EFFECT OF HYDROGEN PEROXIDE TREATMENT ON THE PROTEOME PROFILE OF *MUSCA DOMESTICA* LARVAE

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ABSTRACT

The effects of acute treatment of hydrogen peroxide (LC_{50} = 21.52% (v/v)) on the expression of cytosolic and thiol proteins of housefly (*Musca domestica*) 3rd instar larvae were investigated. Using two dimensional gel electrophoresis, differential analysis was performed to identify protein with moderate (2 to 5 times) and high (more than 5 times) fold changes. In the total proteome, 17 spots and 23 spots were discovered to have moderate fold change respectively in control and treated samples. 3 different identified protein spots were shown increase in high fold change in both treated and non-treated larvae samples respectively. Qualitatively, there was presence of extra expression in 2 and 4 different protein spots in both control and treated samples respectively. The variation of expression of thiol proteins was also investigated by analyzing the purified thiol proteome upon acute peroxide treatment. 5 thiol proteins and 6 thiol proteins were found to have a moderate fold change in control and treated samples respectively. There were 2 different identified thiol protein spots increased at high fold changes in both treated and non-treated larvae samples. Our work has also shown that qualitatively, a significant presence of 4 protein spots in treated samples which were absent in the control samples. Protein spots with high fold changes were identified using LC-MS/MS based peptide mass fingerprinting and biomarkers related with important biological functions were identified including cytoskeleton (actin and tropomyosin), protein degradation (ubiquitin), odorant binding (PBP/GOBP family protein), energy metabolism (arginine kinase), anaerobic metabolism/gluconeogenesis/TCA cycle (Lactate/malate dehydrogenase), and glycolysis/gluconeogenesis (Fructose bisphosphate aldolase). Arginine kinase and fructose bisphosphate aldolase are high in abundance in thiol proteome profile, suggesting their high tolerance of cysteine residues under acute hydrogen peroxide induced oxidative stress.

ABSTRAK

Kesan rawatan akut hidrogen peroksida (LC_{50} = 21.52% (v/v)) dari segi ekspresi inti sel dan protein thiol larva instar ketiga lalat rumah (*Musca domestica*) telah disiasat. Dengan menggunakan elektroforesis gel 2 dimensi, analisis secara pembezaan dijalankan bagi mengenalpasti tompok protein yang meningkat secara sederhana (2 hingga 5 kali) dan tinggi (lebih daripada 5 kali). Dalam proteome secara keseluruhan, 17 and 23 tompok protein dijumpai menunjukkan peningkatan sederhana masing-masing sampel kawalan dan dirawat. 3 protein yang berbeza menunjukkan peningkat tinggi dalam sampel kawalan dan rawatan masing-masing. Secara qualitatif, sampel kawalan dan sampel rawatan masing-masing menunjukkan 2 dan 4 tompok protein yang muncul secara unik. Variasi ekspresi protein thiol setelah dirawat secara akut oleh peroksida juga disiasat. 5 protein thiol dan 6 protein thiol masing-masing menunjukkan peningkatan secara sederhana dalam sampel kawalan dan rawatan masing masing. Kerja kami turut menunjukkan secara kualitatif, Masing-masing dalam sampel larva kawalan dan dirawat, 2 tompok protein thiol telah dikesan. 4 tompok protein dalam sampel rawatan tidak hadir dalam sampel kawalan. Pengenalpastian peptida melalui kromatografi cecair dan spektrometri jisim selaras dalam tompok protin yang berubah secara tinggi mengaitkan petanda dengan fungsi biologi penting termasuk sitorangka (aktin dan tropomiosin), metabolisme tenaga (kinase arginina), degradasi protein (ubikuitin), pengikatan pengahbau (keluarga protein PBP/GOBP), metabolisme anaerobik/gluconeogenesis/ kitaran TCA (laktat/malate dehidrogenase), dan glikolisis/gluconeogenesis (aldolase fruktos bisfosfat). Kinase arginina dan aldolase fruktos bisfosfat menunjukkan peningkatan tinggi dalam profil protein thiol, mencadangkan bahawa toleransi tinggi cysteine di bawah rawatan akut peroksida hidrogen yang mencetuskan tekanan oksidatif.

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LIST OF ABBREVIATIONS AND SYMBOLS

v/v	volume-to-volume
w/v	weight-to-volume
µl	microlitre
CBB	Coomassie Brilliant Blue
CHAPS	3- ((3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
g	gram
HCl	hydrochloric acid
hrs	hours
IEF	isoelectric focusing
IPG	immobilized pH gradient
L	litre
LC-MS/MS	liquid-chromatography tandem mass spectrometry
M	molarity
mA	milliampere
MDA	malondialdehyde

mg	miligram
ml	mililitre
mM	micromol
mm	milimetre
NaCl	sodium chloride
NL	non-linear
PCR	polymerase chain reaction
SDS	sodium-dodecyl-sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris-HCl	Tris-hydrochloric acid
V	volts
W	watt

1.0 LITERATURE REVIEW

1.1 Introduction

Houseflies (*Musca domestica*) are one of the wonderfully evolved organism of the Diptera clade since Jurassic. An estimated 150,000 of species of Diptera have been described (Thompson, 2004).

A notorious vector, houseflies are associated with more than 100 pathogens (Scott *et al*., 2009), and resistance towards insecticides of houseflies have been reported all over the world. According to Scott *et al*., *Musca domestica* is suitable as a model organism for resistance studies and development of new insecticides.

The knowledge on cellular metabolism in recent years has been expanded to understand the metabolic workings upon oxidative stress. Cellular metabolism, particularly in oxygen metabolism, produce reactive oxygen species like superoxide anion ($O_2^{-\bullet}$). Different response system had been developed to sense and respond towards oxidative stress (Groitl and Jakob, 2014).

Cysteine residue in amino acid reactive site undergoes oxidative changes (Biswas *et al*., 2006) and oxidative modification of proteins containing cysteine residue have been shown to play a role during oxidative stress (Song *et al*., 2002; Azevedo *et al*.,2003; Georgiou and Masip, 2003; Alonso *et al*., 2004; Enoksson *et al*., 2005; Winter *et al*., 2005; Biswas *et al*., 2006; Brandes *et al*., 2007; Brandes *et al*., 2009).

In *Musca domestica* alone, a few family of proteins have been more or less associated with oxidative stress response : glutathione –s-transferases (Ketterer, 1998; Strange *et al*., 2000; Eaton and Bammler, 1999; Yin *et al*., 2000), superoxide dismutase (den Hartog *et al*., 2003; Tang *et al*., 2012), glutathione peroxidases (Simmons *et al*., 1987; Simmons *et al*., 1989), and catalase (Allen *et al*., 1983).

Combination of thiol protein trapping techniques (Hu *et al*., 2010), the prowess of two dimensional gel electrophoresis (O' Farrell, 1975 and Klose, 1975) and the liquid chromatography tandem mass spectrometry (LC-MS/MS) identification is possible to probe and observe cytosolic proteins. From that we will be able to provide information on how *Musca domestica* larvae survive under oxidative stress at proteomic level.

1.2 *Musca domestica*

Musca domestica (1758, Linnaeus) (Diptera: Muscidae), commonly known as housefly, is a synanthropic insect living and interacting with human and its environment, especially in urban areas.

Figure 1.1: Adult house fly, *Musca domestica* Linnaeus (1758, Linnaeus) (Adapted from http://diptera.info/forum/viewthread.php?thread_id=42656n Photograph by Jim Kalisch, University of Nebraska-Lincoln.)

The survival of a wild type adults usually spans from 15 to 25 days, which they exhibit the same behavior during our laboratory cultivation. Where during this stage adult housefly undertakes complete metamorphosis from of an egg, larval, pupal, and finally to adult stage.

Houseflies breed extremely fast when compared to other species of flies. Female houseflies lay 500 eggs, in 5 to 6 batches throughout her lifespan, with 75 – 100 eggs deposited each batch in the span of just 3 to 4 days. Females are able to do so by just on fertilization of one male. The white and oval eggs are laid into a moist environment such as animal feces, excrement and garbage, preferably the ones which exposed under light. Larvae weighs from 0.008 to 0.02g at birth.

Larvae develops in 5 days, surviving best in compost mixtures of decaying vegetables enriched with animal material or dung. Throughout this investigation, our cultivation aims to create such similar environment by distilled water-moistened mouse feed for larval cultivation (Hewitt, 1914; Robinson, 2005; Marshall, 2006).

Figure 1.2: Life cycle of the house fly, *Musca domestica* (1758, Linnaeus)

(Clockwise from left: eggs, larva, pupa, adult.)

(Adapted from

http://www.forestryimages.org/browse/detail.cfm?imgnum=1234161 Photograph

by Clemson University - USDA Cooperative Extension Slide Series, Bugwood.org)

Classifications:

Domain: Eukarya

Kingdom: Animalia

Phylum: Arthopoda

Class: Insecta

Order: Diptera

Family: Muscidae

Genus: *Musca*

Species: *domestica*

1.3 Health impacts of *Musca domestica*

Naturally houseflies' main ecosystem role is to decompose and recycle organic material. Houseflies is a synanthropic insect in urban areas where high densities of human waste as their food source. (Dahlem, 2003; Marshall, 2006; Robinson, 2005).

