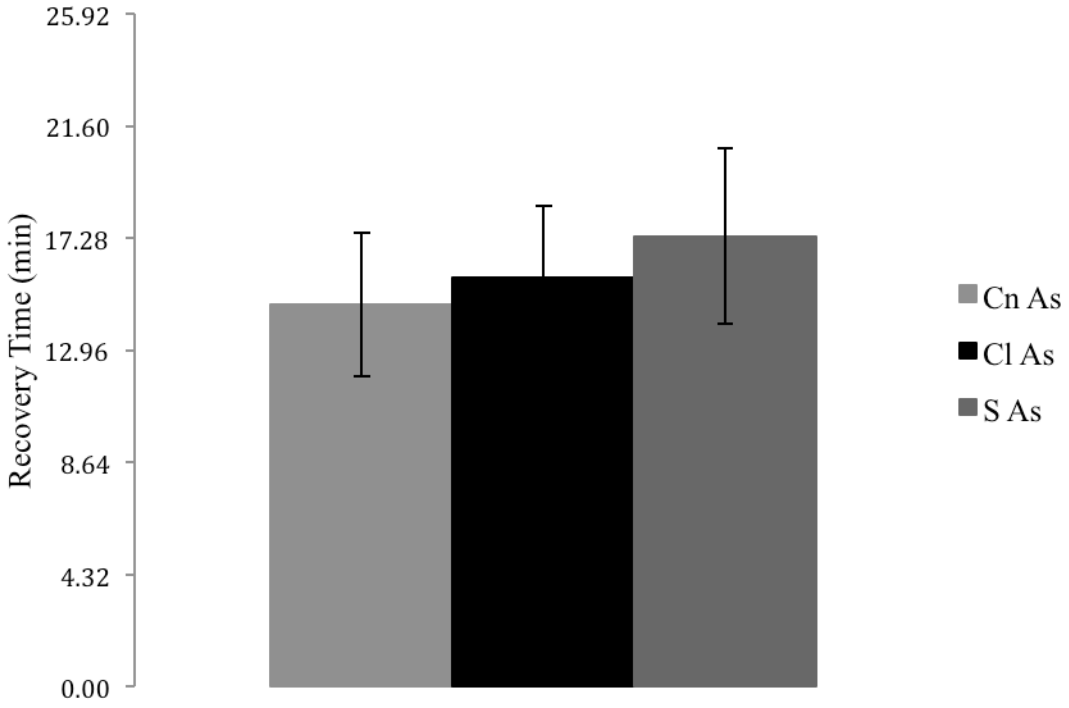

Figure 17. A comparison of total recovery time for autoclaved spores (As) injected female flies from the selected and two control lines of chill coma recovery assay. The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines

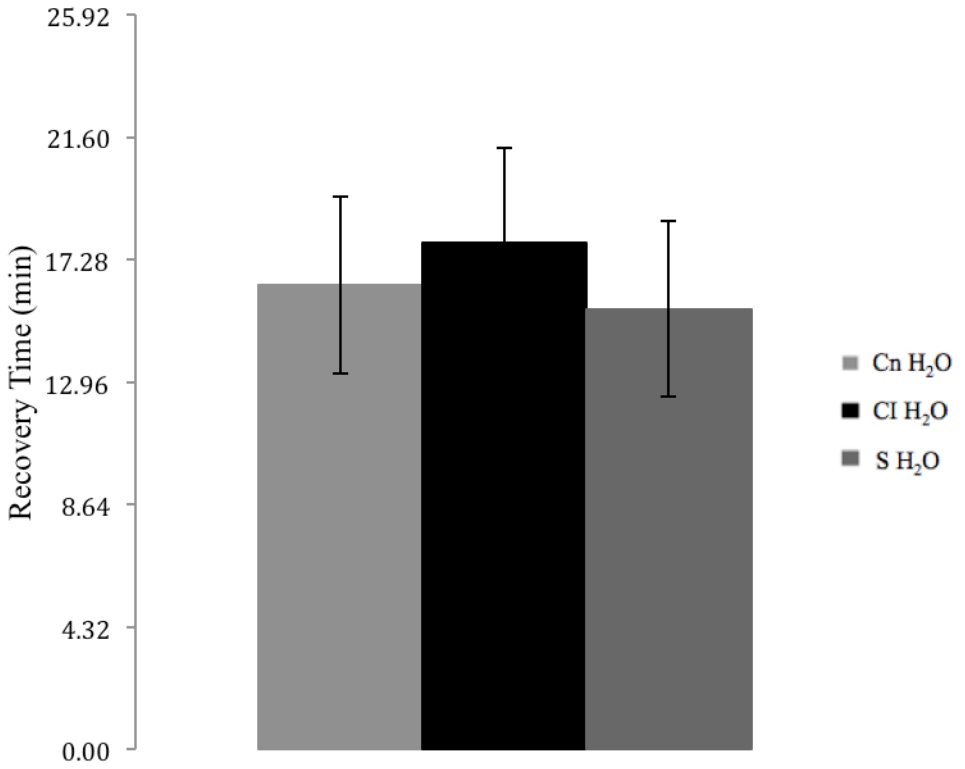

Figure 18. A comparison of total recovery time for H_2O injected (H_2O) female flies from the selected and two control lines for chill coma recovery assay. The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines

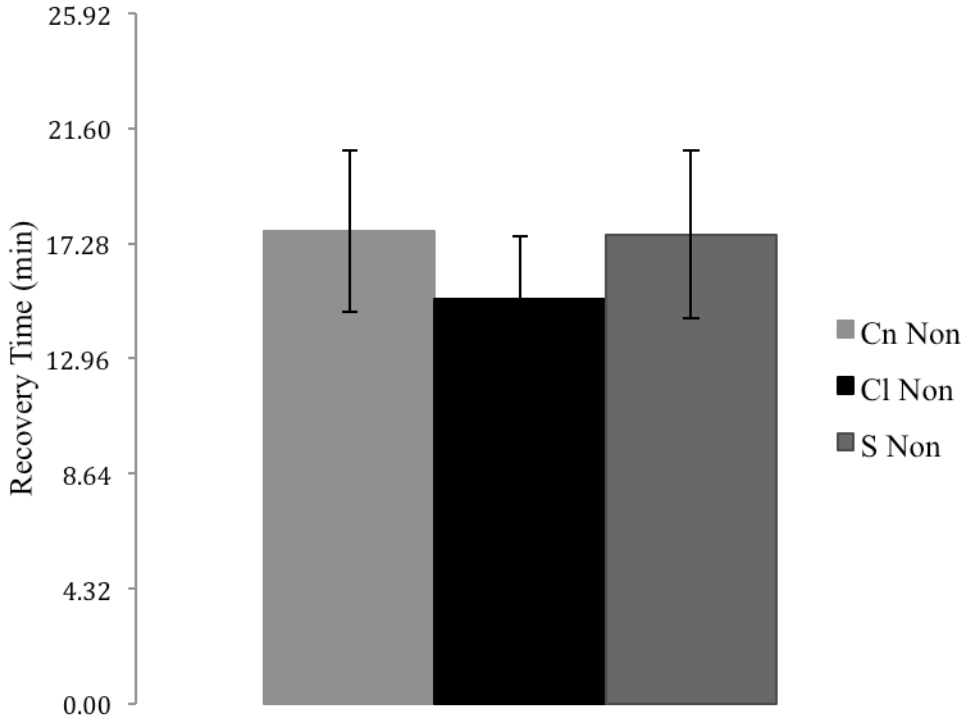

Figure 19. A comparison of total recovery time for female flies without any treatment (Non) from the selected and two control lines for chill coma recovery assay. The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines

