Mjerenje razine oksidativnog stresa u vinske mušice pomoću ekspresije gena Hsp70 i Hsp22 obilježenih zelenim fluorescencijskim proteinom

Butuči, Melina

Master's thesis / Diplomski rad

2010

Degree Grantor / Ustanova koja je dodijelila akademski / stručni stupanj: **University of Zagreb, Faculty of Science / Sveučilište u Zagrebu, Prirodoslovno-matematički fakultet**

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:217:699607

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Download date / Datum preuzimanja: **2024-03-29**

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Levels of chemical and social oxidative stress in *Drosophila melanogaster* measured by expression of Green Fluorescent Protein tagged to genes HSP70 and HSP22

Graduation thesis

Los Angeles, 2010.

This diploma thesis was made at the University of Southern California, College of Letters, Arts and Sciences, Department of Biological Sciences, Molecular and Computational Biology, Los Angeles, California under supervision of Dr. Sergey Nuzhdin, in collaboration with a post-doctoral researcher, Brad Foley, and the Computational Biology department.

Ovaj rad, izrađen na University of Southern California, College of Letters, Arts and Sciences, Department of Biological Sciences, Molecular and Computational Biology, Los Angeles, California, pod vodstvom Prof. dr.sc. Sergeya Nuzhdina u suradnji sa Dr.sc. Bradom Foleyjem i računalnim zavodom predan je na ocjenu Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu radi stjecanja zvanja prof. biologije.

ACKNOWLEDGEMENTS

First, I thank my supervisor, Prof.dr.sc. Sergey Nuzhdin, for giving me this amazing chance to work and learn in the Nuzhdin lab at University of Southern California and for believing in me all the way. I wouldn't be here without you.

A special thanks goes to Brad Foley, a post-doctoral researcher at USC, who was the project leader, the heart and the soul of it and to Reza Dehestani Ardekani, a Ph.D. student at USC, who developed the software MicroPicAnalyzer and for being a friend.

Thanks to my co- supervisor Prof.dr.sc. Mirjana Pavlica for guiding me through my thesis, for having a lot of patience and for giving me her precious time, when none of us had it. Thank you!

Another special thanks goes to Prof.dr.sc. John Tower for letting us use his equipment and the lab space, being always incredibly helpful and full of answers to my questions and for having a lot of patience leading me into *Drosophila* secrets.

Many thanks goes to Prof.dr.sc. Michelle Arbeitman for answering my questions and advising me.

Also thanks to all of my friends and co-workers at the Nuzhdin and other labs as well for interesting discussions and being fun to be with, to Joe and Peter, who were always there for me and Courtney for having tons of patience for me and teaching me a bunch (and for your delicious cookies as well).

I would like to thank to all of my overseas friends who were beside me when I needed them at most, for friendship and for love: Aaron, Patricia, Daniel and Ravi.

Finally, thanks to my closest friends who were always there for me when I needed them, especially to Petra and Marinela, my guardian angels and Dr.sc.Duje, the last- minute saviour.

And lastly, most special thank goes to my granny and grandpa for their astonishing patience, encouragement and love!

BASIC DOCUMENTATION CARD

University of Zagreb
Faculty of Science
Department of Biology

Graduation Thesis

LEVELS OF CHEMICAL AND SOCIAL OXIDATIVE STRESS IN *Drosophila melanogaster* MEASURED BY EXPRESSION OF GREEN FLUORESCENT PROTEIN TAGGED TO GENES HSP70 AND HSP22

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Summary

The free radical theory of aging postulates that the production of intracellular reactive oxygen species (ROS) is the major determinant of the life span. Heat- shock proteins (Hsp) are increasingly being implicated in aging phenotypes and control of life span across species as well.

Green Fluorescent Protein (GFP) is frequently used as a reporter of a gene expression. Here, GFP is fused with the hsp70 and hsp22 genes that are known to be expressed during oxidative stress. It is possible to measure the fluorescence / gene expression in the fruit flies. In this work the levels of chemical and social oxidative stress in fruit flies were measured by expression of GFP tagged to the hsp70 and hsp22 genes. The flies are conditioned in two different ways: first, the oxidative stress was caused by hydrogen peroxide; second, the flies were organized in different social groups, in various ratios males to females.

Results showed that there are significant effects of the social group and chemical environment in causing oxidative stress, especially in female fruit flies. In general, flies on hydrogen peroxide had higher Hsp expression. However, the actual genotype never came out as an important factor.

(54 Pages, 16 figures, 68 references, original in: English)

Thesis deposited in Central Library of Department of Biology, Faculty of Science, University of Zagreb, Roosveltov trg 6, Zagreb, Croatia

Keywords: GFP, Hsp, ROS, oxidative stress, social environment

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Reviewers: Prof.dr.sc. Mirjana Pavlica
Prof.dr.sc. Zlatko Liber
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Thesis accepted: June 30, 2010

TEMELJNA DOKUMENTACIJSKA KARTICA

Sveučilište u Zagrebu
Prirodoslovno- matematički fakultet
Biološki odsjek Diplomski rad

MJERENJE RAZINE OKSIDATIVNOG STRESA U VINSKE MUŠICE POMOĆU EKSPRESIJE GENA HSP70 I HSP22 OBILJEŽENIH ZELENIM FLUORESCENCIJSKIM PROTEINOM

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Sažetak

Temeljno načelo teorije slobodnih radikala bazira se na pretpostavci da je unutarstanična produkcija reaktivnih intermedijera kisika (ROS) odlučujući faktor u određivanju životnog vijeka. Proteini toplinskog šoka (Hsp) također su sve više umiješani u fenotipove koji pokazuju starenje te u kontrolu životnog vijeka u raznih vrsta organizama.

Zeleni fluorescencijski protein (GFP) često se koristi kao reporter ekspresije gena. U ovom istraživanju GFP je spojen s genima hsp70 i hsp22 genima, čiju ekspresiju aktivira oksidacijski stres. Moguće je pratiti/ mjeriti fluorescenciju tj. ekspresiju gena u vinskih mušica. U ovom radu mjerena je razina oksidativnog stresa u vinske mušice pomoću ekspresije gena hsp70 i hsp22 obilježenih GFP-om. Oksidacijski stress u mušica izazvan je: 1. vodikovim peroksidom i 2. Različitom organizacijom socijalnih grupa mušica s obzirom na omjer spolova.

Rezultati su pokazali da postoji značajan utjecaj socijalnih grupa i kemijskog okoliša u nastanku oksidacijskog stresa, posebice u ženki vinske mušice. Općenito, vinske mušice na vodikovom peroksidu pokazale su veću ekspresiju Hspa. Međutim, nije uočena razlika u ekspresiji između različitih genotipova.

(54 stranica, 16 slika, 68 literaturnih navoda, jezik izvornika: engleski)

Rad je pohranjen u Središnjoj knjižnici Biološkog odsjeka Prirodoslovno-matematičkog fakulteta, Sveučilište u Zagrebu, Roosveltov trg 6, Zagreb, Hrvatska

Ključne riječi: zeleni fluorescencijski protein (GFP), proteini toplinskog šoka (Hsp), reaktivni intermedijeri kisika (ROS), oksidacijski stres, socijalne grupe.

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Dr. sc. Zdravko Dolenec, prof.

Rad prihvaćen: 30. lipnja, 2010.

Abbreviations

GFP- Green Flourescent Protein

wtGFP- wild type Green Flourescent Protein

Hsp- Heat-Shock Protein

ROS- Reactive Oxygen Species

HSF- Heat Shock Factor

HSE- Heat Shock Element

IIS- Insulin/Insulin-like growth factor (IGF)-1-like signaling pathway

FOXO- Forkhead box O

SOD- Superoxide Dismutase

g1Sum- the sum of the pixels in the lowest bin

g1ave- g1sum value averaged over the number of flies in the picture

g1log- $\log_{10}$(g1ave)

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1. Introduction

1.1. Fruit fly (*Drosophila melanogaster* Meigen, 1830)

The common fruit fly is normally a yellow brown (tan) color, and is only about 3 mm in length and 2 mm in width (Patterson *et al*., 1943). The shape of the common fruit fly's body is what one would normally imagine for a species of the order Diptera. Similar to all insects *Drosophila* is covered in a chitinous exoskeleton; has three main body segments; and has three pairs of segmented legs. It has a rounded head with large, red, compound eyes; three smaller simple eyes, and short antennae. Its mouth has developed for sopping up liquids (Patterson and Stone, 1952). The female is slightly larger than the male (Patterson *et al.,* 1943). There are black stripes on the dorsal surface of its abdomen, which can be used to determine the sex of an individual. Males have a greater amount of black pigmentation concentrated at the posterior end of the abdomen (Patterson and Stone, 1952) and sex combs on their front legs (Figure 1). It has been theorized that these sex combs might be used for mating (Patterson *et al*., 1943). Like other flies, *Drosophila melanogaster* has a single pair of wings that form from the middle segment of its thorax. Out of the last segment of its throax (which in other insects contains a second pair of wings) develops a set rudimentry wings that act as knobby balancing organs. These balancing organs are called halteres (Raven and Johnson, 1999).



Figure 1**. Normal adult female (left) and male fruit flies, *Drosophila melanogaster*** (Source: http://berkeley.edu/news/media/releases/2002/07/03_paras.html).

Reproduction in *Drosophila* is rapid. A single pair of flies can produce hundreds of offspring within a couple of weeks, and the offspring become sexually mature within one week (Lutz, 1948). *Drosophila* mature through complete metamorphosis, as do all members of the order Diptera. As in all insect species *Drosophila melanogaster* lays eggs. The eggs are placed on fruit, and hatch into fly larvae (maggots), which instantly start consuming the fruit on which they were laid (Patterson and Stone, 1952). Under laboratory conditions, typically a temperature of 25 °C and a relatively high humidity, *D. melanogaster* has a life cycle of 10 days (Figure 2). There are three larval instars, and this is the period of development in which all growth occurs. This times spent in the three major stages are: a) Embryonic development: 24 hours; b) Larval development: 96 hours; c) Pupal development: 96-120 hours. Adult flies typically emerge according to a circadian rhythm and females are unreceptive to the attention of males for about the first 8-10 hours of their lives. This is of great practical importance, because it means that flies separated by their sex during this period will be virgin and can be used in controlled crosses. Under "typical" laboratory conditions the life span of *D. melanogaster* is 45-60 days. There have been very extensive studies of the environmental and genetic influences on life span and this species is extensively used for studies of aging (Patterson and Stone, 1952; Partridge and Tower, 2008).