It has known to be vectors of various diseases of over 30 bacteria, protozoan, viruses and helminth eggs (Greenberg *et al*., 1970, Kobayashi *et al*., 1999). It also transfers viruses such as polioviruses (Kettle, 1990) and Coxackie viruses, as well as numerous bacteria such as *Campylobacter jejuni*, *Heliobacter pylori* (Grubel *et al*., 1997), *Salmonella* sp. (Greenberg *et al*., 1970), *Listeria* sp., Yersinia pseudotuberculosis (Zurek *et al*., 2001), *Shigella* sp. (Ugbogu *et al*., 2006), *Escherichia coli* (Szalanski. *et al*., 2004), and *Vibrio* sp. (Kettle, 1990). Flies may also be vectors of protozoan flies such as *Giardia* and *Entameba* (Ugbogu *et al*., 2006) and eggs of several tapeworms (Graczyk *et al*., 2005). In 2010, there were further proof on transmission of Newcastle disease virus (NDV - Paramyxoviridae), a highly infectious virus shed in the faeces in infectious birds (Barin *et al*., 2010) with *Musca domestica* as vector in both field and laboratory. More recently, *Musca domestica* were also reported to carry antibiotic resistant bacteria such as methicillin resistant Staphylococcus and tiarcilin resistant *Pseudomonas* (Boulesteix *et al*., 2005), which possess threat in hospitals and healthcare facilities (Graczyk *et al*., 2001). In Africa, houseflies transmit pathogens that is responsible for trachoma (Keiding, 1986). Flies causing 6 million cases of childhood blindness each year (http://www.who.int/topics/trachoma/en/).

Musca domestica also create implications in economical ways, costs of pesticides were estimated at more than US$200 million yearly in the United States (Geden *et al*.,1994) and US$1.6 million in 1998 (Crespo *et al*.,1998).

1.4 Insecticide control and resistance of *Musca domestica*

Types of insecticides used to control houseflies on field are adulticides and larvicides (www.flycontrol.norvatis.com). Adulticides are carbamates (e.g. propoxur and methomyl), organophosphates (e.g. fenitrophon, azamethiphos and dimethaoate), pyrethroids (e.g. cyfluthrin, deltamethrin and permethrin) and recently neocotinoids (e.g. imidacloprid, thiamethoxam)(Kaufman *et al*., 2006). Larvicides are insect growth regulators (IGRs) (e.g. triflumuron, diflubenzuron, cyromazine (Kočišová *et al*.,2004)), and nonvaluron (Cetin *et al*.,2006)) and juvenile hormone synthetic analogues (e.g. methoprene, fenoxycarb, pyriproxyfen (Kočišová *et al*.,2004)) (www.flycontrol.novartis.com).

Since 1st case of DDT-resistance is reported on the housefly (Saccà, 1947), resistance of adult *Musca domestica* towards various insecticides in various sites (agricultural, wild, and urban) is a fast-growing global issue. There has been an increasing resistance profiles report from various places in the world.

In United Kingdom a resistance risk-assessment done by Learmount *et al*. (2002) shown that although farmers claimed they had reduced using insecticides (a measure to reduce selective stress on field housefly strains), there were no sign of decrease of housefly resistance towards piperonyl butoxide-synergized pyrethrins.

In Denmark, Keiding (1976) discovered high fenitrothion and dimethoate resistance in field. In 1997, Kristensen *et al*. confirmed increase in pyrethroid-resistant strains and widespread of azamethiphos-resistant strains in 21 different farms all over Denmark.

In Argentina, a first insecticide survey was reported by Acevedo *et al*. (2009). *Musca domestica* populations there were found to be permethrin, dichlorovinyl dimethyl phosphate (an organophosphate) and cyromazine resistant. In the neighbouring Brazil,

Pinto and Prado (2001) led a first evaluation of cyromazine resistance of houseflies in 5 different sites and 3 out of the 5 sites indicated cyromazine resistance.

One finding of insecticide tolerance in tsunami-hit villages in India, emphasizing on post-disaster pest control is done by Srinivasan *et al*. (2008). With hygiene is at minimum provision, immediate fly control was imposed by spraying 76% dichlorvos and LD_{90} of adult housefly was 3.5-3.9 times higher than the flies from control sites.

As in the United States, Scott *et al*. (2000) tested against nine insecticides, flies from caged-layer poultry facilities across New York. The fly strain showed high resistances in tetrachlorvinphos, permethrin and cyfluthrin while in southeastern Nebraska, houseflies are shown to be moderately resistant to permethrin yet extremely resistant to stirofos and methoxychlor (Marçon *et al*., 2003).

In China, Cao *et al*. (2006) discovered deltamethrin resistance in urban garbage dump of cities of Beijing, Tianjin and Zhangjiakou.

Back at Malaysia, Nazni *et al*. (1999) has been evaluating resistance of housefly from a garbage dump, poultry farm and agricultural farm. Five insecticides were evaluated for the purpose. Garbage dump and poultry farm fly samples were more resistant than agricultural farm. Bong and Zairi (2010) has also worked on two poultry farms in the state of Penang against malathion, propoxur and DDT, with resistance ratio have been found with strong correlations against relative humidity, which is a first in field discovery.

While on housefly larvae, resistance assessment has been relatively scarce with only a handful of feeding and toxicity tests done. Other than Acevedo *et al*. (2009) and Pinto and Prado (2001) mentioned above, in Denmark, Kristensen and Jespersen (2003) reported an increase on field in diflubenzuron resistance and new-found cyromazine-resistant strain. A dip test-emergence test of *Musca domestica* third instar larvae on eucalyptol extracts has been done by Sukontason *et al*. (2004), with LD_{50} values of

118mg/fly and 177mg/fly on male and female flies respectively. By the means of feeding assay, Cetin *et al*. (2009) firstly reported existence of low-level IGR-resistance housefly strain in Turkey.

1.5 Insecticide resistance and oxidative stress research

For oxidative stress induced insecticide resistance research, rats (Lukaszewicz-Hussain and Moniuszko-Jakoniuk,1999, 2001 and 2003; El-Demerdash *et al*.,2011), humans (Ranjbar *et al*.,2005), fresh water fish *Brycon cephalus* (Monteiro *et al*.,2009), and black tiger shrimp *Penaeus monodon* (Dorts *et al*.,2009) has been used as model to investigate insecticide inflicted oxidative stress. Insecticides including pyrethroids (Giray *et al*., 2001; Gupta *et al*., 1999; Kale *et al*., 1999), organophosphates (Akhgari *et al*., 2003; Fortunato *et al*.,2006; Lukaszewicz-Hussain, 2010), and organochlorines (Latchoumycandane *et al*., 2002; Koner *et al*., 1998) have known to be inducing oxidative stress. Lukaszewicz-Hussain *et al*. (2003) reported that there were changes in activities of the antioxidative enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and in GSH level changes both in liver and erythrocyte homogenate.

Pre-2014, genomics work has been extensively conducted in scale to evaluating insecticide resistance in a molecular basis. Molecular resistances are consisted of target site resistance and metabolic-based resistance (Perry *et al*., 2011). Yet, most of the works, as far as *Musca domestica* is concerned, these genomic works have been more in top-down approach. With genome sequencing was still ongoing for that time being, specific gene family is identified and sequenced before getting into expression studies . With other fly species such as the dipteran *Drosophila melanogaster* and *Anopheles* genome as comparable reference database, Ranson *et al*. (2002) concluded 3 groups of gene superfamilies that involved metabolic-based resistance i.e.: glutathione S-transferases,

cytochrome P450s, and acetylcholinesterase. Resistance on target site such as kdr-resistance and ligand-gated ion channels (ffrench-Constant *et al*., 2004) are target gene of interest.

In cytochrome P450s, work from Zhu *et al*. (2008) revealed that 3 P450s genes, CYP4D4v2, CYP4G2, and CYP 6A38 were up-regulated in response to permethrin treatment on permethrin resistant ALHF strains. Kristensen *et al*. (2010), by PCR technology, demonstrated constant overexpression CYP 6A1, CYP 6D1 and CYP 6D3 in neocotinoid-resistant strains in Denmark during thiomethoxam challenge. CYP6D1 was also found to be implicating more than 5000-fold of cypermethrin resistance in Learn pyrethroid-resistant strain found in New York (Scott and Georghiou, 1985).

In glutathione S-tranferases, (GSTs; E.C 2.5.1.18), Nazni *et al*. (1999) also evaluated non-specific esterases and glutathione S-transferases and non-specific esterases has significant increase. A remarkable drop on GST activity has been reported on a DDT-resistant strain 698ab was reported by Kristensen (2005).

Point mutation is the blame on insecticide sensitivity in the case of acetylcholineesterases (E.C 3.1.1.7) (Fournier, 2001). Oppenorth *et al*. (1976) in fact discovered 20-fold insensitivity of acetylcholinseesterases in houseflies, alongside with findings of high GST expression is in correlation with de-alkylated and hydrolysation of parathion and methylparaoxon.

As far as metabolic-based resistance is concerned, there still much more questions to be addressed. Scott *et al*. (2000) stated that there is very little known about the mechanism of the pyrethroid resistance (monooxygenase/CYP450s), although pathways have been elucidated via genomic means. Between two different strains tested by Scott *et al*.: Lean Dairy and caged-layer strains, both have opposite effects on pyrethrins. Cao *et al*. (2006) showed a significant correlation between kdr allele (i.e. genes reducing the sensitivity of the nervous system to pyrethroids) frequencies and the levels of knockdown

resistance by deltamethrin via a PCR-based assay. Kristensen (2005) suggested that there had been leading to altered substrate specificity, and did not rule out the potential of GSTs mediating pyrethroid resistance. Learmount *et al*. (2002), from their findings, suggested that behavioral resistance might be playing a role in contributing such resistance and such traits are still being inherited in the field. Possibility of an up-regulation mediated by changes to trans-acting factors is pointed, which appears these mechanisms were underlying in some cases of resistances of P450s, GSTs, and acetylcholineesterases (Feyereisen, 1999 and Hemingway, 2000).