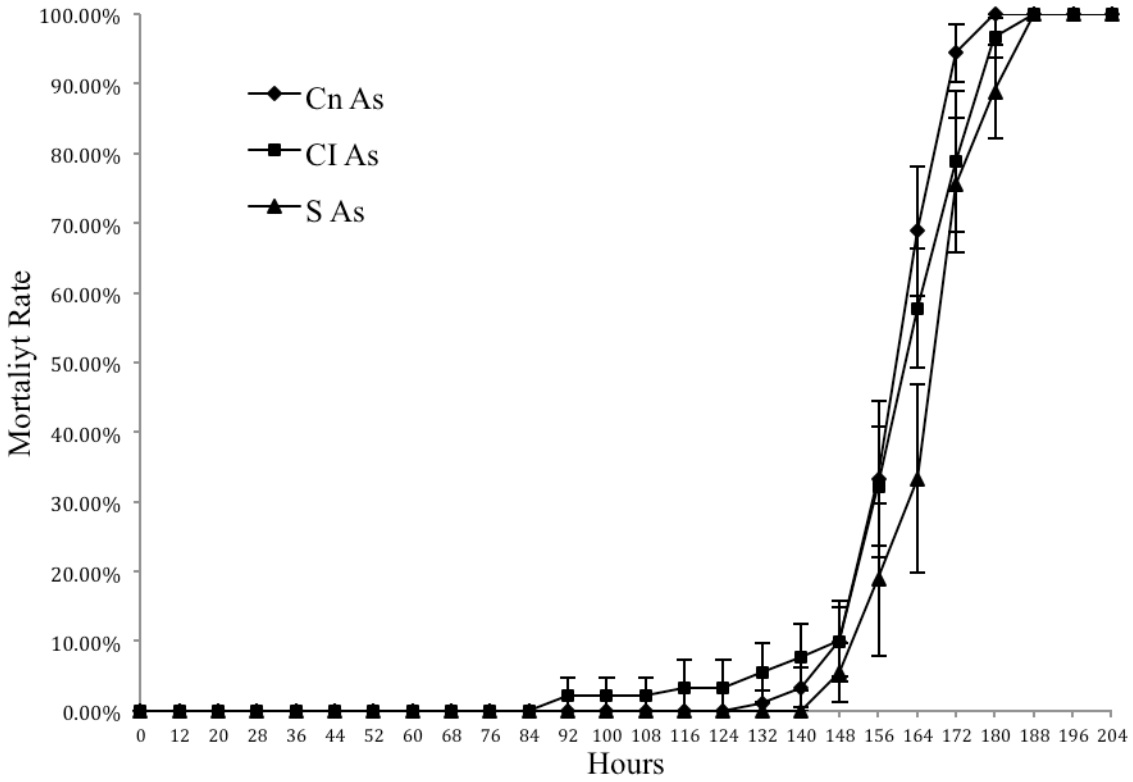

Figure 20. A comparison of average mortality rate for autoclaved spores (As) injected male flies from the selected and two control lines of oxidation assay. The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

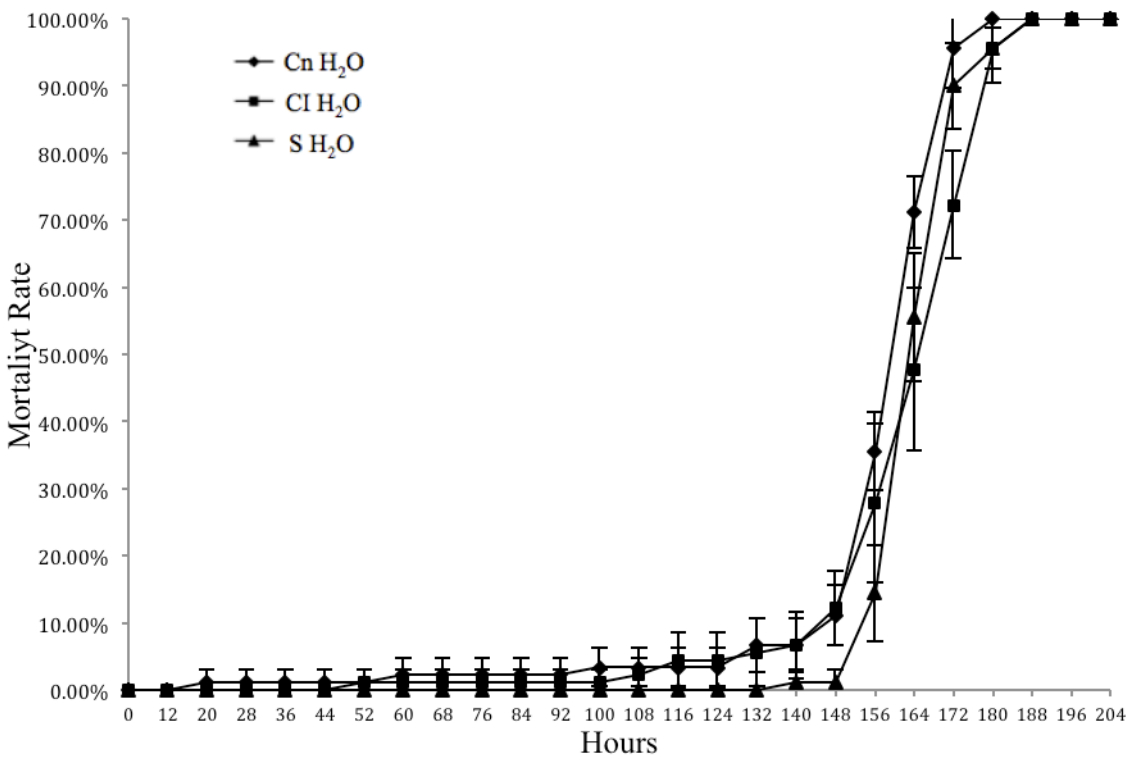

Figure 21. A comparison of average mortality rate for H_2O injected (H_2O) male flies from the selected and two control lines of oxidation assay. The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

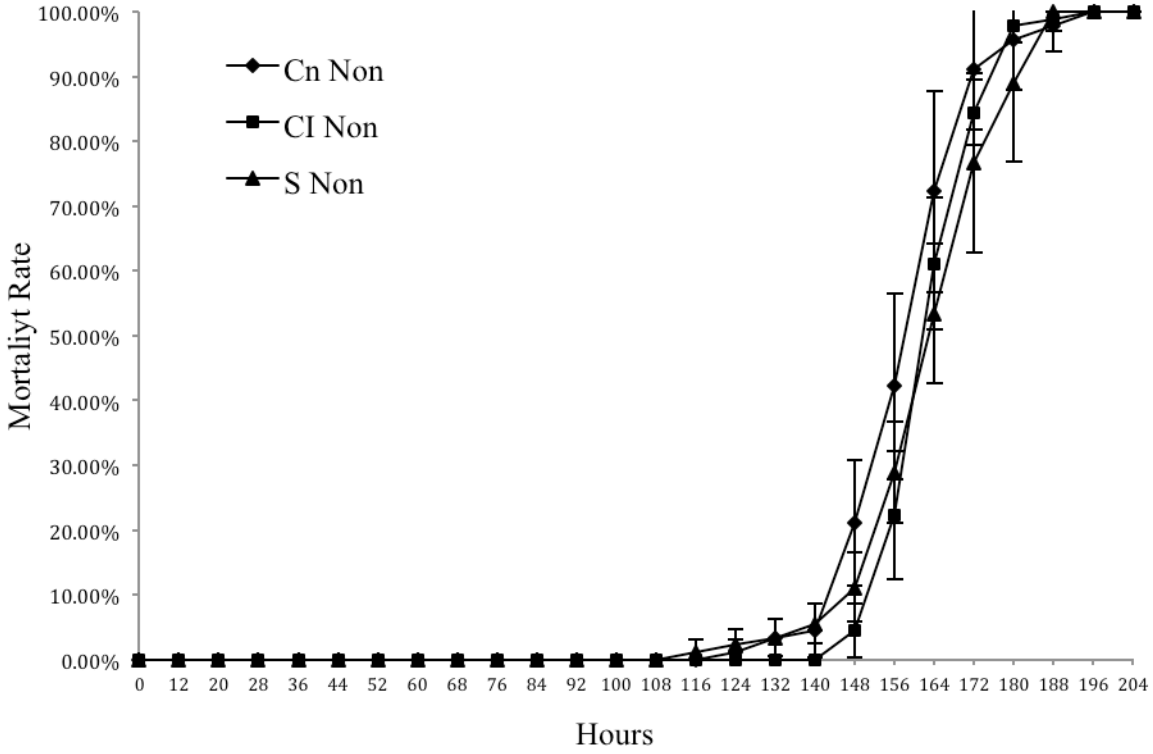

Figure 22. A comparison of average mortality rate for control male flies without any treatment (Non) from the selected and two control lines for oxidation assay. The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