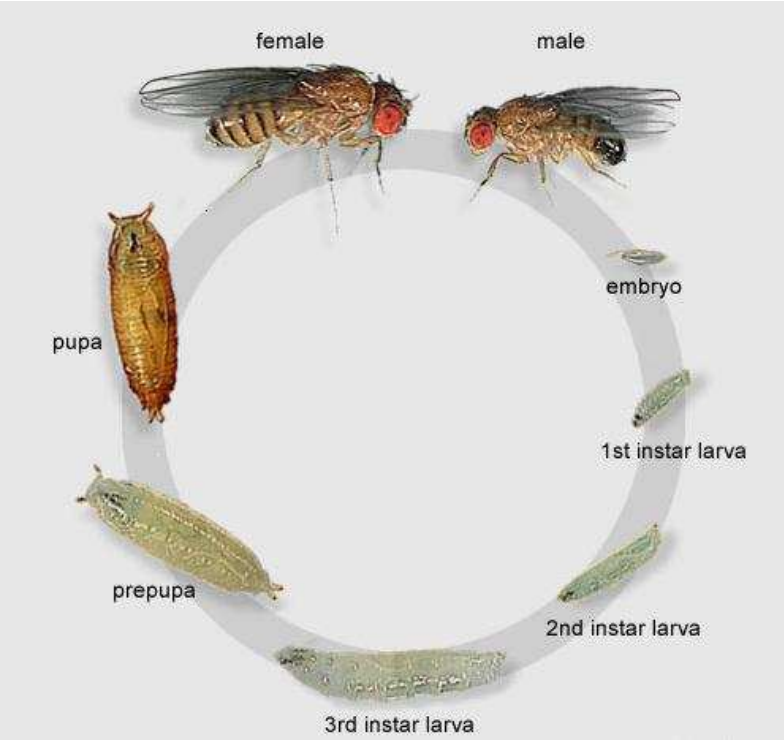


Figure 2. **The life cycle of *Drosophila melanogaster*** (Source: http://itcamp.teacher.org.hk/2009CU2/GEN6/experiment/).

As the name implies, the fruit flies lives primarily on plant material. The adults thrive on rotting plants, and fruits; while eggs are usually laid on unripened/slightly ripened fruit, so by the time the larva develop the fruit will have just started to rot, and they can use the fruit that the egg was laid on as their primary source of nutrition. *Drosophila* are considered major pests in some area of the world for this reason (Lutz, 1948; Demerec, 1950). *Drosophila melanogaster* has been introduced to every continent of the world with one exception, Antarctica. On other continents its range is limited only by mountain ranges, deserts, and high lattitudes (Demerec, 1950). The natural range of *D. melanogaster* is throughout the Old World tropics. Humans have helped to spread *Drosophila melanogaster* to every other location which it inhabits (Demerec, 1950; Patterson and Stone, 1952). The world-wide distribution of this fly is relatively recent, however. Its ancestral home is thought to be tropical West Africa. From there it spread to Euroasia, perhaps 6,000-10,000 years ago. It spread to the Americas only 500 or so years ago, probably on trans-Atlantic slave ships (Keller, 2007).

1.2. Heat Shock Proteins (Hsps)

Heat shock proteins are an evolutionary conserved family of proteins whose expression increases in response to a variety of different metabolic insults. Despite their designation, most of the heat shock proteins are constitutively expressed and perform essential functions. Most notable is their role as molecular chaperones, facilitating the synthesis and folding of proteins throughout the cell. In addition, heat shock proteins have been shown to participate in protein assembly, secretion, trafficking, protein degradation, and the regulation of transcription factors and protein kinases. Increased levels of heat shock proteins after stress plays a central role in cellular homeostasis. All organisms exhibit homeostatic-like responses when subjected to rapid changes in their environment. The ability of the organism to successfully adapt or acclimate to its new environment is critical to its survival, and likely represents an integral driving force in evolution. One well studied response to sudden adverse environmental changes is the so-called heat shock or stress response. When confronted with physiologically relevant increases in temperature, cells from all organisms respond similarly by rapidly increasing the synthesis of a select group of proteins, the heat shock proteins (Hsps). Changes in the expression of the heat shock proteins are controlled by a set of transcription factors referred to as heat shock factors (HSF) 1-4. The resultant increase and accumulation of the Hsps now gives the stressed cell added protection, thereby allowing for

continued cell survival. In addition to increased temperatures, other insults also result in increased Hsp expression. These include exposure of cells to various metals, amino acid analogues, hypoxia, and a large number of agents/treatments which result in reduced ATP levels. Because so many adverse conditions lead to increased Hsp expression, the heat shock response now is commonly referred to as the "stress response". Despite their designation as Hsps or stress proteins we now know that almost all of these proteins are in fact synthesized in cells grown under normal conditions (i.e. constitutive) and that their expression increases after metabolic stress. Under conditions of stress, where protein folding/assembly events may be compromised, the increased expression and accumulation of the stress proteins facilitates the ability of cells to both repair and synthesize new proteins to replace those that were damaged after the particular metabolic insult.

The Hsp70 family represents one of the largest stress protein families with related members distributed throughout the cell. In times of stress, usually whenever the cell finds itself under conditions that are unfavorable for protein folding, members of the Hsp70 family are expressed at higher levels. Increased expression of the chaperones help in the repair of proteins damaged by the particular stress event as well as guide the synthesis of new polypeptides needed to replace those irreparably damaged. Elevated levels of hsp70 proteins have been linked with inhibition of apoptosis as well as the resistance of cells to various chemotherapeutic agents. In addition, numerous studies continue to demonstrate that changes in the levels of the different hsp70 family members may prove clinically useful for the diagnosis of many important human diseases (Hartl and Hayer-Hartl, 2002; Kleizen and Braakman, 2004; Walsh *et al.,* 2004; Haslbeck *et al.,* 2005; Bukau *et al.,* 2006; Pearl and Prodromou, 2006; www.assaydesigns.com).

The small hsps (e.g. hsp22) are perhaps the most widespread but least conserved members of the heat shock protein family. While bacteria and single-cell eukaryotes express only one or two members, *Drosophila melanogaster* expresses 16, humans 10, and plants as many as 19.

The function(s) of each small heat shock protein is unknown. DmHsp22 is shown to localize in mitochondria both in *D. melanogaster* S2 cells and after heterologous expression in mammalian cells. Fractionation of mitochondria indicates that DmHsp22 resides in the mitochondrial matrix, where it is found in oligomeric complexes. The mitochondrial localization of this small hsp22 of *Drosophila* and its high level of expression in aging suggests a role for this small heat shock protein in protection against oxidative stress (Genevieve et al., 2000).

Increased expression and activity of hsps upon heat stress is regulated primarily at the level of increased transcription by heat-shock factor (HSF) (Morimoto, 2008). HSF is constitutively expressed in the cytoplasm and is maintained in an inactive state by association with Hsps, including Hsp70, Hsp90 and Hsp40 (Voellmy, 2004). Stresses that cause protein unfolding in the cell, such as heat and oxidative stress, titrate the Hsps away from HSF, enabling HSF to undergo trimerization, activation and translocation to the nucleus, where it binds to conserved heat-shock elements (HSEs) in the promoters of Hsp genes and activates their transcription. Once Hsp levels have risen sufficiently and/or the stress is removed, HSF is titrated back into its inactive monomeric form by association with Hsps, enabling a feedback loop that terminates the response (Figure 3). HSF is also regulated by posttranslational modifications such as phosphorylation, sumoylation and acetylation (Voellmy, 2004; Morimoto, 2008). Cellular stress that causes protein unfolding, therefore, will cause changes in the amount and availability of Hsps that will be transduced to their networks of clients, thereby coordinately altering signaling pathways, protein localization and stability and affecting global changes in the physiology of the cell. In this way, chronic stress can lead to deleterious effects, such as those associated with aging (Soti and Csermely, 2007). The insulin/insulin-like growth factor (IGF)-1-like signaling (IIS) pathway (Figure 3) has been found to be a key regulator of life span and aging phenotypes across species (Kenyon, 2005). IIS acts through a conserved transcription factor, Forkhead box O (FOXO), the targets of which include Hsps; therefore, Hsps might be key mediators of IIS effects on aging (Cohen *et al.,* 2006). Additional pathways co-regulate stress resistance, life span and Hsp expression. For example, the Drosophila JNK-signaling pathway promotes oxidative stress resistance and expression of the Hsp70-class protein Hsp68, and both JNK signaling and Hsp68 overexpression were reported to extend fly life span (Wang *et al.,* 2003). In summary, several stress-response pathways, including HSF, IIS and JNK, converge upon the activation of Hsp gene expression and can increase both stress resistance and life span (Tower, 2009).

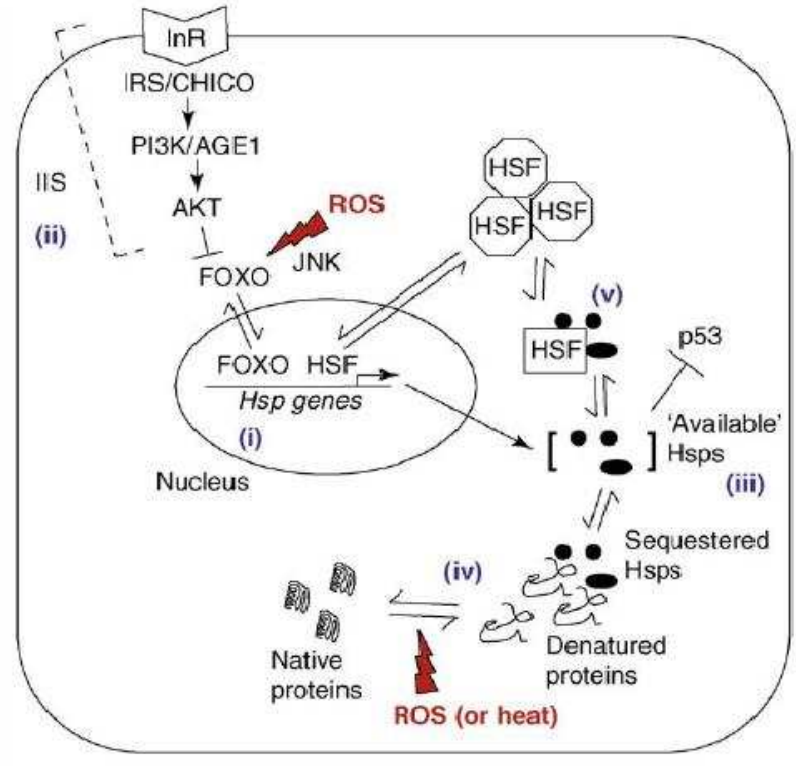


Figure 3**. Regulation of Hsp genes by HSF and FOXO** (Source: Tower, J. (2009): Hsps and aging, Cell press, Trends in Endocrinology and Metabolism 20 (5)).