1.6 Reactive oxygen species and hydrogen peroxide in living organisms.

Free radical is defined as any chemical species that contains one or more unpaired electrons. Reactive oxygen species are activated oxygen species including oxygen radicals (e.g. superoxide anion radical, $O_2^{-\bullet}$, hydroxyl radical, $^{\bullet}OH$) and non-radicals (hydrogen peroxide, H_2O_2, hypochlorous acid, HOCl), which can be easily converted to radicals (Hülya, 2005; Demidchik, 2015).

Living organism deals and generates reactive oxygen species *in vivo* and under such understanding, hydrogen peroxide is not an obscure molecule in a living system. The discovery opened up when Chance (1952) observed that catalase was saturated with intermediates of hydrogen peroxide in high concentrations in bacteria *Micrococcus lysodeikticus*. In 1969, McCord and Fridovich had purified and demonstrated the catalytic activity of superoxide dismutase, which transforms superoxide anion radical $O_2^{-\bullet}$, into hydrogen peroxide. A year later, Sies and Chance (1970) showed that difference in absorbance on the steady state of catalase compound I when glycolate, a hydrogen peroxide generating substrate is inserted into rat liver. Oshino *et al*. (1975) examined the H_2O_2 production of rats under different conditions such as starvation, glycolate and

octanoate diffusion. Loschen *et al*. (1974) drew the relation that products of superoxide dismutase, the superoxide anion is the precursor of hydrogen peroxide.

Oxygen metabolism is one of the essential processes in an aerobic cell and a common site for ROS generation, better known as the 'electron leak' at the electron transport chain in mitochondria. Electron leak is the phenomenon of passing the electrons to oxygen instead of water, causing the reduction of oxygen to superoxide (Jastroch *et al*., 2010) in mitochondria. Numerous locations in mitochondria were found to be generating superoxides and hydrogen peroxides, including outer/inner side of complex III ubiquinol–cytochrome c reductase (Cadenas *et al*., 1977; Turrens, 1985; Han *et al*., 2001), complex I NADH dehydrogenase (Turrens and Boveris, 1980; Kushnareva *et al*., 2002), complex II succinate dehydrogenase (Lenaz *et al*., 2001), external NADH dehydrogenase (Fang and Beattie, 2003) , dihydroorotate dehydrogenase (Forman and Kennedy, 1976) glycerophosphate dehydrogenase (Drahota *et al*., 2002) and mono amino oxidase (Hauptmann *et al*., 1996; Cadenas and Davies, 2000).

There was also a plethora of other enzymatic and non-enzymatic generation of ROS generation than mitochondria of superoxide anion. NAD(P)H oxidase in vascular cell, produce superoxide anion and hydrogen peroxide under stimulation of growth factors and cytokine (Griendling *et al*., 2000). In human dermal fibroblasts, superoxide was observed to be released in a controlled manner (Meier *et al*., 1989). Xanthine oxidase produces hydrogen peroxide directly other than superoxide (Kelley *et al*., 2010). Cytochrome P-450 dependent oxygenases produce superoxide anion and hydrogen peroxide in the catalytic cycle (Zangar *et al*., 2004).

What happened when there is an excess of reactive oxygen species *in vivo*? Sies (1991) coined the term "oxidative stress" by the definition of "an imbalance between oxidants and antioxidants in the favour of the oxidants". A practical definition of oxidative

stress by Betteridge *et al*. (2000) will be "*a disturbance* in the balance between the production of reactive oxygen species (free radicals) and antioxidant defences".

1.7 Enzymatic removal of cellular hydrogen peroxide

Due to their constant metabolism on oxygen and releasing ROS as discussed in Section 1.6, Halliwell and Gutteridge (2006) suggested that aerobic organisms survive due to their evolved antioxidant capability. In this section we hope to provide an overview of the enzymatic mechanisms that remove cellular hydrogen peroxide.

Catalase (EC 1.11.1.6) was discovered in tobacco extracts by Loew (1900). Catalase detoxifies H_2O_2 into water and oxygen (Keilin and Hartree, 1938). Catalase is one of the well described enzyme and it is a class of enzyme including the iron-heme enzyme, catalase-peroxidases and a small group of manganese enzymes (Nicholls, 2012).

Superoxide dismutase (EC 1.15.1.1) is well known enzyme against oxidative stress. SOD1, the first superoxide dismutase to be identified, uses free radical as a substrate (McCord and Fridovich, 1969). A metalloenzyme, superoxide dismutase catalyzes the dismutation of superoxide anion ($O_2^{-\bullet}$) to hydrogen peroxide and oxygen, as the first defense line against oxidative stress (Fridovich, 1995). They are also known to exhibit additional peroxidase activity when hydrogen peroxide level is at its large. It has been suggested removal of superoxide anion will reduce SOD1's alternate toxic behaviour (den Hartog *et al*., 2003).

Copper-zinc and manganese SODs scavenge and dismutate superoxide anion in mitochondrial electron transport systems. Guidot *et al*. (1993) demonstrated that a manganese superoxide dismutase deficient yeast thrived in hyperoxia conditions (95% oxygen, 5% carbon dioxide) under the removal of electron transport system. Sturtz *et al*. (2001) characterized copper-zinc SOD1 in baker's yeast at intermembrane space of mitochondria.

Glutathione peroxidase (EC 1.11.1.9) utilizes reduced glutathione (GSH) to decompose hydrogen peroxide (Mills, 1959; Forstrom *et al*., 1978; Ursini *et al*., 1995). This enzyme was discovered by Mills (1957), and identified as selenocyseteine enzymes at first (Rotruck et. al., 1973 and Chance *et al*., 1979), better known as GPx1. The three consecutive selenocysteine GPxs - GPx2, GPx3 and GPx4 were found by Chu *et al*. (1993), Takahashi *et al*. (1987) and Ursini *et al*. (1985) respectively from mammals. Later Ghyselinck and Dufaure (1990) discovered a catalytic cysteine residue on rat, known as GPx5, and followed by GPx6 (Dear *et al*., 1991), which is a selenocysteine proteins in humans but not in rats or mice (Kryukov *et al*., 2003). Mammalians GPx7 and GPx8 were the last to be elucidated but have a low GPx activity (Brigelius-Flohe and Maiorino, 2012).

Peroxiredoxins (EC 1.11.1.15) is another group of enzyme worth mentioning when discussing about oxidative stress in cellular organisms. Discovered by Kim *et al*. (1988), peroxiredoxins are a family of antioxidants enzymes. Highly specific in reducing hydrogen peroxide (Peskin *et al*., 2007), its cysteine residue makes up the active site of peroxiredoxins, which in turn being oxidized to sulfenic acid and recycled back to thiol, via sulfiredoxins (Woo *et al*., 2005). They also control cytokine-induced peroxide levels which in turn, mediates signal transduction in mammalian cells (Hofmann *et al*., 2002).

1.8 Cysteine residues, thiol proteins and its redox mechanism and modification during oxidative stress

Cysteine residues, the only sulfhydryl/thiol (−SH) - bearing amino acid, has a number of unique properties. Thiol group deprotonates itself into thiolate groups, with the pKa of 8.5 (Poole, 2014) (Fig 1.3). Thiol active site of this residue have high reactivity, unique redox properties, and notably −SH are able to coordinate metal ions to form key catalytic components of enzyme on the active site (Barford, 2004). In an evolutionary

genetics point of view, cysteine is one of the four most conserved amino acid residue within proteins (Marino and Gladyshev, 2010), highlighting its importance in protein functions, thus triggering its selection pressure favouring the preservation of this residue.

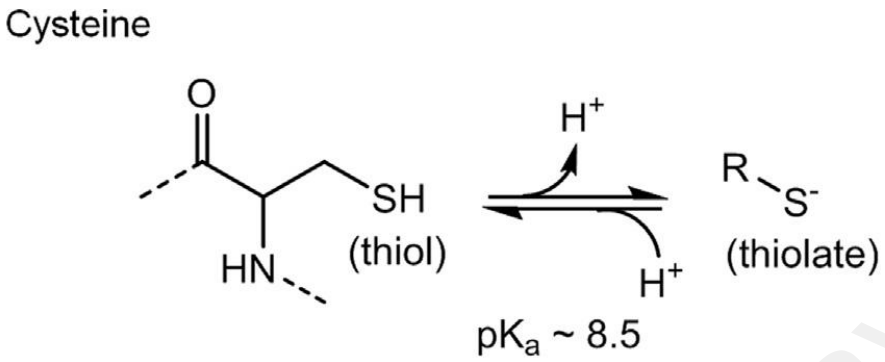


Figure 1.3: The reversible deprotonation of cysteine thiol side chain to thiolate, and protonation vice versa. Adapted from Poole (2015).

Due to the highly reduced environment in the cytoplasm (mammalian and bacteria) (Fahey, 1977; Mallick, 2002), cysteines residues exist in free thiols. The first-off oxidation by hydrogen peroxide on a sulfhydryl group would be to a sulfenic group (−SOH) (Luo *et al*., 2005). Such two electron oxidation of the sulfenic acid is reactive, and short lived (Poole *et al*., 2004). This regenerated moiety raised the interest of a possible involvement in redox modification, where it undergoes reversible and irreversible thiol modifications with reactive oxygen species (Ghezzi, 2005).