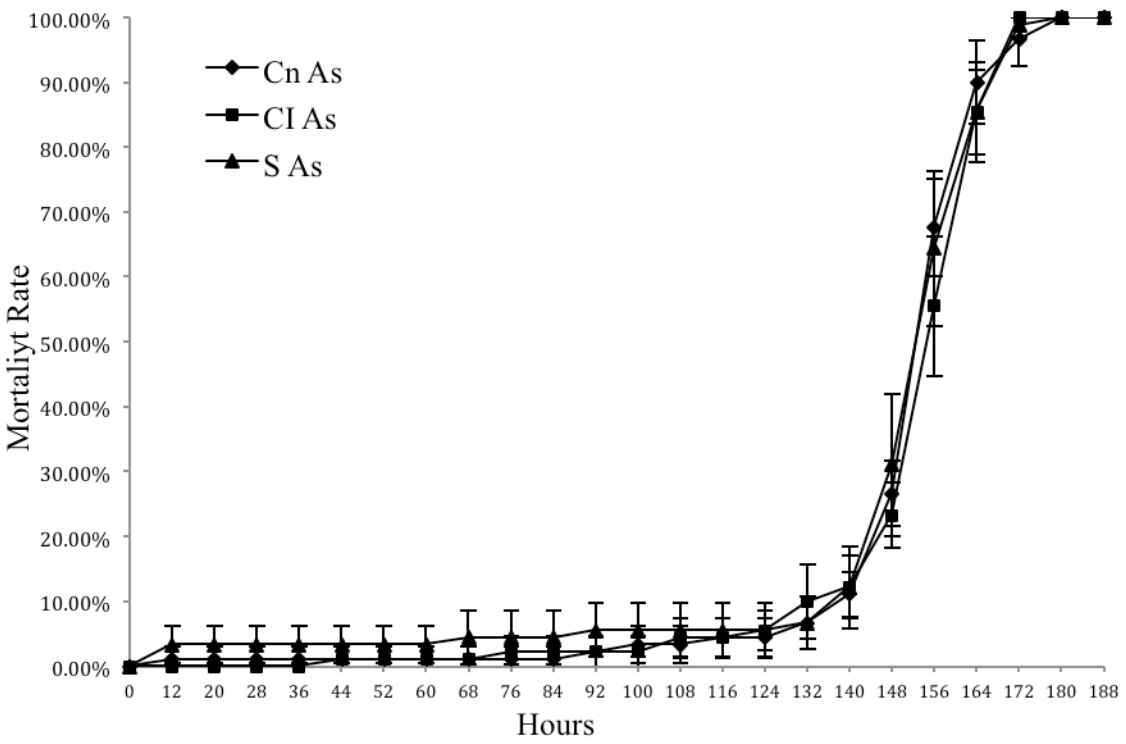

Figure 23. A comparison of average mortality rate for autoclaved spores (As) injected female flies from the selected and two control lines of oxidation assay. The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

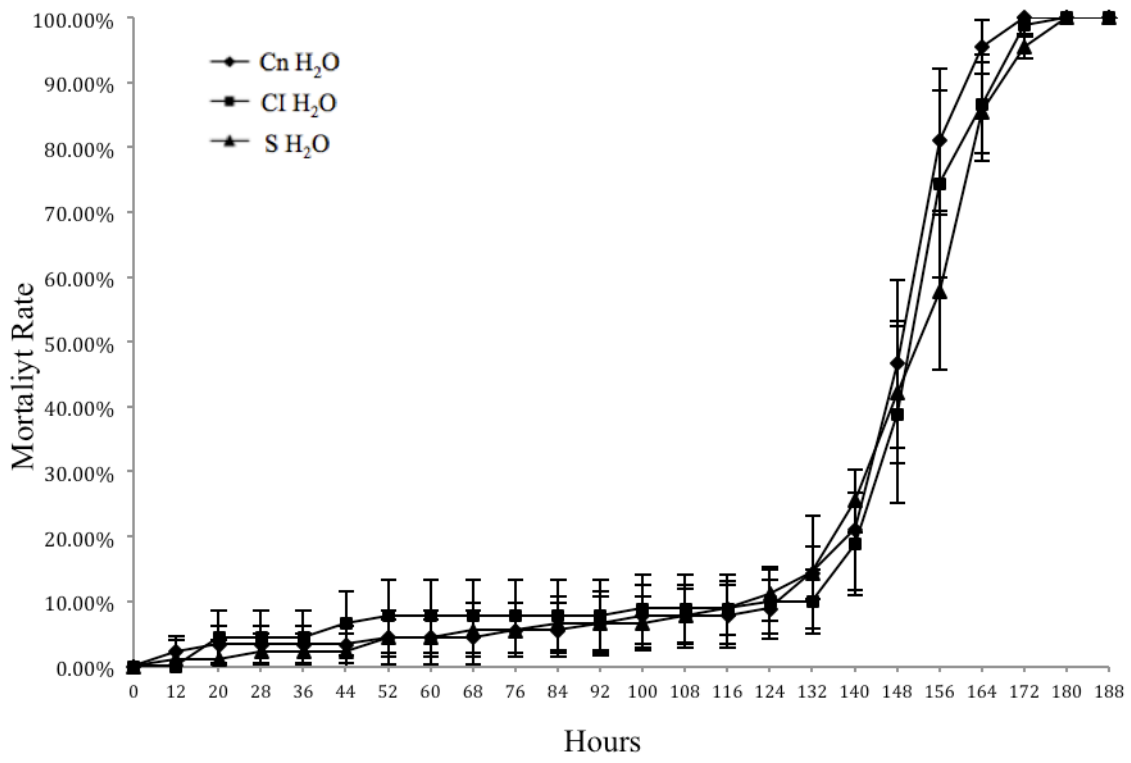

Figure 24. A comparison of average mortality rate for H_2O injected (H_2O) female flies from the selected and two control lines of oxidation assay. The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

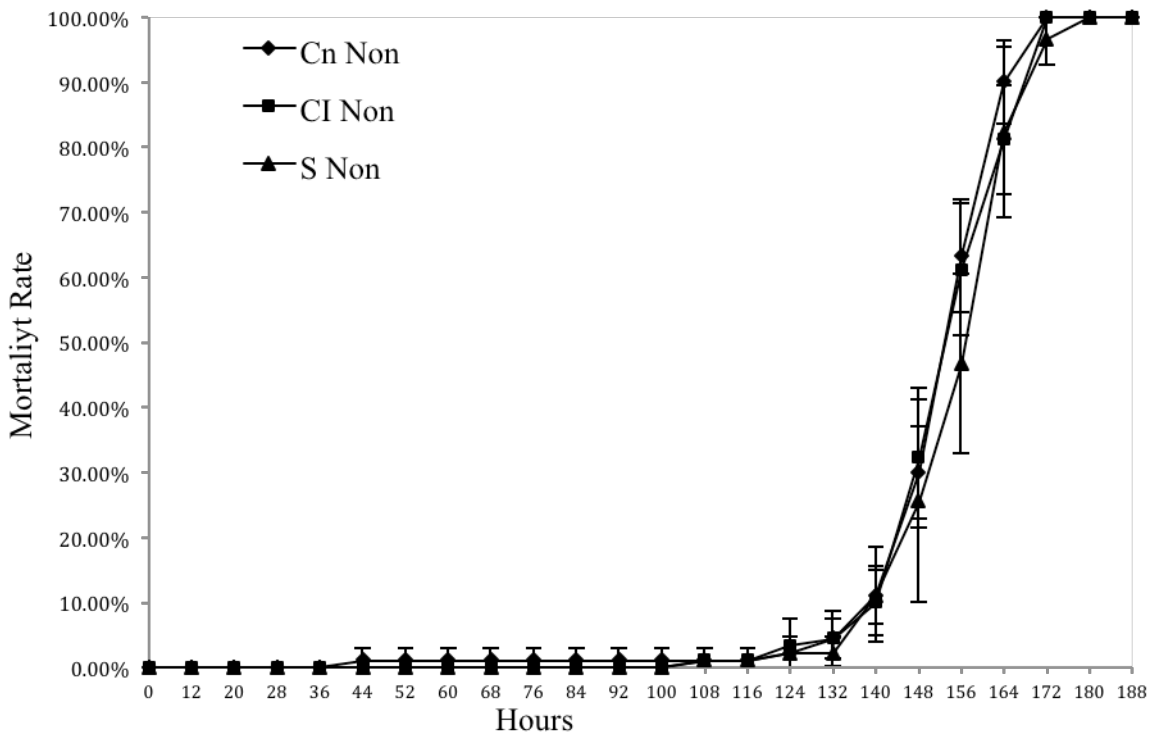

Figure 25: A comparison of average mortality rate for control female flies without any treatment (Non) from the selected and two control lines for oxidation assay. The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