Aging is associated with characteristic changes in gene expression, in particular that of the Hsps. During normal aging of an otherwise unstressed animal, Hsp expression increases in a variety of tissue-specific and disease-specific patterns, consistent with aging and disease as intrinsic stress states (Morrow and Tanguay, 2003; Landis and Tower, 2005; Macario and Conway de Macario, 2005). For example, upregulated hsp22 and hsp70 gene expression during *Drosophila* aging requires functional HSE sites in the promoter. One likely mediator of increased Hsp expression during aging is increased oxidative stress (Landis and Tower, 2005; Muller *et al*., 2007). Several Hsp genes, including hsp22 and hsp70, are upregulated during normal Drosophila aging (Landis and Tower, 2005). Recently, both hsp22 and hsp70 have been found to be predictive biomarkers of *Drosophila* life span and mortality: expression of hsp22-GFP and hsp70-GFP transgenes in young (approximately one week old) flies was negatively correlated with remaining life span (Yang and Tower, 2009), and during aging, both genes were observed to spike in expression in the hours preceding death of the animal (Grover *et al.,* 2008).

1.3. Oxidative stress and aging

Aging is thought to evolve due to the decreasing force of natural selection acting on older individuals (Rose, 1991; Partridge and Barton, 1993; Charlesworth, 1994; Kirkwood, 1995; Kirkwood and Austad, 2000). Repair and maintenance of somatic tissues appears unable to keep pace with endogenous and exogenous sources of damage, leading to progressive deterioration of structure and function with age. Accumulating evidence suggests that oxidative damage may be a key cause of aging in species ranging from *C. elegans* to *Drosophila* to humans (Harman, 1956; Stadtman, 1992; Wallace, 1999; Finkel and Holbrook, 2000; Hekimi and Guarente, 2003). Reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, hydroxyl radical and others are produced as toxic byproducts of normal cellular metabolism (Figure 4). Among the most important defenses against oxygen radicals are the superoxide dismutase (SOD) enzymes (Fridovich, 1995). ROS damage each of the four classes of cellular macromolecules (lipids, nucleic acids, carbohydrates and proteins), and oxidative damage products of each have been found to accumulate during aging (Yu, 1993; Meli *et al.,* 2003). In many organisms aging has been found to be associated with the accumulation of ‘‘abnormal’’ proteins. These include conformationally altered and inactive enzymes and proteins that are oxidatively damaged (Gershon and Gershon, 1970; Rothstein, 1983; Rothstein, 1989; Finch, 1990; Stadtman, 1992; Finkel and Holbrook, 2000; Stadtman and Levine, 2003).

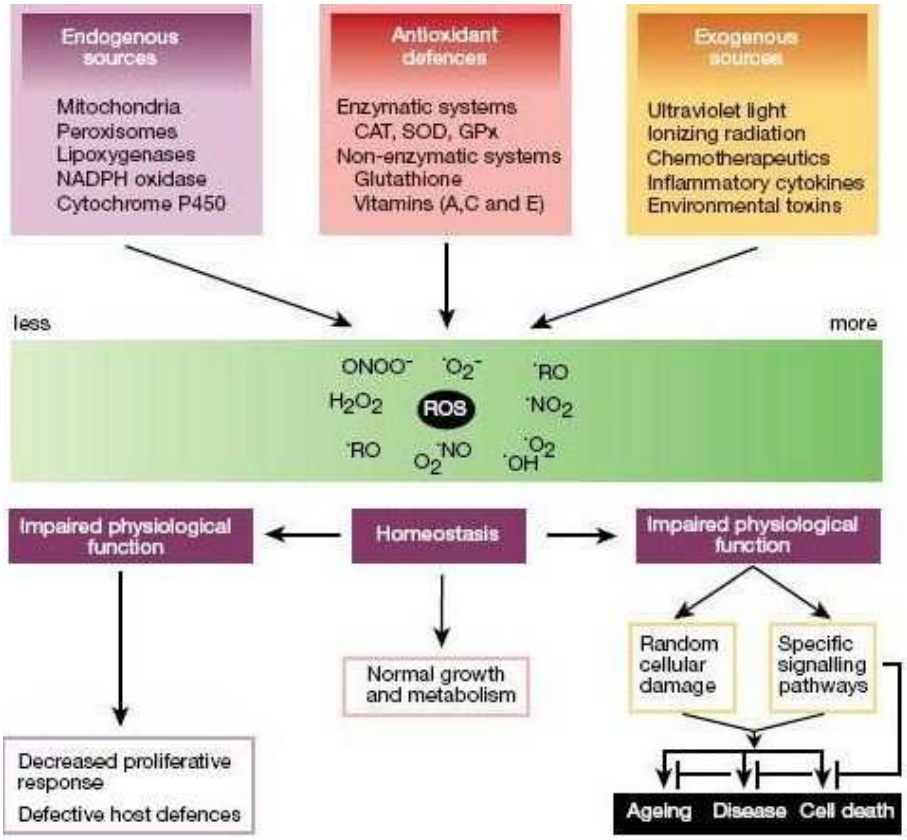


Figure 4**. The sources and cellular responses to reactive oxygen species (ROS)** (Source: Finkel T., N.J. Holbrook (2000): Oxidants, oxidative stress and the biology of aging. Nature 408: 239-247).

1.4. Free radical theory of aging

In the mid-1950s, Denham Harman articulated a free-radical theory of ageing, speculating that endogenous oxygen radicals were generated in cells and resulted in a pattern of cumulative damage (Harman, 1957). The free radical theory of aging postulates that the production of intracellular reactive oxygen species is the major determinant of life span (Figure 5) (Balaban *et al.,* 2005). ROS are generated in multiple compartments and by multiple enzymes within the cell. Important contributions include proteins within the plasma membrane, such as the growing family of NADPH oxidases (Lambeth 2004); lipid metabolism within the peroxisomes; as well as the activity of various cytosolic enzymes such as cyclooxygenases. Although all these sources contribute to the overall oxidative burden, the

vast majority of cellular ROS (estimated at approximately 90%) can be traced back to the mitochondria (Figure 6).

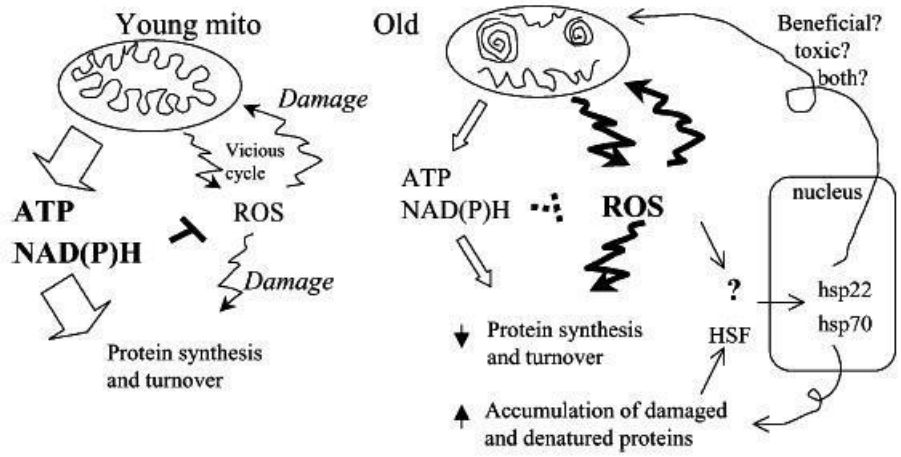


Figure 5. **Model of ROS damage during aging.** In young animals the mitochondria produces ATP and reducing equivalents with low amounts of ROS byproducts. The ROS damage mitochondria, resulting in a vicious cycle of increasing ROS production. The damaged mitochondria are less efficient and produce less ATP and reducing equivalents and more ROS. Decreased ATP production results in decreased protein synthesis and turnover, in turn increasing protein half life and the chances for oxidative damage. Damaged proteins and mitochondria signal induction of hsps that are beneficial in the short term, but may accumulate to toxic levels in old animals (Source: Landis G. N., Tower J. (2005): Mechanisms of Aging and Development 126: 365-379).

The generation of mitochondrial ROS is a consequence of oxidative phosphorylation, a process that uses the controlled oxidation of NADH or FADH to generate a potential energy for protons across the mitochondrial inner membrane. This potential energy is in turn used to phosphorylate ADP via ATPase (Nemoto *et al.,* 2000; Werner and Werb, 2002; Dada *et al.,* 2003). The production of mitochondrial superoxide radicals occurs primarily at two discrete points in the electron transport chain, namely at complex I (NADH dehydrogenase) and at complex III (ubiquinone–cytochrome c reductase). Under normal metabolic conditions, complex III is the main site of ROS production (Turrens, 1997). Evidence indicates that*, in vitro*, mitochondria convert 1–2% of the oxygen molecules consumed into superoxide anions (Boveris and Chance, 1973). Given that these initial estimates were made on isolated mitochondria in the presence of high, non-physiological concentrations of oxygen, *the in vivo* rate of mitochondrial superoxide production is undoubtedly considerably less. Whatever the

absolute amount of mitochondrial ROS, given their potentially harmful effects, it is likely that numerous protective mechanisms have evolved to limit oxidant production and release. The balance between ROS production and antioxidant defences determines the degree of oxidative stress (Finkel and Holbrook, 2000).