Further irreversible oxidation of the sulfenic oxysulfur acid group give rise towards sulfinic ($-SO_2H$) and sulfonic acid ($-SO_3H$) (Kice, 1980). Hamann *et al*. (2011) estimated 5% of cysteine residues in protein occur in sulfinic and sulfonic form.

There is one exception in the irreversible oxidation department. Human inactivated 2-Cys peroxiredoxins due to sulfinic acid oxidation were able to reduce by sulfiredoxins (Jönsson *et al*., 2005).

Glutathione disulfide (GSSG) and free glutathione (GSH) couple indicated the redox condition in the cell as they form mixed disulfide to reduce and return protein thiols (Schafer and Buettner, 2001). Similarly, in the event of oxidative stress, protein thiols can conjugate with GSSG in the process of S-glutahionylation. However, the cellular redox state would have been in an extremity to promote such conjugation (Gallogly and Mieyal, 2007).

Gilbert (1984) discovered that under oxidative stress, S-glutathionylated protein made up to 20 – 50% of total glutathione content. During respiratory burst in neutrophils, 17% of the GSH might be protein-bound (Seres *et al*., 1996). In the two-electron pathway, sulfenic acid moiety undergoes S-glutathionylation with free GSH (Nagy and Ashby, 2007) as shown in Equation 1.

$$\text{Protein–SOH} + \text{GSH} \rightarrow \text{Protein–SSG} + H_2O \qquad (1)$$

Reactive sulfenic ions also form intermolecular or intramolecular disulfide bond with other proximate protein-thiols. This has been shown by few protein biomarkers e.g. OxyR transcriptional factor bacteria (Zheng *et al*., 1998) and Hsp33 (Jakob *et al*., 1999). Sulfenylamide was demonstrated to undergo S-glutathionylation by van Monfort *et al*. (2003). Although there are evidences of S-glutathionylation during oxidative stress, the mechanism *in vivo* is still yet to be understood (Dalle-Donne *et al*., 2009).

Sulfenic acid is further oxidized to sulfenamide with other amino acids in proximity as demonstrated by Salmeen *et al*. (2003) and van Monfort *et al*. (2003) in protein tyrosine phosphatase 1B.

The oxidative thiol modification described above is summarized in Figure 1.4.

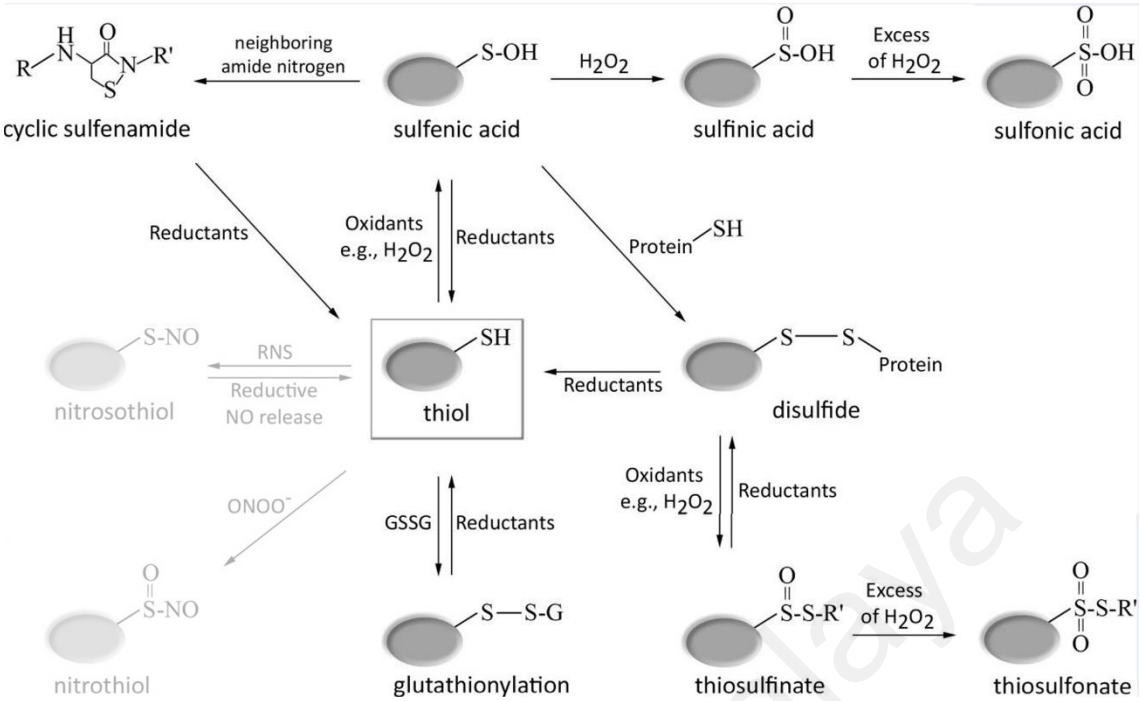


Figure 1.4: Oxidative thiol modification. Adapted from Brandes *et al.* (2009) (The reactive nitrogen species modification were masked as not in the context of the review)

Thiol modified proteins are involved in a series of physiological process in living things as a whole, such as metabolism, signalling, cell growth, gene expression, transcription factor activation, differentiation, senescence and apoptosis (Brandes *et al*., 2009).

In the protein expression department, Yap1p is an AP-1 like transcription factor in *Saccharomyces cerevisiae*, which play a vital role in the regulation of multiple cellular processes, including proliferation, differentiation, stress response, and apoptosis (Azevedo *et al*., 2003). In yeast, Yap1p is the regulator for over 70 genes for most of the antioxidant enzymes and component of the cellular thiol reducing pathway such as thioredoxins, glutaredoxins, glutathione peroxidase, superoxide dismutase and catalase (Carmel-Harel *et al*. (2001), Inoue *et al*. (1999), Kuge and Jones, 1994). This particular transcription factor activates during oxidative stress conditions and reduced during non-

stress conditions. Thiol peroxidase is the hydroperoxide sensor to activate the Yap transcription factor as further demonstrated by Delaunay *et al*. (2002).

Thiol proteins also shows their works from at the cellular metabolism level. One of the notable candidate to mention here is glyceraldehyde-3-phosphate dehydrogenase (GapDH). GapDH sits right at the center of the energy metabolism pathway of glycolysis. GapDH tetramers have highly conserved Cys149 and active site residues at Cys150 (Brandes *et al*., 2007), which are heavily susceptible towards modification upon oxidative stress. At the event of oxidative stress, the highly inhibited GapDH will trigger redirection of glucose-6-phosphate to pentose phosphate pathway (Ralser *et al*., 2007). Pentose phosphate pathway as known is central for NADPH production which in turn increase the restoring capability of thioredoxins and gluthathione reductases. Reduced glutathione and thioredoxin restores GapDH as the oxidative attack subsides (Leichert and Jakob, 2004).

Thiols proteins involved in posttranstional modification as well, for example PTP-1B (protein tyrosine phosphatases-1B), a protein involved in many signal transduction cascades, catalysing dephosphorylation reactions in the cell (Alonso *et al*., 2004). Catalytic cysteine residue Cys215 is present in active site, and modified from sulfenic acid to cyclic sulfenamide on the event of oxidative stress, thus inhibiting the enzyme by altering the structure of the active site (van Monfort *et al*., 2003).

Hsp 33 (Heat shock protein 33) is another common chaperone observed in eukaryotes (Winter *et al*., 2005). After heat shock regulated translation, Hsp 33 is regulated under oxidative stress (Jakob *et al*., 1999). Hsp33's active site contains four cysteines tetrahedrally arranged and coordinating zinc with high affinity (Jakob *et al*., 2000). Oxidation of disulphide bonds in active site release the Zn^{2+} ions and Hsp33 dimer is partially unfolded for binding. When the reducing mechanisms kicks in the Hsp33 dimer is back folded and inactivated (Graf *et al*., 2004).

p53 tumour suppressing factor is inhibited during oxidative stress. Velu *et al*. (2007) discovered that S-glutathionylation in either Cys-124 and -141 will inhibit p53 DNA binding. S-glutathionylation of thimet oligopeptidase in mouse suggestively triggered oligomerization of the enzyme in the event of oxidative challenge (Demasi *et al*., 2008).

In the peroxiredoxins as an example, Georgiou and Masip (2003) discovered that other than the aforementioned reducing and antioxidant features, eukaryotic peroxiredoxins are regulator of H_2O_2-mediated cell signalling in cancer and neurodegenerative diseases.

Human glutaredoxin 1, apart from restoring thiol side chain to reduced form, as discovered to have an influence in apoptosis signalling by binding to apoptosis-signalling kinase-1 (ASK-1) (Song *et al*., 2002). Glutaredoxin 2 exhibits pronounced protective effect on mitochondria mediated apoptosis (Enoksson *et al*., 2005).

Thioredoxins are involved in cellular phenomena such as reduction of ribonucleotide reductase, thioredoxin peroxidase, cell proliferation, thiol-dithiol exchange between cysteine residues of key transcription factors and protection against exogenous oxidants (Watson *et al*., 2004) There are a number of transcription factor which is sensitive to thiols, such as p53, NF-kB, AP-1 and Nrf2, and thioredoxins might act on these transcription at a certain level (Biswas *et al*., 2006).