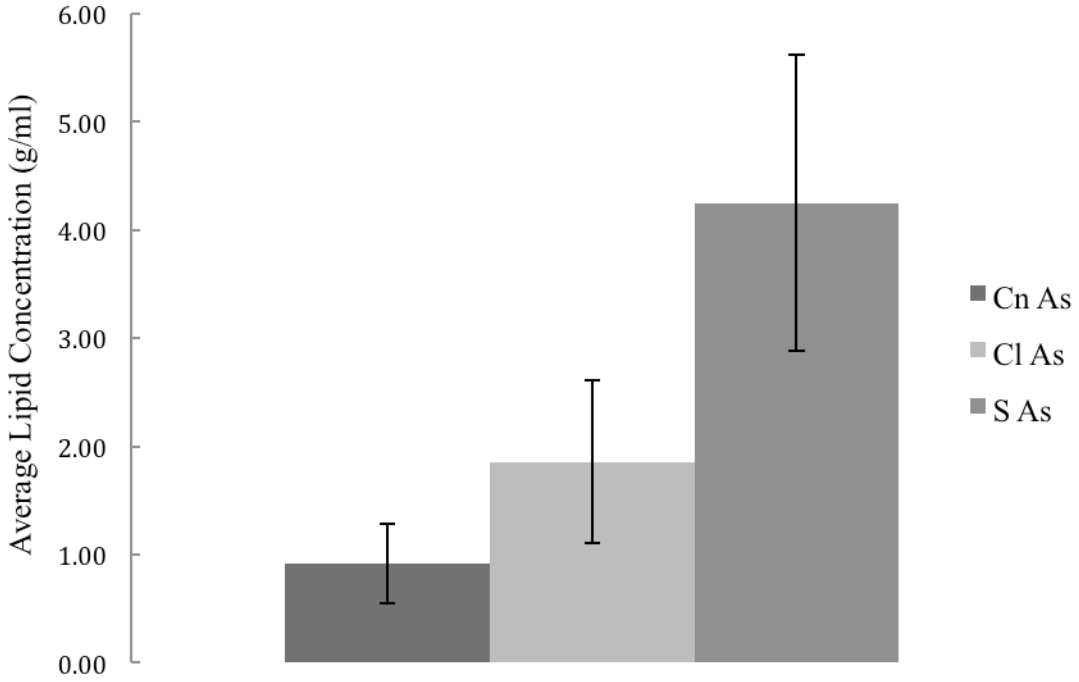

Figure 26. A Comparison of male average lipid concentration between selected and two different control lines with autoclaved spores injection (As). The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

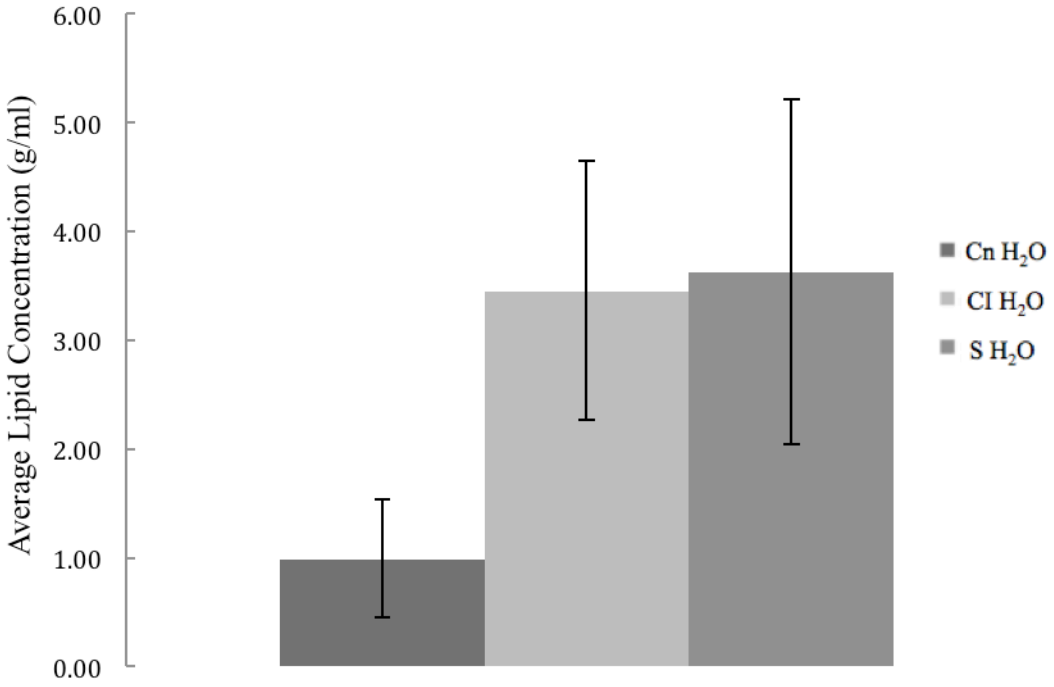

Figure 27. A Comparison of male average lipid concentration between selected and two different control lines with H_2O injection (H_2O). The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

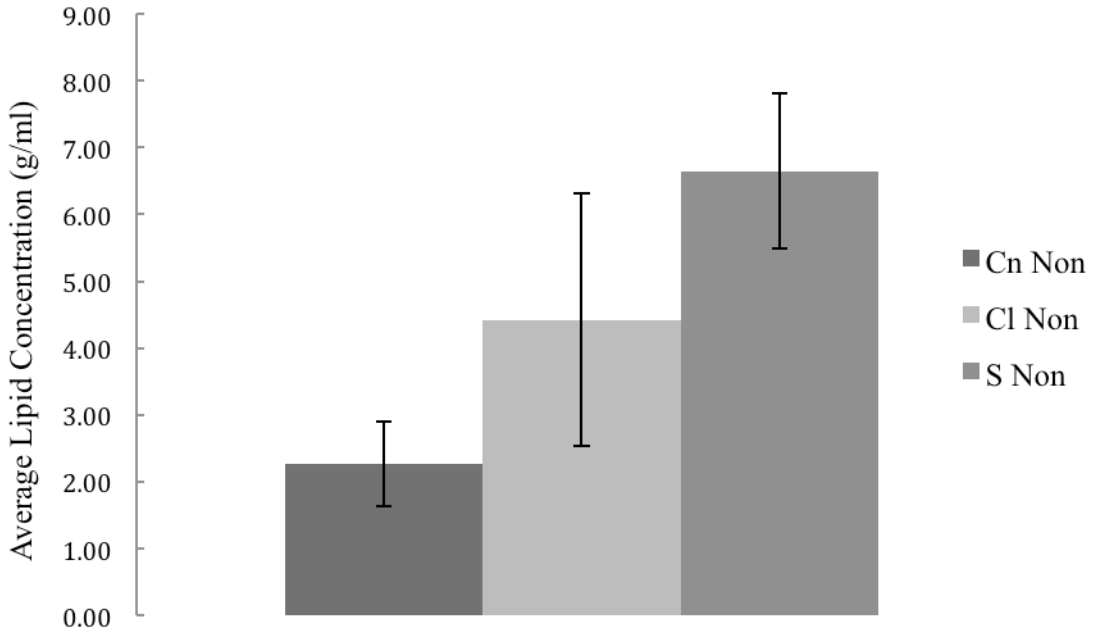

Figure 28. A Comparison of male average lipid concentration between selected and two different control lines without any treatment (Non). The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

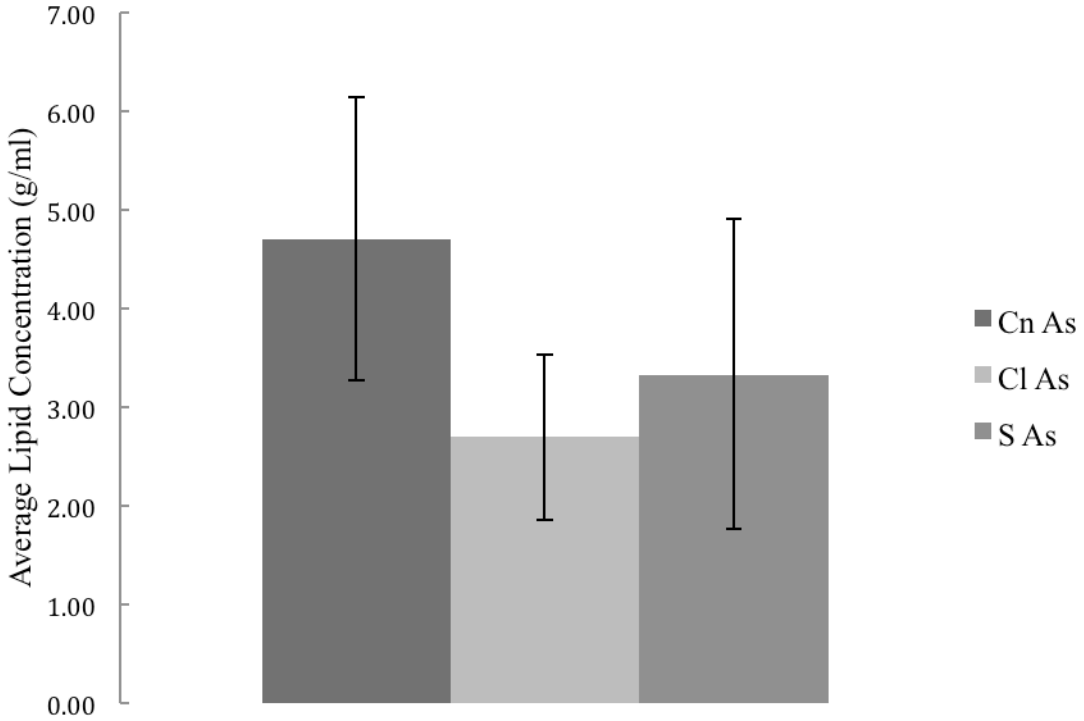

Figure 29. A Comparison of female average lipid concentration between selected and two different control lines with autoclaved spores injection (As). The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