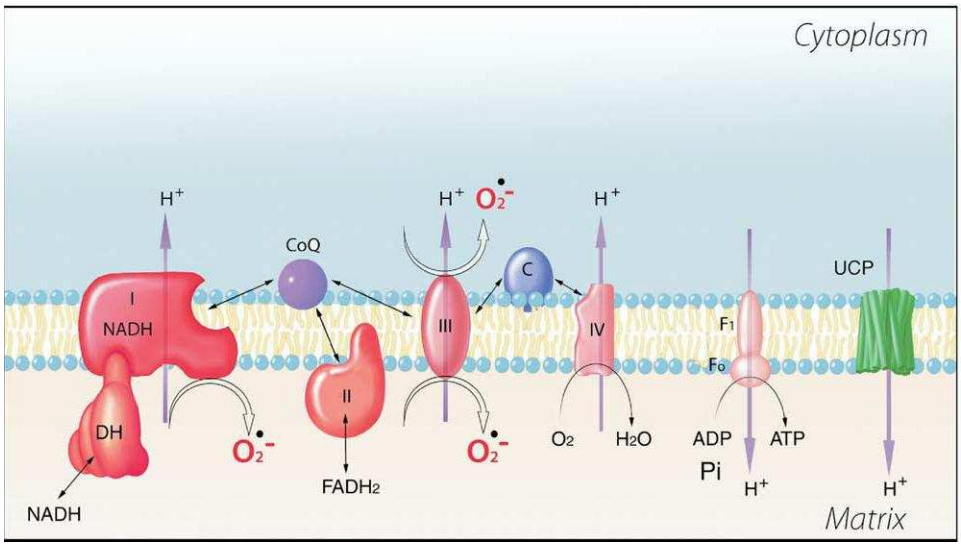


Figure 6. **A Schematic Model of ROS Generation in the Mitochondria** (Source: Balaban *et al.* (2005): Mitochondria, Oxidants and Aging. Cell 120: 483-495).

1.5. Green Fluorescent Protein (GFP)

The green fluorescent protein (GFP) is a protein composed of 238 amino acids (26.9kDa), which exhibits bright green fluorescence when exposed to blue light (Prendergast and Mann, 1978; Tsien, 1998). Although many other marine organisms have similar green fluorescent proteins, GFP traditionally refers to the protein first isolated from the jellyfish *Aequorea victoria.* The GFP from *A. victoria* has a major excitation peak at a wavelength of 395 nm and a minor one at 475 nm. Its emission peak is at 509 nm which is in the lower green portion of the visible spectrum. The GFP from the sea pansy (*Renilla reniformis*) has a single major excitation peak at 498 nm. In cell and molecular biology, the GFP gene is frequently used as a reporter of expression (Phillips, 2001). In modified forms it has been used to make biosensors, and many animals have been created that express GFP as a proof-of-concept that a gene can be expressed throughout a given organism. The GFP gene can be introduced into organisms and maintained in their genome through breeding, injection with a viral vector, or cell transformation. To date, the GFP gene has been introduced and expressed in many bacteria,

yeast and other fungi, fish (such as zebrafish), plant, fly, and mammalian cells, including human (Tsien, 1998). In the 1960s and 1970s GFP, along with the separate luminescent protein aequorin, was first purified from *Aequorea victoria* and its properties studied by Osamu Shimomura (Shimomura *et al.,* 1962). In *A. victoria*, GFP fluorescence occurs when aequorin interacts with Ca^{2+} ions, inducing a blue glow. Some of this luminescent energy is transferred to the GFP, shifting the overall color towards green (Morise *et al.,* 1974).

GFP has a typical beta barrel structure (Figure 7), consisting of one β-sheet with alpha helix(s) containing the chromophore running through the center (Ormö *et al.,* 1996; Yang, 1996). Inward facing sidechains of the barrel induce specific cyclization reactions in the tripeptide Ser65–Tyr66–Gly67 that lead to chromophore formation. This process of post-translational modification is referred to as maturation. The hydrogen bonding network and electron stacking interactions with these sidechains influence the color of wild type GFP (wtGFP) and its numerous derivatives. The tightly packed nature of the barrel excludes solvent molecules, protecting the chromophore fluorescence from quenching by water (Yang, 1996).

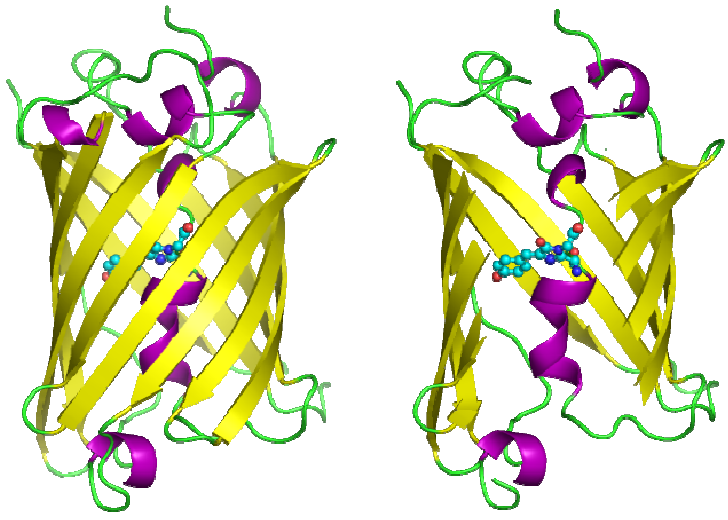


Figure 7. **Gfp and fluorophore.** GFP molecules drawn in cartoon style, one fully and one with the side of the beta barrel cut away to reveal the chromophore (highlighted as ball-and-stick) (Source: http://en.wikipedia.org/wiki/File:Gfp_and_fluorophore.png).

1.6. Goals of the project

It is known that oxidative stress induces the expresion of the heat shock proteins. In this project I wanted to see the effects of different extrinsic factors on the oxidative stress intensity in fruit fly. The objective was to test whether there are any significant differences in the fluorescence, hence the expression of the heat shock proteins between the single flies of two different genotypes that were put on the normal food and on the 2% hydrogen peroxide food. Furthermore, I wanted to investigate whether there are differences between different social groups of flies as well as the diferences between sexes. I was wondering if the females in the group with only one male or with more males are more stressed? Similar, would males be more stressed in the group with only one female or with more of them? Here I used two different genotypes tagged with the GFP, hsp22-GFP and hsp70-GFP. I was interested to see if the genotypes are different in their responses, or they would not show any significant difference.

2. Materials and methods

2.1. Materials

2.1.1. Fly lines

- Hsp22→ yacw; hsp22GFP(3)1mI1/ TM3, Sb
- Hsp70→ yacw; hsp70GFP(3)1mI2/ TM3, Sb

y-ac-w (yellow- acre- white) → Placed in line on the second chromosome
Sb→ Stubble
TM3→ Balancer on the third chromosome

A balancer chromosome is a genetic tool used to prevent crossing over (genetic recombination) between homologous chromosomes during meiosis. Balancers are most often used in *Drosophila melanogaster* genetics to allow populations of flies carrying heterozygous mutations to be maintained without constantly screening for the mutations. TM3, Sb balancer is a balancer chromosome that stabilizes the third chromosome and carries a mutant Sb (Stubble) gene as a marker. All flies containing the TM3, Sb balancer will have shortened (or stubbly) hairs on the back of the fly, which are easily seen when viewed through a microscope (Greenspan, 2004).

2.1.2. Fly food (recipe)

Ingredients:

Water	22 L
Light corn syrup	1.5L
Corn meal	2100 mL
Non-active yeast	600 mL
Soy flour	500 mL
Agar	250 mL
Malt extract	1475 mL

Acid Mix A 225 mL

Tegosept Acid Mix 130 mL

All reagents were purchased from Sigma-Aldrich.

Instructions:

Corn meal is measured and put into plastic bag. All other dry ingredients are measured and put into a plastic bag. Because the malt is sticky, it is measured last. The vat is filled with water. The steam valve (~ 4 ¼ turns) and stirring rod are turned on and the stirring set to medium speed. When the water is almost to a boil, corn syrup is added. Once the corn syrup is dissolved and water is boiling, corn meal is slowly added boiled for 45 minutes. The stirring speed is increased and the stirring direction is changed to prevent clumping. After the cornmeal has boiled for 45 minutes, the rest of the dry ingredients are slowly added boiled for 30-35 minutes. Acid Mix A and Tegosept Acid Mix are added slowly and boiled for 10 more minutes. Steam is turned off and stir rod is turned to slowest speed. Food is pumped into vials and needs to be cooled down for about 24 hours (Figure 8).



Figure 8**. Fly food in vials with fruit flies** (Source: Melina Butuči).

2.1.3. Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is naturally produced in organisms as a by-product of oxidative metabolism. Nearly all living things (specifically, all obligate and facultative aerobes) possess enzymes known as peroxidases, which harmlessly and catalytically decompose low concentrations of hydrogen peroxide to water and oxygen (Halliwell *et al.,* 2000).
Hydrogen peroxide is a good candidate for a behavior regulator as it is the most stable and diffusible of ROS species and has been shown to function as a cellular signaling molecule in several other processes (Balaban *et al.,* 2005).

2.2. Methods

2.2.1. Fly lines construction

Both of the fly lines were constructed in Dr. John Tower's lab, University of Southern California.

2.2.1.1. DNA constructs

Plasmids for making transgenic GFP reporter flies were derived from pGreen pelican. phsp22p-Gpel was a derivative of pGreen pelican in which the sequence between the Kpn I and Bgl II sites was replaced by the sequences from − 312 to +10 of the hsp22 gene of *D. melanogaster*, and in phsp70-Gpel, these sequences were replaced by the sequences from − 194 to +10 of the hsp70 gene. The hsp gene sequences used in cloning were generated using the following oligonucleotides containing engineered Bgl II and Kpn I sites: hsp22 forward primer CC AGA TCT TCA ATG TGT CTC TCT GCG, hsp22 reverse primer CC GGT ACC TTT GAA CTG AGA GCG TAG, hsp70 forward primer CC AGA TCT CTC GAG AAA TTT CTC TGG, and hsp70 reverse primer CC GGT ACC GAA TTG AAT TGT CGC TCC. The template for amplification of hsp70 sequences was plasmid pBS70Z, and the template for amplification of hsp22 sequences was plasmid " hsp22 5 ′ D (− 314)" (Yang and Tower, 2009).