1.9 Oxidative stress related proteins in *Musca domestica*

Allen *et al*. (1991), determined the activities of possible candidates of oxidative stress defense: superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione S-transferases (GST), GSSG reductase, thiol transferases, gamma glutamylcysteine synthetase, and glucose-6-phosphate dehydrogenase. The team looked in to the concentrations of H_2O_2 and reduced and oxidized glutathione as well across the various

developmental stage of houseflies as well, and revealed there was a massive change in H_2O_2 level towards pupation. Their work discovered that housefly, from egg to adult, increases in terms of cellular oxidizing equivalents and loses ground of cellular reducing capacity. Such work helped to raise our attention in looking into the proteome of the larvae.

Oxidative stress hypothesis is evident on aging and always been raising questions from researchers. *Musca domestica* (Yan *et al*., 2000), *Drosophila melanogaster* (Magwere *et al*., 2006 and Rebrin and Sohal, 2006), and *Caenorhabditis elegans* (Leiers *et al*., 2003) are made as model tested on hyperoxia conditions. Aging is resulted from oxidative damage from cellular macromolecules (Magwere *et al*., 2006). Sohal and Weindruch (1996) stated that the main prediction of this hypothesis is the rate of aging cannot be slowed down without corresponding attenuation of oxidative damage/stress. Though this research has no interest of unlocking secret of longevity, the models above will leave an important reference on understanding how oxidative stress will fare in *Musca domestica* larvae.

GSTs gene family and their isoforms have been discovered to participate in oxidative stress pathway. Overexpression and peroxidase activity of GSTs in peroxide treatment were observed (Veal *et al*. 2002). Thiol oxidoreductase activities of an GSTO-1 isoform were shown during insecticides treatment (Burmeister *et al*., 2002). Li *et al*. (2013) discovered in carcinoma cells, overexpression of GSTs increased resistance to insecticides. Other than oxidative stress resistance, GSTs detoxifies xenobiotics, protects from tissue-damage, participates in Jun-kinase signaling pathway, and as non-catalytic carrier proteins (ligandins) in the intracellular transport of hydrophobic compounds (Ketterer, 1998; Strange *et al*., 2000; Eaton and Bammler, 1999; Yin *et al*., 2000). Glutathiones (GSHs) are responsible in the antioxidant defense as the dominant non-protein sulphydryls in the cell (Ketterer, 1982), forming conjugates non-enzymatically or

more by the catalysis and mediation of GSTs. H_2O_2 oxidizes thiolate group in cysteine residues (-S-) into thiols (-SOH), which is present in the exposing active site.

Reaction against peroxidants is also energy consuming due to inhibition of oxidative phosphorylation (Milatovic *et al*., 2006), deprives energy to maintain the recycling of NADPH during pentose phosphate pathway and glucose 6-phosphate dehydrogenase, making cells hyperglycemic (Rahimi and Abdollahi, 2007) and able to topple the condition of cell redox levels in levels of lactate/pyruvate ratio (Lukaszewicz-Hussain *et al*., 1997). Most of the cases above were investigated towards organophosphates and pyrethroids.

Tang *et al*. (2012) have been successfully characterizing and identifying two novel superoxide dismutase genes from *Musca domestica*. In cadmium ion treatment, concentration ranging from 0.2 to 5mM in the medium, widely known to enhance reactive oxygen species in cell increases the levels of superoxide dismutase (Dabas *et al*., 2012).

Simmons *et al*. (1987), documented the activity of glutathione peroxidase for the first time in houseflies and in insects. Lowering the intake of selenium via diet increases the events of a peroxidative injury. Simmons *et al*. (1989), further purified the selenium-independent glutathione peroxidase, and suggested this enzyme and the related pathways should be in the picture during the investigation of insect antioxidant defense system.

There was no direct research work on peroxiredoxins with relation to houseflies and its mechanisms and activities *in vivo* are not much of knowledge.

However, Simmons *et al*. (1987) discovered in their work that there was no increase in catalase activity even though the diet of selenium in *Musca domestica* was lowered. Another investigation by Allen *et al*. (1983) in houseflies revealed that total inhibition of catalase also did not affect the survival of the flies, although slight increase in the level of SOD activity were observed.

Hence in this experiment we hope through proteomic means we recover more markers related to protein response to oxidative stress and signaling in *Musca domestica* larvae.

1.10 Other genomic and transcriptomic works

Despite such remarkable immunity and rising insecticide tolerance exhibited by *Musca domestica*, and being such prominence as model for biochemistry and insect physiology, no genome project has been launched till 2009 (Scott *et al*., 2009). More importantly, in the best of our knowledge, only a handful of *Musca domestica* related proteomics work has been reported as well.

However, in this last 5 years there is an increasing interest unravelling the inner molecular workings of this insect. A genome project was launched and efforts coerced by Scott *et al*. in 2010 successfully in 2014, sequencing the full genome of *Musca domestica*.

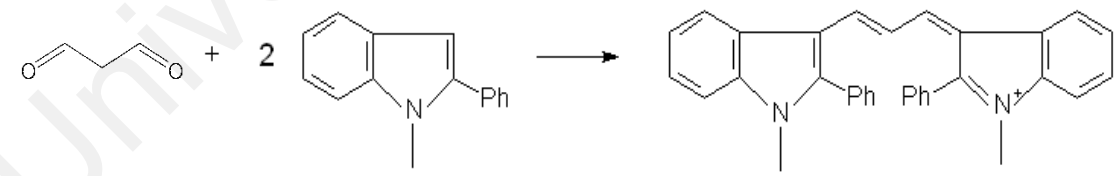
The sequenced genome is 691 MB, some gene sequences notably 771 putative immune-related, 86 CYP450s-related, 33 glutathione S-transferase (GST), and 92 are predicted to have esterase activities. In comparison, this genome contained a plethora of shared and novel sequences than its *Drosophila* counterparts, supporting the fact of an exemplary ability of *Musca domestica* of associating closely with numerous amounts of pathogens and living in a septic environment.

Pioneering transcriptomic works have been done on *Musca domestica* larvae, by massive cDNA parallel pyrosequencing by Liu *et al*. (2012). The unique sequences are compared with Swiss-PROT and NCBI non-redundant protein database. A gene ontology map was done based on the sequence similarity, classified into 14 major biological processes. Liu *et al*. has successfully found a peptidoglycan recognition protein SC (PGRP-SC), a protein important in sensing microbial infection involving pathways such as Toll-like receptor and Imd (immune deficiency).

With the aid of qPCR technology, an up-regulation of expression was shown during *Staphylococcus aureus* and *Escherichia coli* infection. Liu *et al*. also suggested potential antioxidant enzymes and proteins, such superoxide dismutases, glutathione S-tranferases, glutathione peroxidases and glutathione reductases, can be unravel in the future. Such findings, although is only one of many to come, it has opened the door in proteomics investigation especially in protein identification based on the sequence databases.

1.11 Lipid peroxidation detection

Lipids are the most susceptible during peroxidant attack in cellular levels. And malondialdehyde (MDA) is the by products produced during an oxidative event (Esterbauer and Cheeseman, 1990), due to attack of free radical species on polyunsaturated fatty acids of cellular membrane (Alessio ,2000). For the detection of the lipid peroxidation markers i.e. in this case malondialdehyde and 4-hydroxynonenal, reacts with 1-methyl-2-phenylindole to form a stable cyanin chromophore with a maximal absorbance at 586nm is used (Gerard-Monnier *et al*., 1998).



Malondialdehyde 1-methyl-2-phenylindole cyanin chromophore

Figure 1.5: Reaction of malondialdehyde with 1-methyl-2-phenylindole forming a stable cyanin chromophore with maximum absorbance at 586nm.

1.12 Purification of thiol proteins.

The condition of −SH and −S groups in the cysteine-containing protein is a good measurement on how *Musca domestica* larvae counter oxidative stress in vivo. Activated Thiol Sepharose® 4B (Sigma-Aldrich) is a commercially available resin. It binds covalently to thiol groups by employing a thiol-disulfide interchange between protein thiol groups and the glutathione-2-pyridyl-disulfide conjugate of the affinity resin (Caldas *et al*., 1998). The particular resin for the activated group anchoring is CNBr-activated Sepharose 4B. Hu *et al*. (2010), worked on activated thiol sepharose to select thiol-containing proteins from control- and menadione-treated *Escherichia coli*.

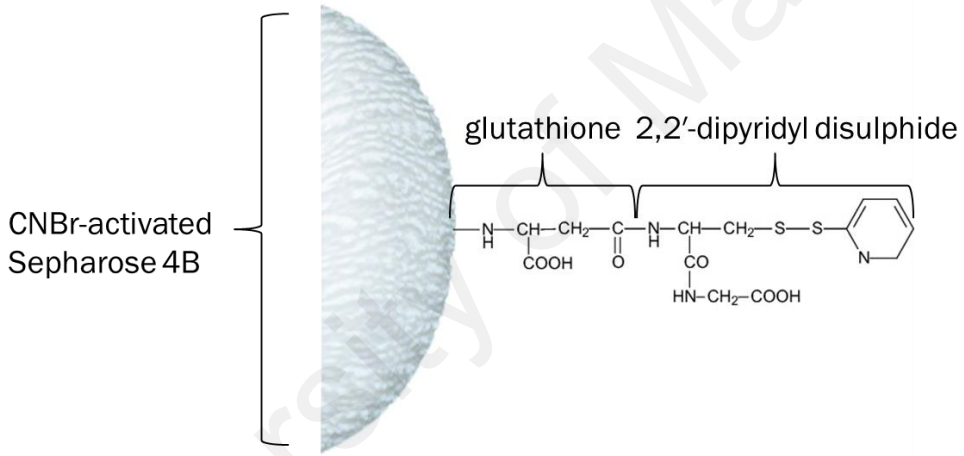


Figure 1.6: The structure of Activated Thiol Sepharose® 4B (Sigma-Aldrich)

1.13 Proteomics, two dimensional gel electrophoresis and LC-MS/MS identification

Proteome, described as the entire set of proteins expressed by a genome, cell, tissue or organism, more specifically, a set of expressed proteins in a given type of cells or an organism at a given time under defined conditions. (Wasinger *et al*., 1995)

2-dimensional gel electrophoresis was described simultaneously by O' Farrell (1975) and Klose (1975) with iso-electric focusing for separation of protein based on pI (1st dimension) and second separation is done with SDS-PAGE electrophoresis based on molecular weight (2nd dimension).