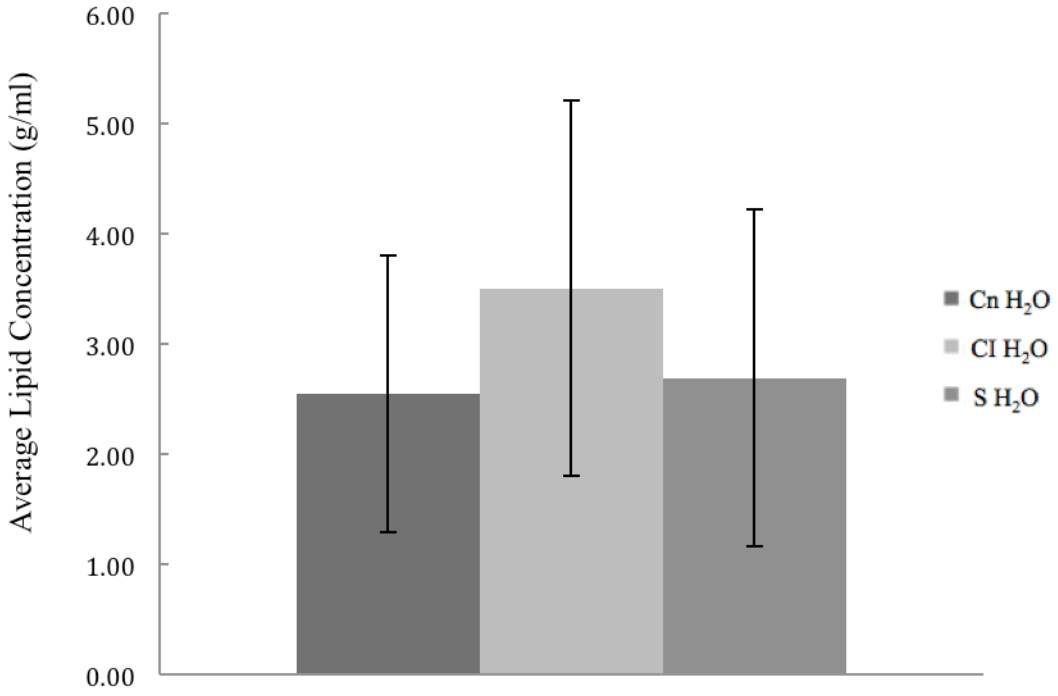

Figure 30. A Comparison of female average lipid concentration between selected and two different control lines with H_2O injection (H_2O) . The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

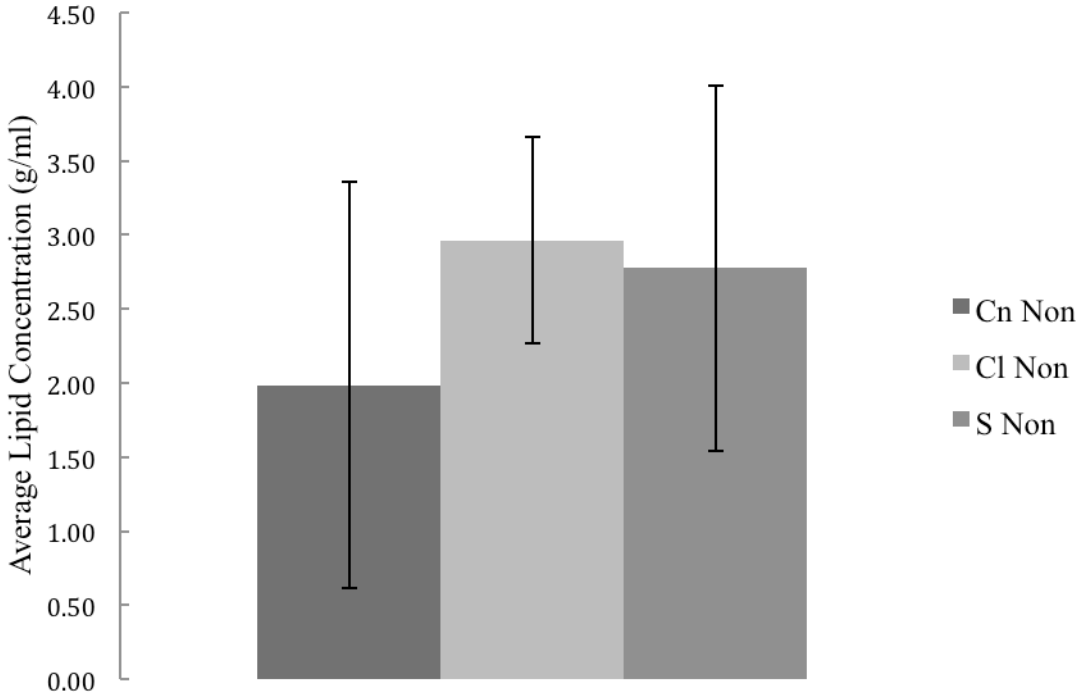

Figure 31. A Comparison of female average lipid concentration between selected and two different control lines without treatment (Non). The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H2O (wound control), Cn-no perturbation lines.

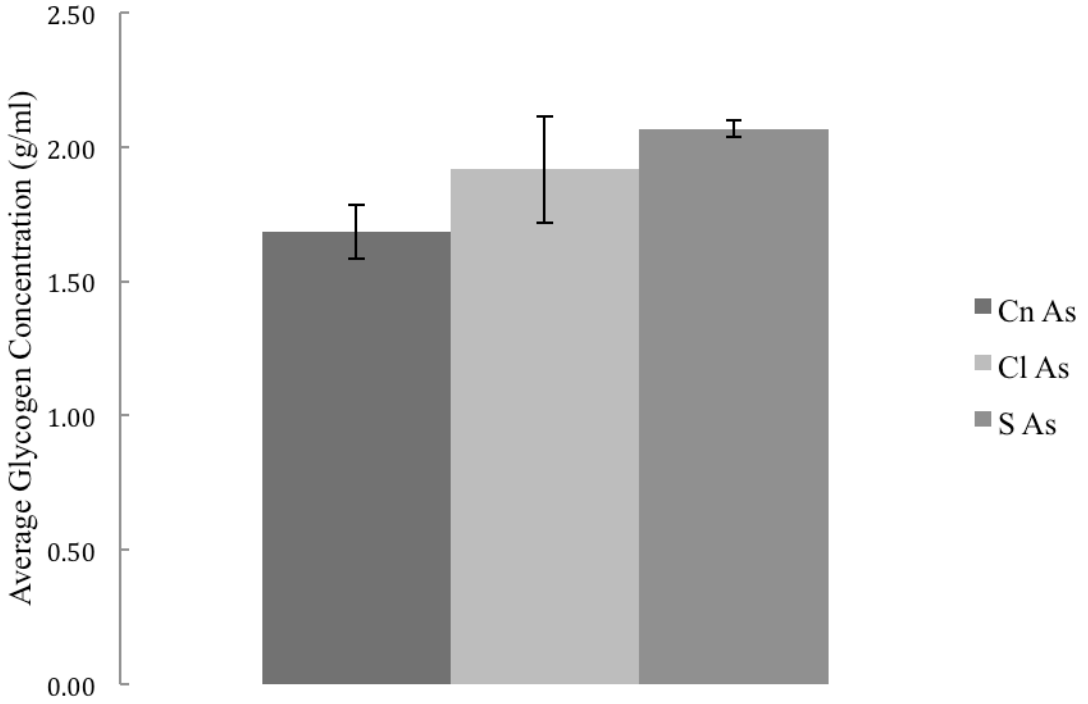

Figure 32. A Comparison of male average glycogen concentration between selected and two different control lines with autoclaved spores injection (As). The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