2.2.1.2. *Drosophila* P element – mediated transformation

Transgenic fly strains were generated by microinjection of the P-element constructs into fly embryos along with delta2 – 3 “ turbo ” helper plasmid (pUChspD2-3wc) as a source of transposase, using standard methods. Strains with multiple inserts were generated in order to increase reporter expression levels, as follows: to generate strains with multiple inserts, strains with single inserts were crossed to the delta2-3 transposase source to mobilize the inserts, and chromosomes bearing multiple inserts were first identified by increased expression of the mini-white+ marker gene, and then insert copy number was confirmed using genomic Southern blotting (Yang and Tower, 2009).

2.2.1.3. Generation of PEPCK-GFP reporter flies

A strain of flies was generated where GFP expression is under the control of a promoter that is not induced by heat or oxidative stress, in this case the PEPCK gene promoter. A gene-trap line of genotype w[1118]; P{w[+mGT] = GT1} BG02569 was obtained from Bloomington Drosophila stock center and produces GAL4 protein under the control of PEPCK gene regulatory sequences; this strain is hereafter referred to as PEPCK-GAL4 . A strain was constructed containing multiple copies of a construct with a upstream activating sequence (UAS) promoter driving expression of eGFP as follows: starting strains were obtained from Ron Davis (Baylor College of Medicine) that contained the UAS-2XeGFP construct inserted on the second chromosome (line U-202-3) and on the third chromosome (line U-307-1). These lines were crossed to a strain bearing the delta2-3 transposase source to mobilize the inserts, and chromosomes were derived that contained multiple copies of the insert on the second and third chromosomes, named UAS-2xEGFP[m5B29] and UAS-2xEGFP[m6B1] , respectively, where the “ m ” stands for multimer derivative chromosome. These second and third chromosomes bearing the multiple inserts were then placed into the same genetic background and made homozygous by appropriate crosses to double-balancer strains, to generate strain w ; UAS-2- xEGFP[m5B29] ; UAS 2xEGFP[m6B1] . This strain was crossed to the PEPCK-GAL4 strain described earlier to generate progeny of genotype w; UAS-2xEGFP[m5B29]/PEPCK- GAL4; UAS-2xEGFP[m6B1] . In these progeny flies, the PEPCK gene regulatory sequences drive expression of GAL4 transcription factor, which in turn binds to the multiple UAS promoter constructs to drive expression of eGFP. Therefore, in these flies

the GFP expression is ultimately driven by the regulatory sequences of PEPCK , and they are hereafter referred to as “ PEPCK-GFP ” reporter flies (Yang and Tower, 2009).

2.2.2. Keeping/Maintaining flies

Figure 9. shows how the fruit flies are being stored for maintenance in the Nuzhdin lab at USC. It is the most important that the vials are properly marked with the fruit fly line and a date. On the left hand side of the picture are showed boxes with flies that are being kept as factories for mating. They are grouped by different fly lines and separated from each other so they do not get mixed up and are called aging vials. The reason why there are many of them is because they are in different stages of life. The midle box contains all of the fly lines we are using prepared for every day virgin collection. In the last box are fly lines back-ups kept only for maintaining the lines, in case something goes wrong.

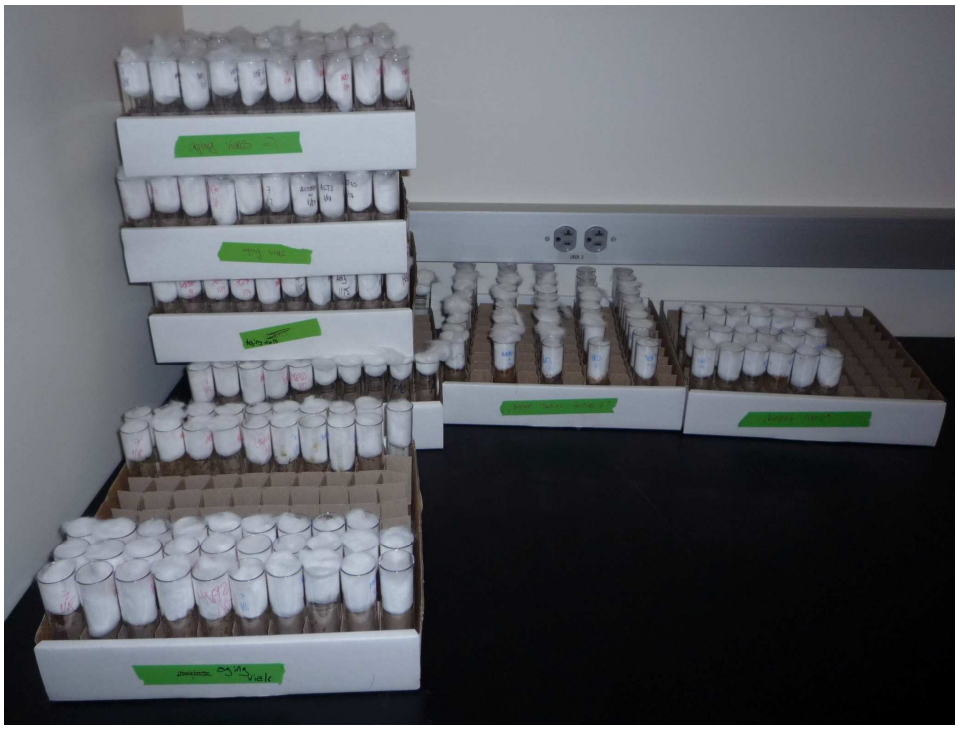


Figure 9. **Maintenance of the fly lines used in this project** (Source: Melina Butuči).

2.2.2.1. Knocking- out flies

- Carbon dioxide (CO_2)

In order to use *D. melanogaster* as a model organism for genetic analysis, it is necessary to maintain cultures of flies for manipulation in crosses and as a backup for any mishaps which may occur. Culturing is very easy and it is recommended to have students maintain their own cultures of flies. As long as students reculture their flies on a regular basis and no mass contamination occurs, flies can be maintained for decades.

The problem with fruit flies is that they fly. Therefore a variety of methods have been developed to anesthetize flies. Included are ether, commercial brands such as Flynap, carbon dioxide and cooling. Each has its strengths and weaknesses. Ether is flammable, has a strong odor and will kill flies if they are over-etherized (and can anesthetize younger students). Flynap (from Carolina Biological) is messy and has an odor that some find offensive. Each of these, however, requires low- cost equipment which can be easily purchased. Carbon dioxide works very well, keeping flies immobile for long periods of time with no side effects, however CO_2 mats (blocks) are expensive and a CO_2 source (usually a bottle) and delivery system (vials and clamps) are necessary, increasing the costs. The least harmful to the flies is either carbon dioxide or cooling anesthetizing. In the Nuzhdin lab at University of Southern California, CO_2 from bottles is being used every day on a regular basis.

2.2.2.2. Transferring flies from one vial to another

Flies should be transferred every 10 to 14 days. A funnel is placed in the mouth of a fresh culture vial that already has media added. In the old vial (the one with flies in it), the flies are gently tapped down by softly tamping the vial on a soft surface, such as a mouse pad. The flies will fall to the bottom and remain there for a few seconds, enough time to quickly take the plug off the vial, invert it into the funnel, and gently tamp, together, the two vials to force flies down into the new vial.

2.2.2.3. Sexing flies

It is quite easy to differentiate males from females and with a little practice one becomes confident of its ability to do so. Males are generally smaller and have a darker and more rounded abdomen. The coloration of the abdomen is the easiest to recognize. In addition, males have tarsal sex combs on their first pair of legs. These are black and very distinctive, but can only be seen under relatively high magnification. With a little practice, by looking at the abdomen one will become proficient in accurately sexing flies. Sexing flies is critical when making crosses, so be sure you are confident in identifying the difference between the sexes.

2.2.2.4. Collecting virgin females

While it's a simple matter of placing virgin females with males, it is important to recognize the time factor involved for obtaining virgins. Females remain virgins for only 8-10 hours after eclosure and must be collected within this time frame. Alternatively, it is quite easy to distinguish virgins from mature flies visually. It is strongly suggested that one obtains extra virgins in case a mistake is made in identification or the fly dies before mating and egg lying can occur. In a strong culture, multiple virgin females should be easily obtained. Although females are able to lay eggs as virgins, they will be sterile and no larvae will be produced. In the removal method all flies need to be removed 8-10 hours before collecting. The surface of food needs to be visually inspected to ensure complete removal of flies. After 8-10 hours all females that are present need to be collected. All will be virgins. They are placed in a fresh culture vial and we need to wait 2-3 days to look for larvae. Virgin females can lay eggs, but they will be sterile. Since they are photoperiod- sensitive, females tend to eclose early in the morning. Therefore early collections will ensure the greatest number of virgins for experimentation. However, collection is possible later in the day. Sometimes it is possible to separate flies by visually looking at them. Being able to recognize virgin females removes the necessity of emptying culture vials on a timely basis. Virgin females are much larger than older females and do not have the dark coloration of mature females. In addition, in the early hours after eclosure, there will be visible a dark greenish spot (the meconium, the remains of their last meal before pupating) on the underside of the abdomen.

2.2.3. Conditioning

The flies were collected as virgins. Then they were put into their treatment vials. Flies that were chosen to be solo conditioned were put in male/female pairs for 3 days to mate, then they were separated into solo conditioned vials, so that all females were mated and laying eggs. They were transferred once during their aging process, and were aged for 14 days.
Then, the flies chosen for chemical conditioning with hydrogen peroxide were put on the 2% hydrogen peroxide medium for 24 hours and at the same time the rest of the flies were transferred onto fresh food as well. That was done with a reason that no one gets transferred more than anyone else. For getting hydrogen peroxide medium prepared fly food was taken and microwaved so it melts. Next, it was left to cool down, but not to get too hard and afterwards hydrogen peroxide was added to get 2% solution, depending on the fly food volume. The food with hydrogen peroxide was then transferred into the clean vials and let to completely cool down. The volume of the fly food in the vial is 10 ml.