Two-dimensional gel electrophoresis is a stalwart in proteomics until today (Victor *et al*., 2007). Godovac-Zimmermann (2000) also supported the prowess of proteomics analysis and stated the potential of detecting modifications such as splice variation, proteolytic processing, and post-translational phosphorylation.

LC-MS/MS identification utilizes a high performance liquid chromatography (HPLC) coupled with tandem mass spectrometry to identify proteins. Technique of detection of peptide masses as pieces of puzzle to construct a protein thus identifying it is coined as "peptide mass fingerprinting". Tryptically digested peptide fragment were separated with HPLC before being ionized and detected in mass spectrometer. By searching through the MS/MS spectra alongside optimizing the acquisition of data (flow rate, ionization energy, spectral counting etc.), the identity of the protein can be deduced from the search score of the peptides (Chen and Pramanik, 2009)

1.14 Objective

In this current investigation, we hope to achieve the following goals:

1. To analyze the proteome of *Musca domestica* larvae.
2. To analyze differential expression of the proteome under peroxide treatment.
3. To investigate differential expression of the purified thiol proteome.

CHAPTER 2: MATERIALS AND METHOD

2.0 Materials

2.0.1 Chemicals

10X Tris/Glycine/SDS Running Buffer (Bio-Rad), 1-methyl-2-phenylindole (Sigma Aldrich), 2-iodoacetamide (Merck Chemicals), 30% acrylamide/bis-acrylamide 29:1 (Bio - Rad), 30% hydrogen peroxide (Systerm), 37% HCl (Systerm), Acetone (Systerm), Acetonitrile (Systerm), Activated Thiol–Sepharose® 4B (Sigma Aldrich), Agarose (Bio-Rad), Ammonium persulphate (Bio-Rad), Ammonium sulphate (Systerm), BenchMark™ Unstained Protein Ladder (Invitrogen), Bromophenol Blue (Bio-Rad), Carrier ampholytes pH 3-10 (Bio-Diagnostics Sdn. Bhd.), CHAPS (Merck Chemicals), Coomassie Brilliant Blue R-250 (Bio – Rad), DTT (Bio Rad), EDTA (Sigma Aldrich), Glycerol (Systerm), Immobiline™ Drystrips, pH 3-10 NL (GE Healthcare), Methanol (Systerm), N, N, N', N'-tetramethyl-ethane-1,2-diamine (TEMED) (Bio-Rad), Phenylthiourea (Sigma-Aldrich), Phosphoric acid (Merck Chemicals), PlusOne Drystrip Cover Fluid (GE Healthcare), Protease inhibitor (Sigma-Aldrich), Ramy Feeds (Bengy), SDS (Bio-Rad), Sodium chloride (Systerm), Sodium dihydrogen phosphate (Systerm Malaysia), Thiourea (Sigma Aldrich), Tris-Base (Bio-Rad), Urea (Sigma Aldrich)

2.0.2 Buffers

Binding buffer solution (0.1M Tris-HCl pH 7.5, 0.5M NaCl, 1mM EDTA)

0.1M Tris-HCl pH 7.5, 0.5M NaCl, 1mM EDTA

1.5M Tris-HCl buffer, pH 8.8 (Bio-Rad)

0.5M Tris-HCl buffer, pH 6.8 (Bio-Rad)

Solubilizing buffer (8M urea, 4% CHAPS, 65mM DTT, 3M thiourea)

Rehydration solution (8M urea, 2% CHAPS, 0.2%(w/v) DTT, 2% carrier ampholytes (pH 3-10), 30mM thiourea)

Equilibration buffer 1 (1.5M Tris-HCl buffer, 6M Urea, 4.7M glycerol, 2% (w/v) SDS, 16.2mM DTT)

Equilibration buffer 2 (1.5M Tris-HCl buffer, 6M Urea, 4.7M glycerol, 2% (w/v) SDS, 0.243mM 2-iodoacetamide)

Homogenizing buffer (0.1M NaH_2PO_4 buffer, pH 7.5, 1.3mM EDTA, 0.1mM EDTA, 1% protease inhibitor, and trace phenylthiourea)

2.0.3 Kits

ReadyPrep™ Protein Extraction Kit (Cytoplasmic/Nuclear) (Bio-Rad)

2-D Quant Kit (GE Healthcare)

2.0.4 Equipment

Agilent 1200 HPLC-Chip/MS Interface, coupled with Agilent 6520 Accurate-Mass Q-TOF LC/MS (Agilent)

EPS 3500 XL (GE Healthcare)

Centrifuge machine (Eppendorf)

HG-15D homgenizer (WiseTis ®)

Image Scanner III (GE Healthcare)

Laminar hood

Microwave

Mini-Protean II Tetra Cell (Bio-Rad)

Multiphor II (GE Healthcare)

Multitemp III (GE Healthcare)

Shaking incubator

Sonicator

Vortex mixer

Water bath

2.0.5 Software

Labscan (GE Healthcare)

PD Quest 8.0.1 (Bio-Rad)

2.0.6 Insects

Adult *Musca domestica* samples were obtained from Vector Control Research Unit, World Health Organization, Science University of Malaysia, Penang.

2.1 Methodology

2.1.1 *Musca domestica* adult and larvae cultivation

The flies were reared in modified plastic cages with ensured aeration in room temperature. Sugar and milk powder were mixed on a petri dish in a 1:1 (v/v) ratio as a glucose and protein supply to the adult flies. Water was supplied by moist cotton with distilled water. Water, sugar and milk powder were exchanged every week.

For the medium of housefly larvae growth, the Bengy Ramy Feeds hamster pellet was mixed with distilled water in a 1:1.5 (v/v) ratio and left until all water had been absorbed by the pellet and mushy.

The damp pellet was then placed in the cage for 4 hours for the oviposition by female flies to lay eggs in the moist mouse pellet. The pellet laid with *Musca domestica* eggs were then transferred into a new mouse pellet medium and left for 2-3 days for larvae hatching. A three or four day larvae were collected for analysis.

For cultivation of adult larvae, the larvae were left pupating after 4-5 days. The pupae collected were transferred to a new cage ready with water and feed. New flies emerged approximately another 4-5 days upon pupation.

2.1.2 Peroxidant feeding assay on 3rd instar larvae

Approximately 6 g of mouse pellet was mixed with 10ml of solution of different dilutions of 30% hydrogen peroxide ranging from 0 – 30 %. The mouse pellet was left to absorb the hydrogen peroxide solutions until moist and mushy. 15 individuals of 3-day-old larvae was collected, and placed to each medium with different concentrations and left to feed the medium for 48 hours. 3 – day old larvae is selected as it is grown 3rd instar larvae (Kočišová *et al*., 2004)

2.1.3 Determination of toxicity parameters

The number of mortalities of the larvae was calculated and the lethal concentration for 50% (LC_{50}) of hydrogen peroxide treatment of the particular larvae strain as subjected to PROBIT analysis (Finney, 1947) To obtain the larvae which was resistant on LC_{50}, samples which survived were collected after 48 hours of feeding assay as mentioned at 2.1.2. Larvae knockdown and pupae formation was also noted and recorded.

The concentration of the hydrogen peroxide which caused the LC_{50} of the tested larvae population was calculated and used in peroxidant assay for future investigation.

2.1.4 Determination of lipid peroxidation (Tedesco *et al*., 2010)

Approximately 1g of larvae sample was homogenized in homogenizing buffer (0.1 M NaH_2PO_4 buffer, pH 7.5, 1.3 mM EDTA, 0.1 mM EDTA, 1% protease inhibitor, and traces of phenylthiourea) in HG-15D homgenizer (WiseTis ®) at medium speeds. Samples were centrifuged at 3000 × *g* for 20 minutes and then derivatized in 1ml of reaction mixture containing 10.3 mM 1-methyl-2-phenylindole which was dissolved in acetonitrile:methanol with ratio of 3:1 (v/v) with 32% HCl. The unknowns and standard curve are read at absorbance of 586 nm. The malondialdehyde (MDA) standard curve was produced to determine the internal concentration of MDA in nmol/ g wet weight.

2.1.5 Preparation of homogenous protein lysate.

ReadyPrep™ Protein Extraction Kit (Cytoplasmic/Nuclear) from Bio-Rad was used to extract cytosolic proteins from the homogenate. The larvae samples were homogenized manually with a blue propylene pellet pestle (Sigma - Aldrich) in Eppendorf tubes in ice. For every 15 mg of tissue, 0.75 ml of CPEB from the kit (cytoplasmic protein extraction buffer) was added before homogenization.

After homogenizing the larvae, the lysate including pestle alongside with the Eppendorf tubes was incubated in ice for 1-2 minutes to sediment large tissue and cellular fragments. The supernatant was carefully transferred to a new tube.