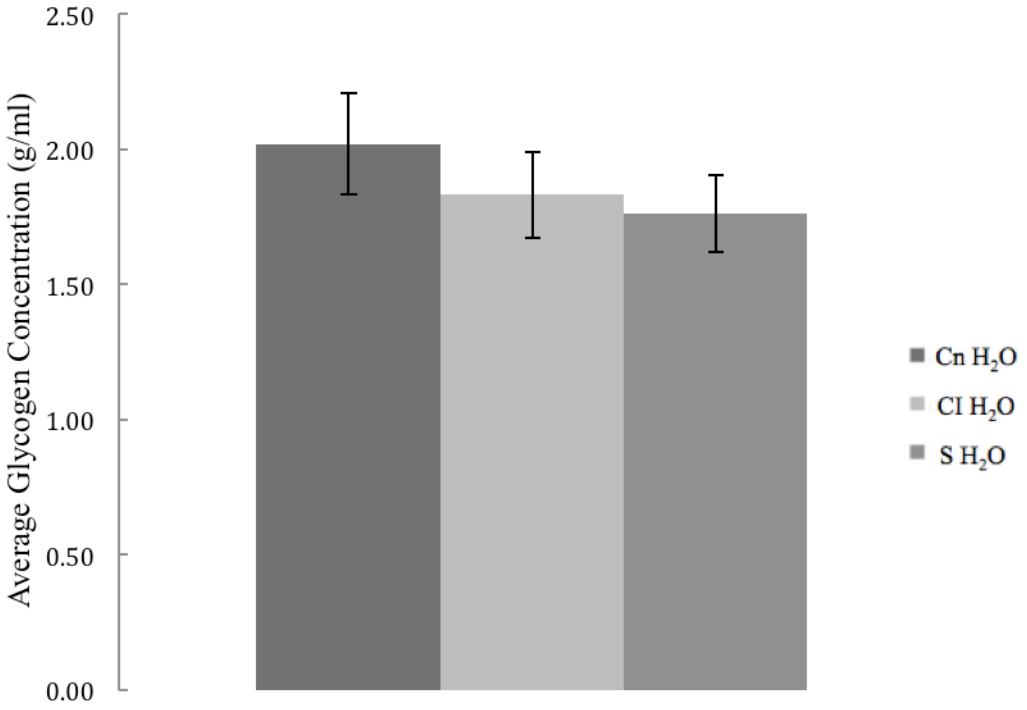

Figure 33. A Comparison of male average glycogen concentration between selected and two different control lines with H_2O injection (H_2O). The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

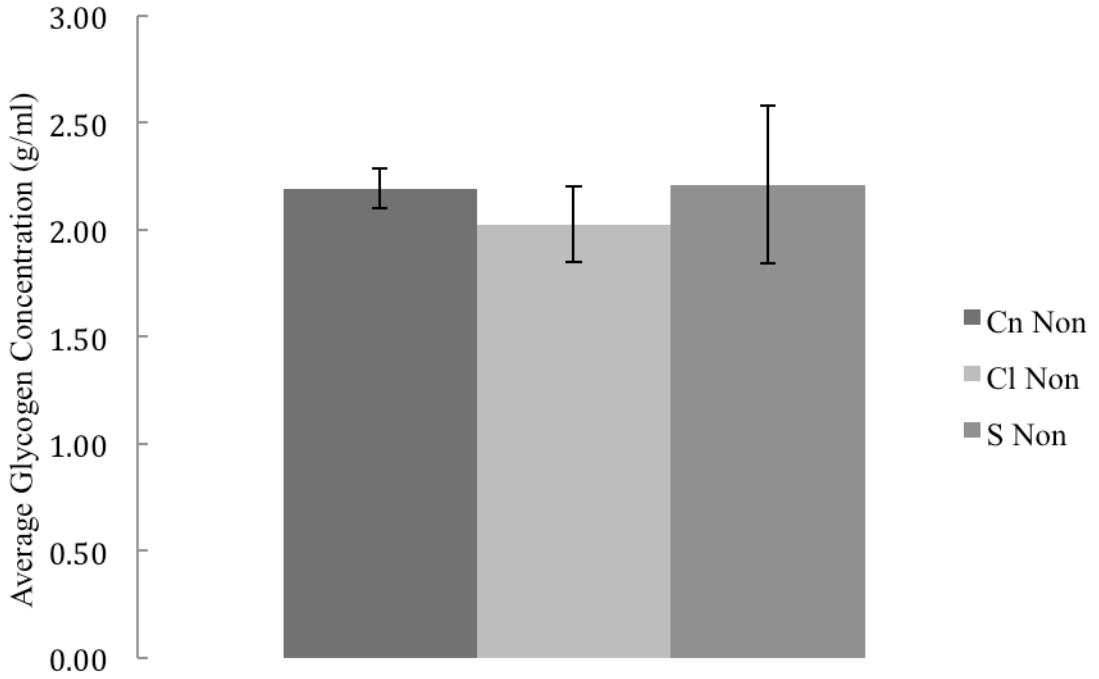

Figure 34. A Comparison of male average glycogen concentration between selected and two different control lines without treatment (Non). The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

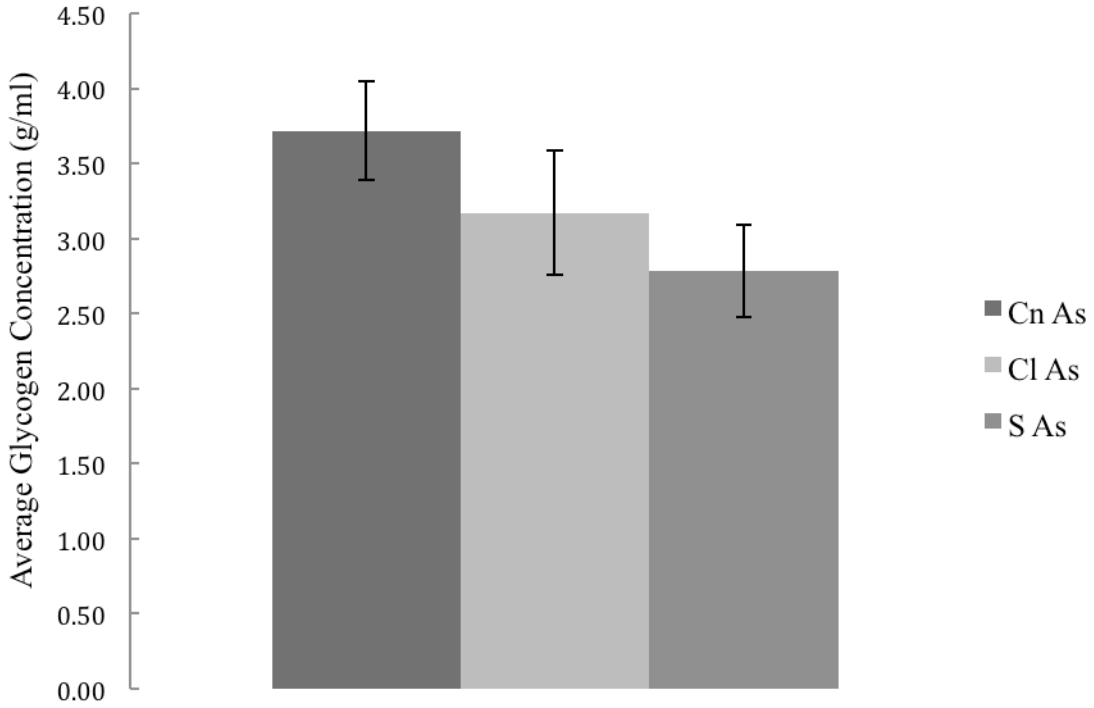

Figure 35. A Comparison of female average glycogen concentration between selected and two different control lines with autoclaved spores injection (As). The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

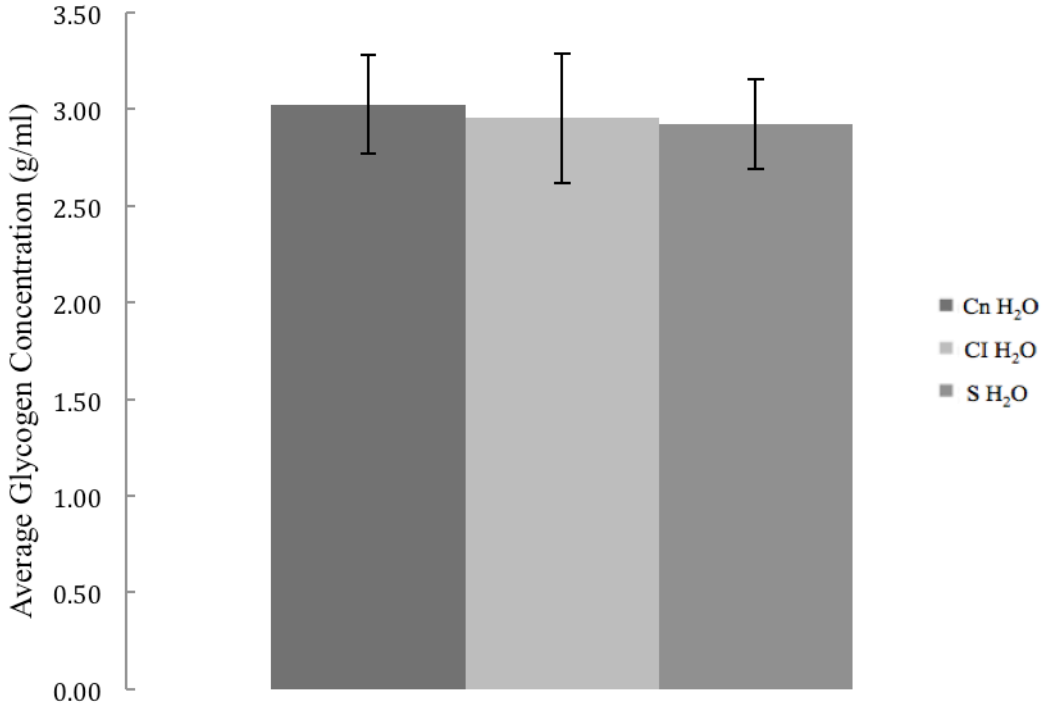

Figure 36. A Comparison of female average glycogen concentration between selected and two different control lines H_2O injection (H_2O). The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