2.2.4. Environment

The easiest way to grow flies is at room temperature. However, the optimum rearing condition is a temperature of 25 °C and 60% humidity. In these conditions generation time is shorter (9-10 days from egg to adult). Unless equipment is readily available this is unnecessary for successful rearing and crossing of flies. It is preferable to keep flies out of drafts and direct sunlight or heat sources. These will rapidly dry the media, necessitating frequent media changes and the potential to dehydrate the flies.
Flies are being kept in the room where the circadian rhythms can be controlled by automatically switching the lights on and off according to the timer. Our flies were timed to have the day light from 9am to 9pm (Figure 10).



Figure 10**. Fly room at the University od Southern California, Nuzhdin lab.** It is the place where the flies are kept at constant environmental conditions, where they are being sexed from each other using CO_2 and transfered from old to new vials (Source: Melina Butuči).

2.2.5. Stereo microscope

After the social and chemical treatment, pictures of flies were taken using Leica MZ FLIII flourescence stereomicroscope with SPOT CCD camera and imaging software (Figure 11).
Stereo microscopes, also called dissecting microscopes, are two compound microscopes which focus on the same point from slightly different angles. This allows the specimen to be viewed in three dimensions. As opposed to compound microscopes, the image is upright and laterally correct (not upside down and backwards). The Leica MZ FLIII with fluorescence filter system enables unprepared fluorescing specimens to be non-destructively inspected, manipulated, sorted and recorded. The fluorescence stereomicroscope has a large field of view and a long working distance, for viewing and manipulating large specimens in

transmission, relection and fluorescence. The magnification zooms from 1x to 10x. A colour CCD camera of moderate quality documents observations. Particularly in molecular biology and in gene technology this new observation technique offers ideal conditions for in-vivo and in-situ investigations of living organisms in real time.

Model:	Leica MZ FLIII Stereomicroscope
Objective Lenses:	Plan-Apo 0.8x to 10x 0.12NA
Illumination Sources:	100W Mercury
	Intralux 5000 halogen
Filter Sets:	470nm Excitation filter set(GFP) and 560nm Excitation filter set(dsRed)
Cameras:	Leica DC 300 F cooled colour CCD camera
Software:	Media Cybernetics Image Pro Express

2.2.5.1. Advantages and Techniques

- Able to image large specimens using reflected, transmitted or fluorescent light
- Large working distance between objective lens and sample
- Great for animal specimens

The digital image recording systems from Leica Microsystems allow rational creation, processing and archiving of digitized images. It is combined with the Leica Image Manager image management system. The IM Image Overlay module delivers perfect results for multiple fluorescence recordings in cell biology or genetics. It has quick switching from 3D to microscopic observation with 10x micro objectives (resolution 0.7 µm, 1320 Lp/mm) or 20x (resolution 0.8 µm, 1260 Lp/mm). Leica stereomicroscopes are designed with two parallel beam paths above a common main objective. This is the most elaborate, but proven the best principle for fatigue-free viewing and for perfect image quality. In addition, the Leica MZ FLIII has the patented separate illumination beam path (TripleBeam™) for the fluorescence illuminator. This unique innovation ensures that, at all zoom positions, the light is guided correctly and utilized fully, and that the background of the field of view is uniformly

dark. Leica's innovation in the design and manufacturing stages pay off with the intense fluorescence and in the detail-rich, reflex-free images with their jet-black backgrounds.

Two novel features enable filters to be changed in a moment: the arrangement of excitation and barrier filters on the same filter carrier, and the creation of a horizontally-rotatable rapid filter changer for four filter combinations. With just one quick movement, the excitation filter is in the illumination beam path and the barrier filter is in the observation beam path. At low magnifications, stereomicroscopes provide a panoramic view of the whole object; at high magnifications, they reveal fine detail. The zoom range of the Leica MZ FLIII, from 8x to 100x with 1x objective and 10x eyepieces, its maximum magnification of 800x and its high resolution of up to 750 line pairs/mm with the 2x planapochromatic objective, take its observation range into that of the classical microscope. Intense UV radiation can cause damage to the retina of the observer's eye. That is why UV barrier filters are permanently installed in the observation beam paths; there is a UV protection screen above the specimen plane and stray-light protection at the lamp housing, and there are dummy filter carriers in the empty filter positions (http://www.oulu.fi/pyokui/tiedostot/laitteet/Leica%20M1-160-0en.pdf).

GFP Filter sets	Excitation filter	Barrier filter
GFP fluorescence	425/60 nm	480 nm
GFP Plus fluorescence	480/40 nm	510 nm
GFP Plant fluorescence	470/40 nm	525/50 nm



Figure 11**. Leica MZ FLIII flourescence stereomicroscope with SPOT CCD camera and imaging software** (Source: http://www.oulu.fi/pyokui/tiedostot/laitteet/Leica%20M1-160-0en.pdf).

2.2.6. MicroPicAnalyzer

The pictures of flies were then processed with MicroPicAnalyzer. The software was developed at University of Southern California, by a Ph.D. student Reza Dehestani Ardekani. Colored pictures have 3 channels: Red, Green and Blue. The color of each pixel is determined by a combination of these 3 channels, for example Red and Green make Y considering one byte of memory for each number. The range of each channel is between 0 to 255 and higher the number means brighter the picture. If r,g,b for a pixel is 0,0,0 the color of that pixel would be black. Same way, if r,g,b is 255,255,255 the color would be white. To quantify GFP, we are looking at the intensity of the green channel. Since checking each pixel in these large pictures (1600x1200) is really tedious, we do it in an automated way by looking at each pixel and making a histogram of their values. We divide each channel to N bins (here the number of bins is N = 4) and count the number of pixels that fall into that bin. For example, for N = 4, the threshold for bins are 0-64, 64-128, 128-192, 192-255. Now for each image, we record the number of pixels that their green's intensity is between 0-64 and also the sum of their values.

We do the same thing for all the other bins as well. Using this method, pictures that have higher number of pixels in higher bins (bin 192-255 is the highest) are more greenish, and since in these pictures the only source of green channel is GFP, they have higher expression of the GFP.
The input file is a picture in jpg format, and the output file are RGB values in Excel.

2.2.7. Data analysis

Data analysis and charts were done in Excel and statistics in SAS proc glm. SAS has several procedures for analysis of variance models, including proc anova, proc glm, proc varcomp, and proc mixed. We mainly will use proc glm and proc mixed, which the SAS manual terms the “flagship” procedures for analysis of variance. The “glm” in proc glm stands for “general linear models.”

3. Results

3.1. Chemical stress in male fruit flies

Statistical analysis was done on the sample size of 23 males. 12 of them were put on the normal food, each one of them separately into a single vial; 11 of them were were put on the food with 2% hydrogen peroxide. The actual genotypes (hsp22 versus hsp70) did not show statisticaly significant importance, which is why they are processed together as a single hsp genotype. Looking at the data we were not able to show that there was a difference of the GFP expression between the flies that were put on the normal food versus flies that were conditioned with 2% hydrogen peroxide. Yet, the values of flourescence are slightly higher for the flies put on the 2% hydogen peroxide food (Figure 12).

$F_{1, 23}$=0.79; P=0.385; mean without H_2O_2= 4.82458; mean with H_2O_2=4.95491;
StDev 1m H_2O_2= 0.345026; StDev 1m= 0.374893.

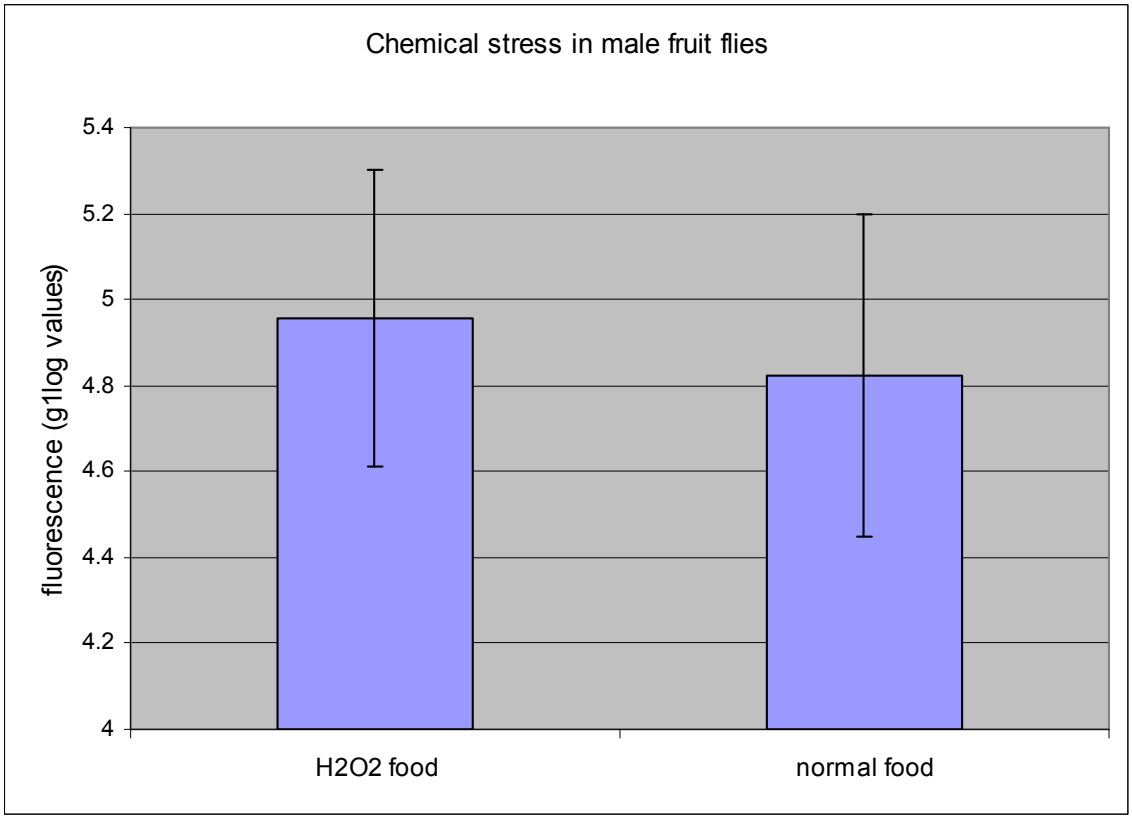


Figure 12. **Chemical stress in male fruit flies.** Results are showed as means of a variable g1log ± SD.