The cell lysate was centrifuged at 1000 × *g* for 10 minutes at 4 ºC. Upon completion the supernatant, which was the cytoplasmic protein fraction, was transferred to a new tube. The original tube was centrifuged for 5 – 10 seconds at 1000 × *g* again and pooled with the protein fraction above.

The prepared cytoplasmic sample protein fraction lysate was later pipetted into aliquots of 50 µl, labeled and stored in PCR tubes, in -80 °C refrigerator until further analysis.

2.1.6 Thiol protein purification (Hu *et al*., 2010)

Protein extract with total protein content of 2.5 mg after peroxidant feeding was reacted with 5 M urea for 10min at room temperature. 20 mg of Activated Thiol–Sepharose® 4B and 200 µl binding buffer solution (0.1 M Tris-HCl pH 7.5, 0.5 M NaCl, 1 mM EDTA) was added. The samples were incubated on ice for 1.5 hours and the mixture is shaken every 15 minutes to ensure complete binding. The samples were washed with 500 µl binding buffer solution 8 times and centrifuged at 11000 × *g* for 3 minutes for each wash. Supernatants were discarded to remove any unbounded proteins. 200 µl buffer, containing 25 mM DTT, was added to sample after the final washing step and incubated on ice for 1 hour with gentle shaking every 15 minutes to release all thiol-containing proteins bound to activated thiol sepharose. The sample was centrifuged at 11000 × *g* for 3 minutes and thiol-containing proteins collected in the supernatant.

2.1.7 Protein quantitation

Protein quantitation of the sample lysate prior to 2-dimensional gel electrophoresis for both total proteome and thiol protein purified samples was done by using GE Healthcare's 2-D Quant Kit.

Copper (II) ions binds towards the protein polypeptide backbone. Protein detection via this kit is based on the principle of binding of a colorimetric agent which binds towards the unreacted cupric ions in an alkaline solution. Hence the color intensity was inversely correlated with the protein content. The protein was mixed with a precipitant and co-precipitant to ensure effective binding on the cupric ions.

The standard curve was obtained by plotting the absorbance of the standard bovine serum albumin provide in the kit against the quantity of the protein. A bovine serum albumin solution of 2 mg/ml was diluted into protein content ranging from 0µg to 50 µg. 500 µl of precipitant was added to each tube, vortexed and incubated for 2-3 minutes at room temperature. 500 µl of co-precipitant was later added and vortexed. The tubes were centrifuged for 10,000 × *g* for 5 minutes to sediment the protein. The precipitant and co-precipitant were decanted and removed. 100 µl of copper solution and 400 µl of deionized water was added and vortexed to dissolve the protein precipitate. 1 ml of the working color reagent, prepared by mixing 100 parts of color reagent A and 1 part of color reagent B of the kit was added into each tubes, inverted and incubated in room temperature for 15-20 minutes. Absorbance of 480 nm was read for each tubes with water as reference.

To determine the protein content of the larvae protein lysate, aliquots of lysate in triplicates were prepared using the kit as described above and the absorbance were read in 480 nm. The content of the protein was determined by comparing the absorbance value on the standard curve.

2.1.8 Two dimensional gel electrophoresis analysis

40 µl of protein lysates was dissolved in 40 µl of rehydration solution (8 M urea, 2% CHAPS, 0.2% (w/v) DTT, 2% carrier ampholytes (pH 3-10)). The mixture of was later mixed with 45µl of solubilizing buffer (8 M urea, 4% CHAPS, 65 mM DTT, 3 M thiourea).

The protein lysates were then loaded into 7 cm Immobiline™ Drystrips, pH 3-10 NL IPG strip (GE Healthcare) in a rehydration tray. Handling of IPG strips was done with forceps sterilized with acetone. The IPG strips were left overnight for the passive rehydration to take place for 18 hrs.

The strip after rehydration containing the protein sample were run for isoelectric focusing on the Multiphor III (GE Healthcare) under the voltage setup:

Step 1(Gradient): Voltage: 200 V; Time: 1 minute; Current: 5 mA; Power: 2 W.

Step 2(Gradient): Voltage: 3500 V; Time: 1.5 hours; Current: 5 mA; Power: 2 W.

Step 3(Gradient): Voltage: 3500 V; Time: 1.5 hours; Current: 5 mA; Power: 2 W.

The temperature of the focusing was controlled by the thermostatic circulator Multitemp III (GE Healthcare) at 16 °C, and the power supply was provided by EPS 3500 XL (GE Healthcare). The isoelectric focusing setup was covered in PlusOne Drystrip Cover Fluid (GE Healthcare).

Equilibration buffer 1 (1.5 M Tris-HCl buffer, 6 M Urea, 4.7 M glycerol, 2% (w/v) SDS, 16.2 mM DTT) and equilibration buffer 2 (1.5 M Tris-HCl buffer, 6 M Urea, 4.7 M glycerol, 2% (w/v) SDS, 0.243 mM 2-iodoacetamide) was prepared.

The IPG strips were shaken in a container containing for 2.5 ml equilibration buffer 1 for 15 minutes and then equilibration buffer 2 for another 15 minutes.

SDS-PAGE was done using the Mini-Protean II Tetra Cell (Bio-Rad). The casting of the gels was done according to the Bio-Rad's manual. All the mixture was sonicated for 15 minutes in degassing mode. Table 2.1 describes the SDS-PAGE gel formulations.

Table 2.1: The SDS-PAGE gel formulations.

Solution	4% Stacking Gel (ml)	12% Resolving Gel (ml)
30% acrylamide/bis-acrylamide (29:1)	3.3	4.3
1.5 M Tris-HCl buffer pH 8.8	-	2.5
1.5 M Tris-HCl buffer pH 6.8	2.5	-
Deionized water	15	3.1
10% (w/v) SDS	0.25	0.1
TEMED	0.005	0.005
10 % (w/v) Ammonium Persulphate	0.125	0.05

To prepare the gel, the resolving solution was quickly loaded into the spaces between plates until the level reaches the green mark before overlaid with distilled water and left to polymerize. When the resolving gel layer was fully polymerized, the water was removed and the water on top of the gel surface was drawn up with filter paper. The stacking gel were poured until the plate level and covered with gel comb and left to polymerize.

The agarose overlay solution was prepared by dissolving 0.5 g was dissolved in 100 ml SDS-PAGE electrophoresis buffer. The IPG strips after equilibration was placed on top of the stacking gel and sealed with the agarose overlay solution above. Extra bubbles were pressed out.

The electrophoresis process was performed as described by the instructions of the Mini-Protean II Tetra Cell (Bio-Rad). The electrophoresis power pack was switched on and the voltage was set to 150 V. Electrophoresis was performed in a descending manner with 1 X SDS-PAGE running buffer (Bio-Rad). BenchMark^{TM} Unstained Protein Ladder (Life Technologies) was concurrently run as standards for estimating molecular weight of protein spots. After the dye front reaches on the bottom of the glass plate, the current was stopped and the power pack was switched off.

After SDS-gel electrophoresis, the gel was removed and stained with Coomassie Brilliant Blue R-250 staining solution (5% (w/v) Coomassie Brilliant Blue, 85% ortho-phosphoric acid and ammonium sulphate). The staining was done placing on orbital shaker for 4 days. After 4 days, the gels were destained with destaining solution. The destaining solution was prepared by 80 ml of distilled water to 20 ml of methanol. The destaining of gel was done for 1-2 days until the gel background was clear and clear spots were observed.

2.1.9 Differential expression analysis

The gel images were obtained from Image Scanner III (GE Healthcare) and Labscan software (GE Healthcare) and the spot analysis were done in PDQuest software (Bio-Rad). The gels replicates, in triplicates and analysis batches were labelled according to name and date.

Spot detection were done in Gaussian distribution. Spot intensities with moderate fold change (2 times to 5 times with 90% significance in t-test), high fold change (more than 5 times with 90% significance in t-test) and presence of spots were picked. Mr-pI values of the picked protein were identified after the Mr-pI grid was drawn on the graph.

The gels containing desired spots were preserved, pooled over replicates if necessary, for the gel spot excision and protein identification via LC-MS/MS.

2.2.0 Spot excision, in-gel digestion, and LC-MS/MS identification by peptide sequencing.

The instrument that were used in this study was Agilent 1200 HPLC-Chip/MS Interface, coupled with Agilent 6520 Accurate-Mass Q-TOF LC/MS+ ESI.

The gel plugs were destained using 15 mM potassium ferricyanide in 50 mM sodium thiosulphate pentahydrate until they were transparent. They were further reduced and alkylated using 10 mM DTT in 100 mM ammonium bicarbonate and 55 mM iodoacetamide in 100 mM ammonium bicarbonate, respectively. Then, the gel plugs were washed with 50% acetonitrile in 100 mM ammonium bicarbonate and 100% acetonitrile and followed with dehydration using vacuum centrifugation. The dried plugs were incubated overnight in 25 µl of 6 ng/µl trypsin in 50 mM ammonium bicarbonate at 37 ^{o}C. Finally, the peptides were dried using a vacuum centrifugation and prepared for ZipTip® desalting and MS analysis.

The samples were then applied into LC-MS/MS. The Information were collected and analysed by Agilent Spectrum Mill MS Proteomics Workbench software. The database selected aims at current *Musca domestica* species database (NCBI and SwissProt) and ALL species if the specific species database returns no significant search. The selection of protein entries was based by the notion that the identified entries' MW to be closest to the approximated MW in the 2D gels, had the highest percentage of amino acid coverage in their respective entry lists.