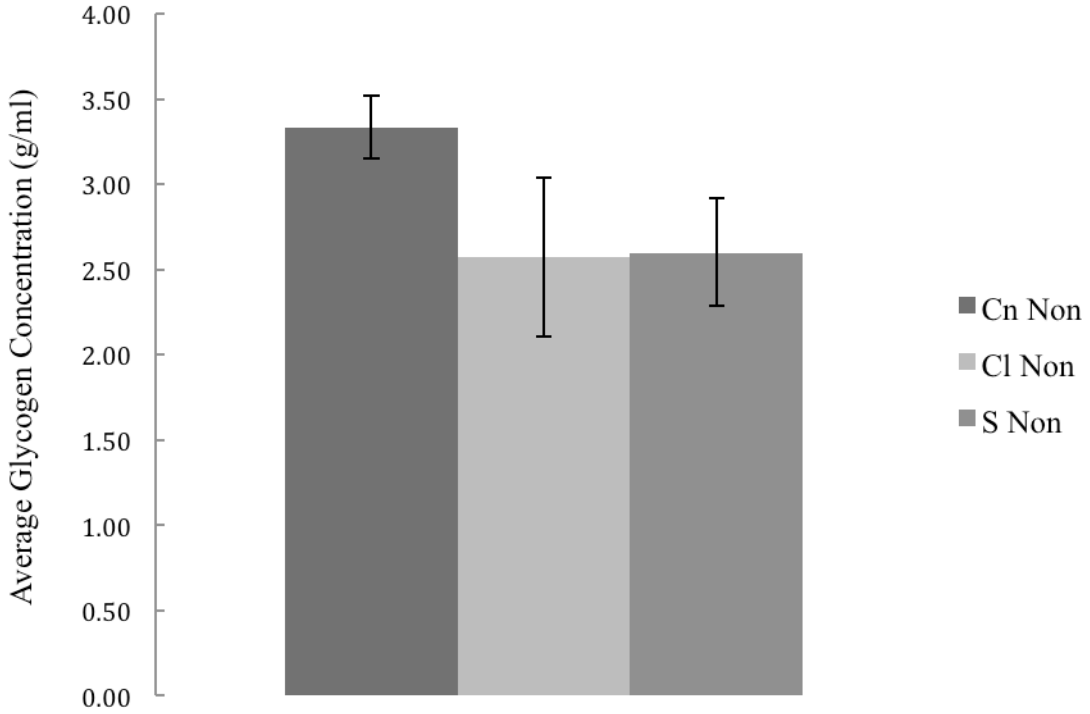

Figure 37. A Comparison of female average glycogen concentration between selected and two different control lines without treatment (Non). The mean was determined from the replicate lines of the same type: S-selected lines, CI-lines punctured with H_2O (wound control), Cn-no perturbation lines.

Table 1. P values of different treatments and lines comparison for each stress response assay * by Generalized Linear Mixed Model in SAS 9.3.

Overall	Comparisons		Starvation		Desiccation		Chill Coma		Oxidation	
			Female	Male	Female	Male	Female	Male	Female	Male
Lines			0.0201	0.0798	0.1758	0.0563	0.4813	0.2529	0.4882	0.0007
Trts**			0.7533	0.0025	<0.0001	<0.0001	0.1654	0.5167	0.0147	0.7129
Lines*trts**			0.8625	0.0204	0.2246	0.0142	<0.0001	0.3906	0.7712	0.7706
	Lines									
	Cn	Trts**	0.4717	0.0029	0.0029	<0.001	0.0012	0.8235	0.1352	0.8401
	CI	Trts**	0.9121	0.2856	0.0046	<0.001	0.0028	0.7452	0.3618	0.5638
	S	Trts**	0.9267	0.0084	0.2484	<0.001	0.0296	0.1080	0.1085	0.6135
	Trts**									
	As	Lines	0.5434	0.1807	0.5253	0.7357	0.0055	0.7739	0.6267	0.0423
	H2O	lines	0.2611	0.1398	0.0260	0.0021	0.0145	0.0773	0.5171	0.0727
	Non	lines	0.0681	0.0088	0.7711	0.0816	0.0035	0.5424	0.6144	0.0645

*Starvation, desiccation and oxidation is compared by hours when flies reach 50% mortality rate; chill coma is compared by average recovery hours.

** Trt–treatments: autoclaved spores(As), sterile H_2O (H_2O) and no treatment (Non).
Lines – line types: selected lines(S), wound control lines(CI) and non-perturbation lines (Cn).

Table 2. A comparison of average survival time for male flies among different line type under starvation assay at 50% mortality rate. Estimate represents the mean comparison between line types under same treatment.

Differences of trt*selection Least Squares Means									
Effect	trt	selection	trt	selection	Estimate	S.E.	DF	t Value	Pr > |t|
trt*Selection	As	CI	As	Cn	-13.9844	7.7151	72	-1.81	0.0741
trt*Selection	As	CI	As	S	-3.8667	7.7151	72	-0.50	0.6178
trt*Selection	As	Cn	As	S	10.1178	7.7151	72	1.31	0.1939
trt*Selection	H_2O	CI	H_2O	Cn	11.0522	7.7151	72	1.43	0.1563
trt*Selection	H_2O	CI	H_2O	S	-3.9067	7.7151	72	-0.51	0.6141
trt*Selection	H_2O	Cn	H_2O	S	-14.9589	7.7151	72	-1.94	0.0564
trt*Selection	Non	CI	Non	Cn	9.7000	7.7151	72	1.26	0.2127
trt*Selection	Non	CI	Non	S	-14.6744	7.7151	72	-1.90	0.0612
trt*Selection	Non	Cn	Non	S	-24.3744	7.7151	72	-3.16	0.0023

Trt–treatments: autoclaved spores(As), sterile H_2O (H_2O) and no treatment (Non).
Selection – line types: selected lines(S), wound control lines(CI) and non-perturbation lines (Cn).

Table 3. A comparison of average survival time for female flies among different line types under starvation assay at 50% mortality rate. Estimate represents the mean comparison between line types under all treatments.

Differences of selection Least Squares Means						
selection	selection	Estimate	S.E.	DF	t Value	Pr > |t|
CI	Cn	9.0285	4.3267	70	2.09	0.0406
CI	S	-2.8974	4.3267	70	-0.67	0.5053
Cn	S	-11.9259	4.3267	70	-2.76	0.0074

Selection – line types: selected lines (S), wound control lines (CI) and non-perturbation lines (Cn).

Table 4. A comparison of average survival time for male flies among different line type under desiccation assay at 50% mortality rate. Estimate represents the mean comparison between line types under same treatment.

Differences of trt*selection Least Squares Means									
Effect	trt	Selection	trt	Selection	Estimate	S.E.	DF	t Value	Pr > |t|
trt*Selection	As	Cl	As	Cn	0.1595	0.5033	259	0.32	0.7516
trt*Selection	As	Cl	As	S	-0.2265	0.5032	259	-0.45	0.6531
trt*Selection	As	Cn	As	S	-0.3859	0.4947	257	-0.78	0.4360
trt*Selection	H_2O	Cl	H_2O	Cn	-1.1484	0.4958	257	-2.32	0.0213
trt*Selection	H_2O	Cl	H_2O	S	-1.7503	0.5001	259	-3.50	0.0005
trt*Selection	H_2O	Cn	H_2O	S	-0.6019	0.4974	258	-1.21	0.2273
trt*Selection	Non	Cl	Non	Cn	1.0703	0.5034	259	2.13	0.0344
trt*Selection	Non	Cl	Non	S	0.2156	0.4958	257	0.43	0.6640
trt*Selection	Non	Cn	Non	S	-0.8547	0.4980	258	-1.72	0.0873

Trt–treatments: autoclaved spores (As), sterile H_2O (H_2O) and no treatment (Non).

Selection – line types: selected lines (S), wound control lines (CI) and non-perturbation lines (Cn).

Table 5. A comparison of average survival time for female flies among different line type under desiccation assay at 50% mortality rate. Estimate represents the mean comparison between line types under all treatments.