3.2. Chemical stress in female fruit flies

Statistical analysis was done on the sample size of 23 females. 7 of them were put on the normal food, each one of them separately into a single vial; 16 of them were put on the food with 2% hydrogen peroxide. The actual genotypes (hsp22 versus hsp70) did not show statisticaly significant importance, which is why they are processed together as a single hsp genotype. Looking at the data we were not able to show that there was a difference of the GFP expression between the flies that were put on the normal food versus flies that were conditioned with 2% hydrogen peroxide. But the difference between the values of flourescence between the female flies put on normal food and those put on 2% hydogen peroxide food is noticable, showing higher values for those flies put on the 2% hydrogen peroxide food (Figure 13).

$F_{1, 23}$=2.73; P=0.1132; mean without H_2O_2= 4.9242; mean with H_2O_2= 5.17591;
StDev 1f H_2O_2 = 0.374502; StDev 1f = 0.211093.

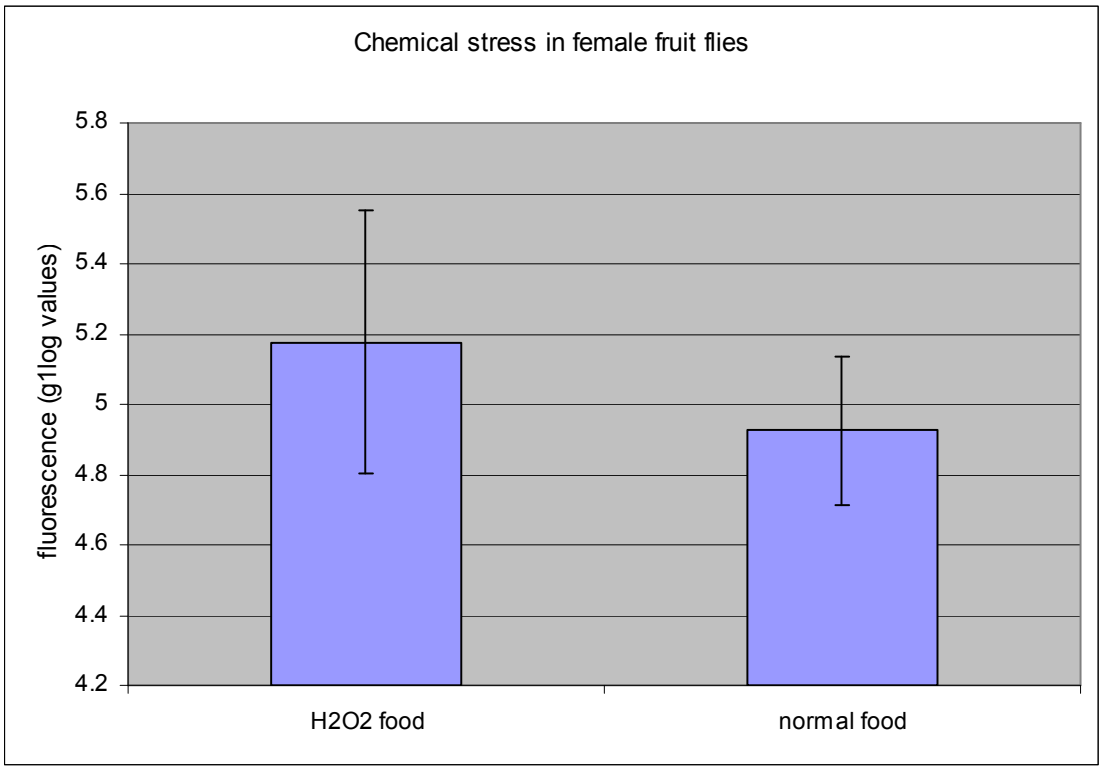


Figure 13. **Chemical stress in female fruit flies.** Results are showed as means of a variable ± SD.

3.3. Chemical stress in both male and female fruit flies together

Although looking at the males and females separately on 2% hydrogen peroxide food showed only a slight difference in flourescence values compared to those from normal food values, it pointed out that females give a stronger flourescence signal than males under the same conditions. Nevertheless, if we look at the overall flourescence in males and females put on the 2% hydrogen peroxide together, the result will show a statistically significant difference between those flies and the ones that were on the normal food (Figure 14).

$F_{1, 46}$=4.39; P=0.0417; mean without H_2O_2 = 4.86129; mean with H_2O_2 = 5.0812; StDev 1m+f H_2O_2 = 0.372587; StDev 1m+f = 0.321216.

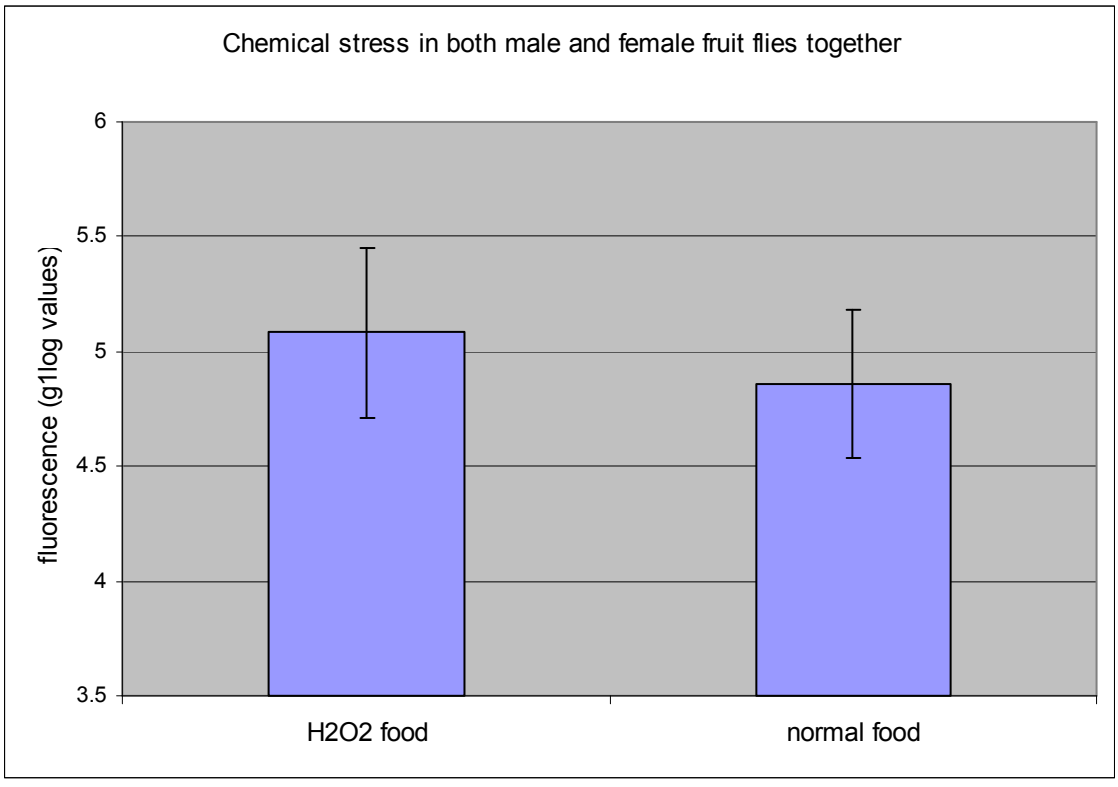


Figure 14. **Chemical stress in both male and female fruit flies together.** Results are showed as means of a variable g1log ± SD.

3.4. Social stress in female fruit flies

Statistical analysis was done on the sample size of 45 females. There were three different social groups . The maximum number of flies per vial was 8. Group one was made out of 1 male and 7 females per vial. From this group we examined 18 females. Second group had an even ratio between sexes, 4 males and 4 females per vial. From this group we examined 10 females. Lastly, the third group was made out of 7 males and 1 female per vial only. Here, we examined 17 females. The highest flourescence value in females showed the third group of 7 males and 1 female per vial, while the least fluorescence value in females showed the first group of 1 male and 7 females. We also compared the fluorescence values between the groups. First group with 1 male and 7 females and the second group with 4 males and 4 females are not significaly different from each other, but both of them are different from the third group with 7 males and 1 female. From this results we can see that overall effect from different social groups on female stress is significant (Figure 15).

$F_{2, 42} = 12.35$; $P<0.0001$; mean 1,7=4.804; 4,4=4.916; 7,1=5.257;
StDev (1,7)f = 0.25572; StDev (4,4)f = 0.320871; StDev (7,1)f = 0.269619.

4,4*7,1 $F_{1, 25}=8.75$; $P=0.0068$;
1,7*7,1 $F_{1, 33} = 26.01$; $P<0.00011$;
7*4,4 $F_{1,26}=1.03$; $P=0.320$.

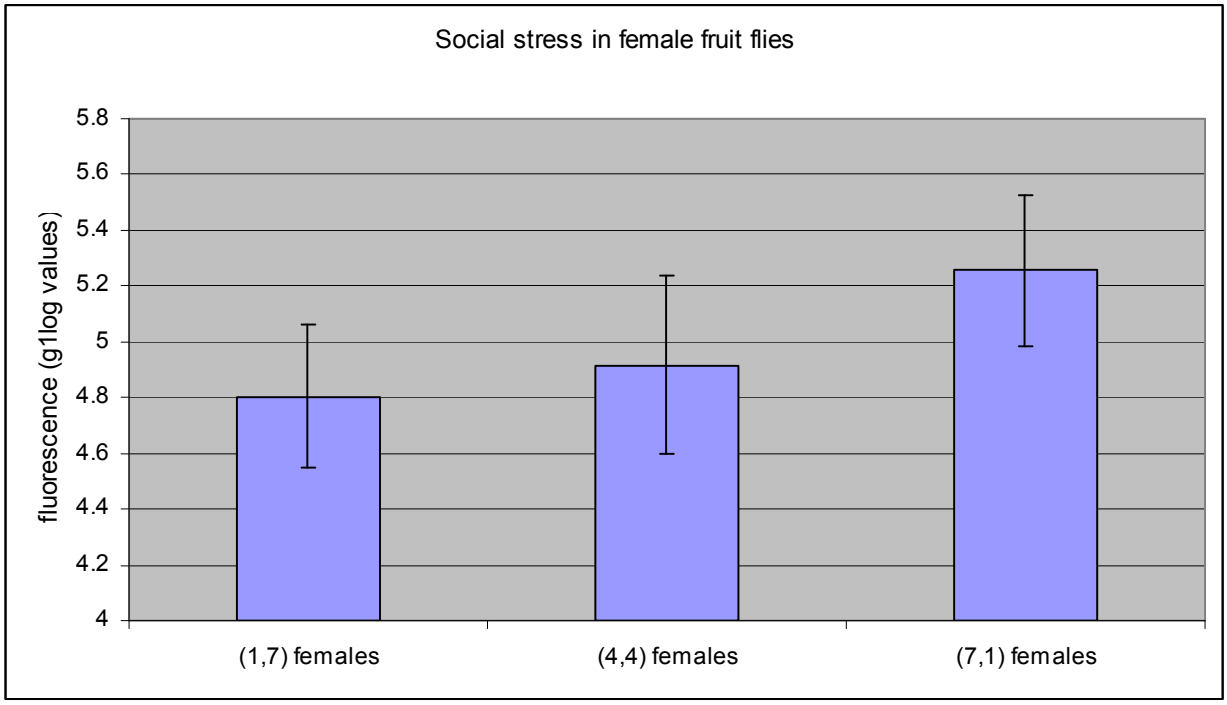


Figure 15. **Social stress in female fruit flies.** Results are showed as means of a variable g1log ± SD.