Differences of Least Squares Means							
Effect	Selection	Selection	Estimate	S.E.	DF	t Value	Pr > |t|
Selection	Cl	Cn	0.8287	0.5059	259	1.64	0.1026
Selection	Cl	S	0.01852	0.5059	259	0.04	0.9708
Selection	Cn	S	-0.8102	0.5059	259	-1.60	0.1105

Selection – line types: selected lines (S), wound control lines(CI) and non-perturbation lines (Cn).

Table 6. A comparison of average recovery time for male flies among different line type under chill coma recovery assay. Estimate represents the mean comparison between line types under all treatments.

Differences of Least Squares Means							
Effect	Selection	Selection	Estimate	S.E.	DF	t Value	Pr > |t|
Selection	Cl	Cn	0.01157	0.6461	636	0.02	0.9857
Selection	Cl	S	-0.9230	0.6461	636	-1.43	0.1536
Selection	Cn	S	-0.9345	0.6461	636	-1.45	0.1485

Selection – line types: selected lines(S), wound control lines(CI) and non-perturbation lines (Cn).

Table 7. A comparison of average recovery time for female flies among different line type under chill coma recovery assay. Estimate represents the mean comparison between line types under same treatment.

Differences of trt*selection Least Squares Means									
Effect	trt	Selection	trt	Selection	Estimate	S.E.	DF	t Value	Pr > |t|
trt*Selection	As	Cl	As	Cn	1.0425	0.8238	634	1.27	0.2062
trt*Selection	As	Cl	As	S	-1.6058	0.8238	634	-1.95	0.0517
trt*Selection	As	Cn	As	S	-2.6483	0.8238	634	-3.21	0.0014
trt*Selection	H_2O	Cl	H_2O	Cn	1.5000	0.8238	634	1.82	0.0691
trt*Selection	H_2O	Cl	H_2O	S	2.3775	0.8238	634	2.89	0.0040
trt*Selection	H_2O	Cn	H_2O	S	0.8775	0.8238	634	1.07	0.2872
trt*Selection	Non	Cl	Non	Cn	-2.5317	0.8238	634	-3.07	0.0022
trt*Selection	Non	Cl	Non	S	-2.2615	0.8238	634	-2.75	0.0062
trt*Selection	Non	Cn	Non	S	0.2701	0.8238	634	0.33	0.7431

Trt–treatments: autoclaved spores (As), sterile H_2O (H_2O) and no treatment (Non).

Selection – line types: selected lines (S), wound control lines (CI) and non-perturbation lines (Cn).

Table 8. A comparison of average survival time for male flies among different line type under oxidation assay at 50% mortality rate. Estimate represents the mean comparison between line types under all treatments.

Differences of Least Squares Means							
Effect	selection	selection	Estimate	S.E.	DF	t Value	Pr > |t|
selection	CI	Cn	3.8963	1.4000	72	2.78	0.0069
selection	CI	S	-1.5222	1.4000	72	-1.09	0.2805
selection	Cn	S	-5.4185	1.4000	72	-3.87	0.0002

Selection – line types: selected lines (S), wound control lines (CI) and non-perturbation lines (Cn).

Table 9. A comparison of average survival time for female flies among different line type under oxidation assay at 50% mortality rate. Estimate represents the mean comparison between line types under all treatments.

Differences of Least Squares Means							
Effect	selection	selection	Estimate	S.E.	DF	t Value	Pr > |t|
selection	CI	Cn	2.0285	1.7161	70	1.18	0.2412
selection	CI	S	0.6767	1.7161	70	0.39	0.6946
selection	Cn	S	-1.3519	1.7161	70	-0.79	0.4335

Selection – line types: selected lines (S), wound control lines (CI) and non-perturbation lines (Cn).

Table 10. The overall mean and standard Error (S.E.) among different treatments and lines comparison for each stress response.

Lines	Trts**	Starvation		Desiccation		Chill Coma		Oxidation	
		Female	Male	Female	Male	Female	Male	Female	Male
CN		112.40	86.7459	12.9713	9.0201	16.2803	16.3761	152.25	159.74
CI		121.43	89.0019	13.8	9.0472	16.2839	16.3876	154.27	163.64
S		124.32	96.4844	13.7815	9.761	16.7805	17.3106	153.6	165.16
	As	121.02	92.6881	12.7130	7.9974	15.9397	16.4069	154.79	163.22
	H_2O	119.38	81.9115	13.0296	9.9432	16.6017	17.1116	150.41	163.14
	Non	117.76	97.6326	14.8102	9.7610	16.8033	16.5559	154.92	162.18
Overall S.E.		5.28	3.15	0.57	0.33	1.0055	0.6861	1.2958	0.99
CN	As	116.72	100.72	12.5972	7.8156	14.7094	16.2024	154.01	160.36
CN	H_2O	112.94	73.2411	11.6806	10.1253	16.3942	16.7775	148.76	159.90
CN	Non	103.57	86.2744	14.6361	9.1193	17.7372	16.1483	153.96	158.96
CI	As	121.32	86.7378	12.2833	7.975	15.7519	16.8685	156.45	162.75
CI	H_2O	119.88	84.2933	13.9417	8.9769	17.8942	16.0433	152.19	165.13
CI	Non	123.09	95.9744	15.175	10.1896	15.2056	16.2511	154.19	163.03
S	As	125.02	90.6044	13.2583	8.2015	17.3578	16.1497	153.9	166.54
S	H_2O	125.31	88.2	13.4667	10.7272	15.5167	18.5139	150.27	164.38
S	Non	122.64	110.65	14.6194	9.974	17.4671	17.2682	156.62	164.55
S.E.		6.83	5.4554	0.76	0.44	1.1123	0.9424	2.1504	1.7147

*starvation, desiccation and oxidation is compared by hours when flies reach 50% mortality rate, chill coma is compared by average recovery hours.

** Trt–treatments: autoclaved spores(As), sterile H_2O (H_2O) and no treatment (Non).
Lines – line types: selected lines(S), wound control lines(CI) and non-perturbation lines (Cn).

Table 11. P values of different treatments and lines comparison for lipid and glycogen assay.

Overall Comparisons	Lipid		Glycogen	
	Female	Male	Female	Male
Lines	0.9897	0.0071	0.0958	0.8379
Trts**	0.6512	0.0857	0.3545	0.1661
Lines*trts**	0.7752	0.8341	0.6905	0.5007

Trts–treatments: autoclaved spores (As), sterile H_2O (H_2O) and no treatment (Non).

Lines – line types: selected lines (S), wound control lines (CI) and non-perturbation lines (Cn).

Table 12. Lipid concentration comparison for male flies among different line types. Estimate represents the difference of average lipid concentration between line types under all treatments.

Differences of Selection Least Squares Means						
Selection	Selection	Estimate	S.E.	DF	t Value	Pr > |t|
CI	Cn	1.8509	0.9492	18	1.95	0.0669
CI	S	-1.5946	0.9492	18	-1.68	0.1102
Cn	S	-3.4454	0.9492	18	-3.63	0.0019

Selection – line types: selected lines (S), wound control lines (CI) and non-perturbation lines (Cn).

Table 13. Lipid concentration comparison for female flies among different line types. Estimate represents the difference of average lipid concentration between line types under all treatments.

Differences of Selection Least Squares Means						
Selection	Selection	Estimate	S.E.	DF	t Value	Pr > |t|
CI	Cn	-0.02478	1.0869	18	-0.02	0.9821
CI	S	0.1211	1.0869	18	0.11	0.9125
Cn	S	0.1459	1.0869	18	0.13	0.8947

Selection – line types: selected lines (S), wound control lines(CI) and non-perturbation lines (Cn).

Table 14. Glycogen concentration comparison for male flies among different line types. Estimate represents the difference of average glycogen concentration between line types under all treatments.

Differences of selection Least Squares Means						
Selection	Selection	Estimate	S.E.	DF	t Value	Pr > |t|
CI	Cn	-0.04444	0.1506	18	-0.30	0.7713
CI	S	-0.09000	0.1506	18	-0.60	0.5575
Cn	S	-0.04556	0.1506	18	-0.30	0.7657

Selection – line types: selected lines (S), wound control lines (CI) and non-perturbation lines (Cn).

Table 15. Glycogen concentration comparison for male flies among different line types. Estimate represents the difference of average glycogen concentration between line types under all treatments.

Differences of selection Least Squares Means						
Selection	Selection	Estimate	S.E.	DF	t Value	Pr > |t|
CI	Cn	-0.4567	0.2666	18	-1.71	0.1039
CI	S	0.1311	0.2666	18	0.49	0.6288
Cn	S	0.5878	0.2666	18	2.20	0.0407

Selection – line types: selected lines (S), wound control lines (CI) and non-perturbation lines (Cn).