3.5. Social stress in male fruit flies

Statistical analysis was done on the sample size of 40 males. There were three different social groups . The maximum number of flies per vial was 8. Group one was made out of 1 male and 7 females per vial. From this group we examined 12 males. Second group had an evan ratio between sexes, 4 males and 4 females per vial. From this group we examined 9 males. Lastly, the third group was made out of 7 males on 1 female per vial only. Here, we examined 19 males. The highest flourescence value in males showed the first group of 1 male and 7 females per vial, while the least fluorescence value in males showed the second group of 4 males and 4 females. We also compared the fluorescence values between the groups. None of the groups of different socials treatments showed to be significant for males, since they do not differ statisticaly from each other (Figure 16).

$F_{2, 37} = 1.09$; P=0.347; mean 1,7=4.846; 4,4=4.595; 7,1=4.706;
StDev (1,7)m = 0.455448; StDev (4,4)m = 0.391052; StDev (7,1)m = 0.348134.

4,4*7,1 $F_{1, 26}$=0.58; P=0.453;

1,7*7,1 $F_{1, 29}$ = 0.93; P=0.342;

1,7*4,4 $F_{1,19}$=1.76; P=0.200.

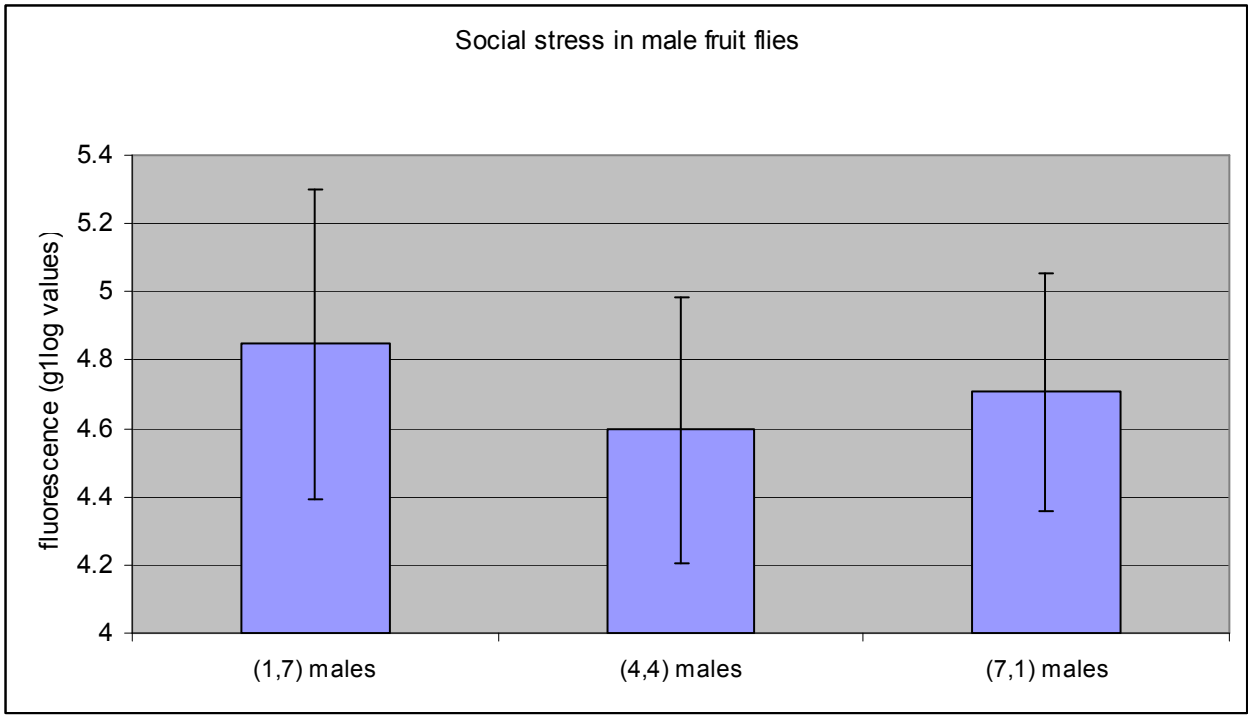


Figure 16. **Social stress in male fruit flies.** Results are showed as means of a variable ± SD.

4. Discussion

It is known that hsps are robustly induced in response to various stresses (both intrinsic and extrinsic) and are key mediators of an organism's resistance to stress (Tower, 2009). In this experiment, we tested the effects of the chemical stress on the expression of the hsps. We found that there is a significant effect only when the results for males and females were taken together. However, looking at the absolute values of the fluorescence in the samples, it is apparent that the fruit flies (both males and in females) that were put on the 2% hydrogen peroxide food showed higher fluorescence values than the flies put on the normal food. Interestingly, in the experiments on the effect of chemical stress it is possible to see that the maximum levels of hsp expression in males on 2% hydrogen peroxide food almost equaled the minimum level of the hsp expression in females on normal food. We have therefore shown that the fruit flies on 2% hydrogen peroxide generate higher hsp expression by measuring the artificially induced increase in the fluoresence of a GFP via the response to oxidative stress by hsp.

In determining how the social environment effects the levels of stress in both males and females, we chose to create three different social groups: 1 male with 7 females, 4 males with 4 females and 7 males with 1 female. Looking at females, the data showed statistical significance only for the females that were placed in the groups of 7 males with 1 female. Lone females in the vial with 7 males experience the most stress, as measured by the highest flourescence values from all the other male/female ratios. This is probably due to the constant male aggressive behaviour correlated with mating impulse. However, the least stressed environment for female flies was group of 7 females and 1 male. Interestingly, what seems to be the lowest value for female stress, seems to be the highest value of all for the male stress values. Although there is not a statisticaly significant difference between the social treatments for males, we can still predict that the most stressed males are the ones from the group of 1 male and 7 females. The least stressed males were the ones from the group where the ratio between males and females is the same, 4 males and 4 females.

Both in social and chemical experiments the statistically least stressed females showed higher flourescence, meaning stronger stress response than the most stressed male fruit flies. One explanation for this results could be the fact that the effects of stress are simply higher in female fruit flies. Or maybe it is only due to the size of females, who tend to be even twice as big as males. There could be another factors involved and to answer this question furter investigations need to be performed.

There are a lot of other questions that arose and haven't been answered yet. For example, does changing the sex ration effect males more than females? Or vice versa? How different the

genotypes really are in their responses to stress? Is the effect consistent across all treatments? Is hsp22 always dimmer and by the same amount across all the treatments? Or is it brighter for some and dimer for others?

In order to give a good answer to those questions further experiments need to be performed. The social treatments need to be expanded and more male/female ratios need to be investigated. It would be useful to have some data from the wild type flies in addition to our GFP-hsp tagged flies and to use those flies as a control group. Expanding the sample size would theoretically result in less noise whereby our results would be much more confident, and our conclusions more realistic. Furthermore, by expanding the sample size, it would enable us to do more complicated and specific analysis and we would generate data from more comparisons between the treatments.

Our next step in this project will be to automate the experiments and monitor fruit flies with the camera system that is being developed at the moment at University of Southern California. The camera system will allow us to track each fly separately in real-time, for as many hours/days as we want, which will show us hour-by-hour hsp expression change and thus for each fly separately.

Within this experiment it has been noticed that the males often, about 50% of the time, had bright spot in their lower right abdomens. It lacks the symmetry and its positioning suggests that it might be related to the digestive system. Although it is still not known what that might be, it is often seen in the wild type flies which makes us exclude the possibility that it is related to the transgenes. This type of glow is all or nothing - either they have it, or they do not. That still was not the problem for our data, since we could chose from the MicroPicAnalyzer at what bins to look at while examining the GFP output. To remedy this situation, we ignored the higher bins in the green channel, and chose only the lowest one. Thereby, lots of low level fluorescence represents widespread hsp expression.

5. Conclusion

Based on the results from this research, whose goal was to see whether different chemical and social environments cause a significant level of oxidative stress in fruit flies, we came to this conclusions:

i. There is a significant effect of 2% hydrogen peroxide on the expression of the hsp genes involved in the response to oxidative stress in fruit fly*, Drosophila melanogaster*. The higher intensity of the fluorescence was noticed in both males and females that were put on the 2% hydrogen peroxide food, but the statisticaly important difference between them was noticed when we looked at the expression in both males and females together.

ii. There was a difference in the hsp genes expression established in the experiments involving different social groups of fruit flies, especially in females the effect was strong. The most stressful group for females was the group of 7 males and 1 female, and the least stressful group was the group of 1 male and 7 females. The most stressful group for males was the group of 1 male and 7 females, and the least stresssful group was the group of 4 males and 4 females.

iii. In all the analyses the actual genotype, hsp22 and hsp70, never came out as an important factor, showing a difference between the two lines used in this experiment.

iv. Interestingly, it has been seen that in the experiments on the effect of chemical stress that maximum levels of Hsp expression in males on 2% hydrogen peroxide food almost equaled the minimum level of the Hsp expression in females on normal food, showing general higher expression of Hsps in female fruit flies.

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