CHAPTER 3: RESULTS

3.1 Probit analysis of peroxide feeding assay.

The probit analysis of peroxide treatment of 3rd instar *Musca domestica* larvae (n=300) was performed and the probit graph was plotted. To figure out the LC_{50}, the $\log_{10}$ of concentration of probit number of 5 was determined. The LC_{50} of 3rd instar larvae were calculated as 21.52% (v/v) hydrogen peroxide.

3.2 Lipid peroxidation determination

The malondialdehyde concentration for control and treated larvae samples was calculated from the standards and they were 1.040±0.612 nmol/ g wet weight and 11.924±4.528 nmol/ g wet weight of 3rd instar larvae respectively.

3.3 Two dimensional gel electrophoresis

Larvae grown in normal feed were labelled as "control" samples and larvae grown in feed with hydrogen peroxide were known as "treated" samples. For larvae total proteome analysis, an estimated amount of 180.30 µg and 193.91 µg of protein were loaded into each control and treated samples respectively. Thiol protein purification yielded 94.407 µg and 94.659 µg of protein respectively for loading into each control and treated gels.

After analysis of visualized gel in PDQuest, a total of 294 spots were present across control and treated proteome gels. For thiol proteome, 125 spots were found across the gels.

3.3.1 Proteome expression profile

3.3.1.1 Protein spots with moderate fold changes

Based on our analysis in the total proteome of 3rd instar housefly larvae during hydrogen peroxide treatment, spots with fold change values from 2 to 5 were considered as the protein with moderate fold change.

In the proteome samples, it was discovered that 17 spots have a moderate fold change in the control samples. On the other hand, 23 spots changed moderately in hydrogen peroxide-treated samples.

Out of 17 spots of abundance in proteome control samples, 10 had a fold change in abundance between 2 to 3 times. 3 protein spots were discovered to be between 3 to 4 times while 4 of them increased in 4 to 5 times in control samples. Results were summarized in Figure 3.1 and Table 3.1 – 3.3.

Meanwhile in the treated samples, out of the 23 spots, 18 of them were more than 2 – 3 times of abundance. 5 spots were found to have fold change between 3 – 4 times (Figure 3.2, Table 3.3 – 3.5).

3.3.1.2 Protein spots with high fold changes

From protein spots which have more than 5 fold of change is considered with high fold changes. A total of 3 protein spots each were more than 5 fold change in control and treated samples each (Figure 3.3 and 3.4). Results were summarized in Table 3.6 and 3.7.

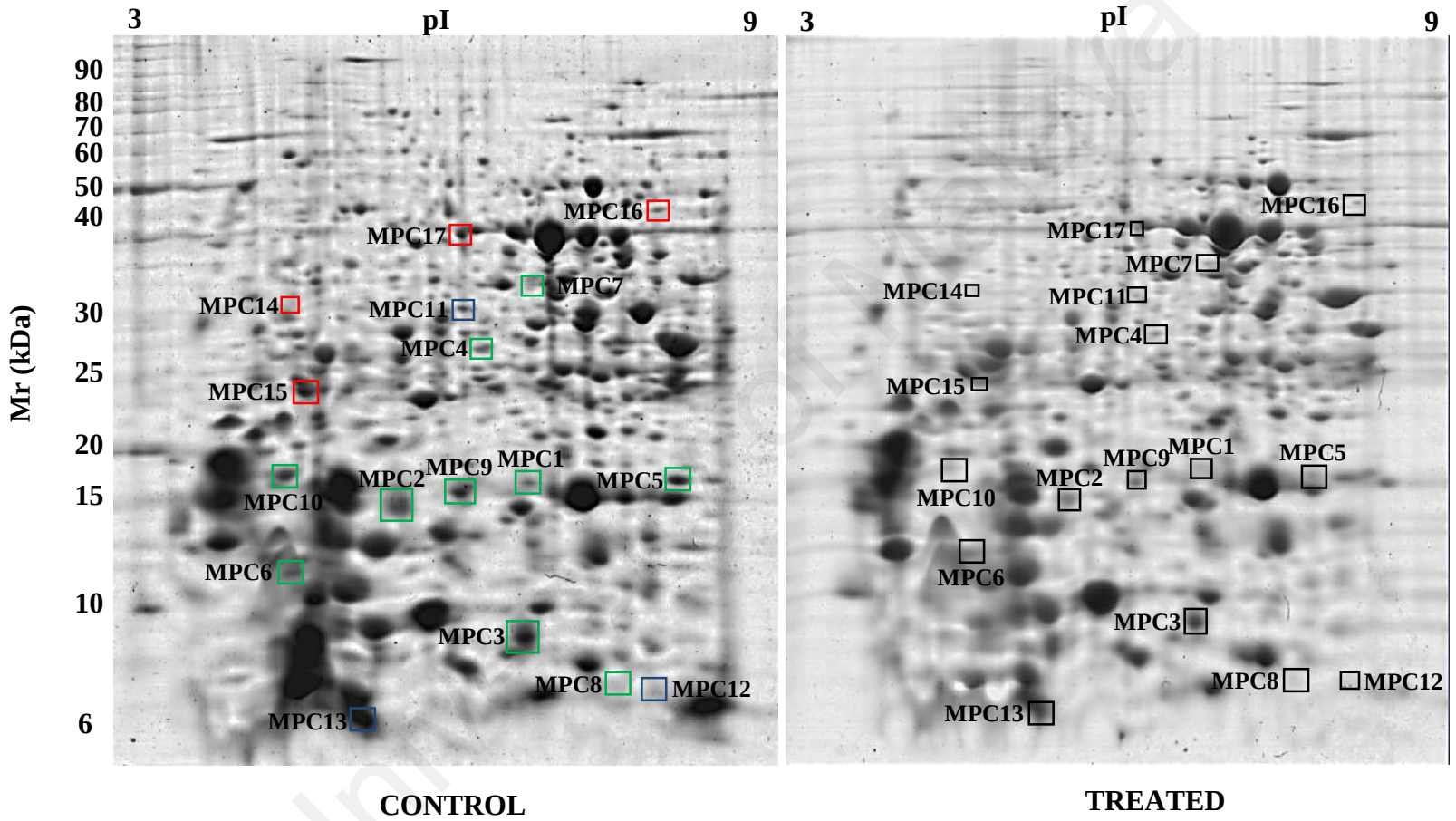


Figure 3.1: Protein spots with moderate fold change in control larvae proteome. Spots boxed in green were in between 2 to 3 fold changes, in blue were in between 3 to 4 fold changes, and in red between 4 – 5 fold change in control samples. Estimated amount of protein loaded in control gel = 180.2955 µg, treated gel =193.9065 µg; Larvae samples were fed in 21.52% (v/v) H_2O_2 in treated samples)

Table 3.1: Summary of protein spots between 2 - 3 fold changes in abundance in control proteome samples. (Note: MPC = moderate proteome control) (Note: All spot intensity values in respective replicates were subjected to t-test and were of 90% confidence; Average spot intensities in replicates with 2 - 3 fold change were shaded.)

SPOT ID	Label	Mr	pI	Average spot intensity		Fold change of control samples
				Control	Treated	
5303	MPC1	15.9	5.9	2618286.33 ↑	1282666.26	2.04
3203	MPC2	14.3	5.3	7211898.67 ↑	3486531.42	2.07
5103	MPC3	8.3	5.9	10168831.00 ↑	4880658.08	2.08
4505	MPC4	27.7	5.7	1216699.19 ↑	533356.81	2.28
8306	MPC5	16.1	8.7	3178349.42 ↑	1381479.15	2.30
1206	MPC6	11.5	4.8	5481872.33 ↑	2361746.38	2.32
6511	MPC7	37.5	5.9	843163.34 ↑	325038.06	2.59
7001	MPC8	6.7	7.2	850803.08 ↑	313346.79	2.72
4301	MPC9	15.1	5.6	5410657.00 ↑	1938733.73	2.79
1302	MPC10	16.6	4.7	3372263.33 ↑	1170814.86	2.88

Table 3.2: Summary of protein spots between 3-4 fold changes in abundance in control proteome samples. (Note: MPC = moderate proteome control) (Note: All spot intensity values in respective replicates were subjected to t-test and were of 90% confidence; Average spot intensities in replicates with 3 - 4 fold change were shaded.)

SPOT ID	Label	Mr	pI	Average spot intensity		Fold change of control samples
				Control	Treated	
4603	MPC11	32.4	5.6	897415.48 ↑	287815.61	3.12
8006	MPC12	6.6	8.0	1832924.13 ↑	561091.80	3.27
2004	MPC13	6.0	5.1	21698006.00 ↑	6033128.50	3.59

Table 3.3: Summary of protein spots between 4-5 fold changes in abundance in control proteome samples. (Note: MPC = moderate proteome control) (Note: All spot intensity values in respective replicates were subjected to t-test and were of 90% confidence; Average spot intensities in replicates with 4 - 5 fold change were shaded.)

SPOT ID	Label	Mr	pI	Average spot intensity		Fold change of control samples
				Control	Treated	
1507	MPC14	35.3	4.8	435566.91 ↑	107874.70	4.04
1404	MPC15	23.4	4.9	5224888.00 ↑	1179168.65	4.43
8708	MPC16	48.8	8.2	628861.37 ↑	137791.29	4.56
4708	MPC17	44.0	5.7	1760356.21 ↑	384355.68	4.58

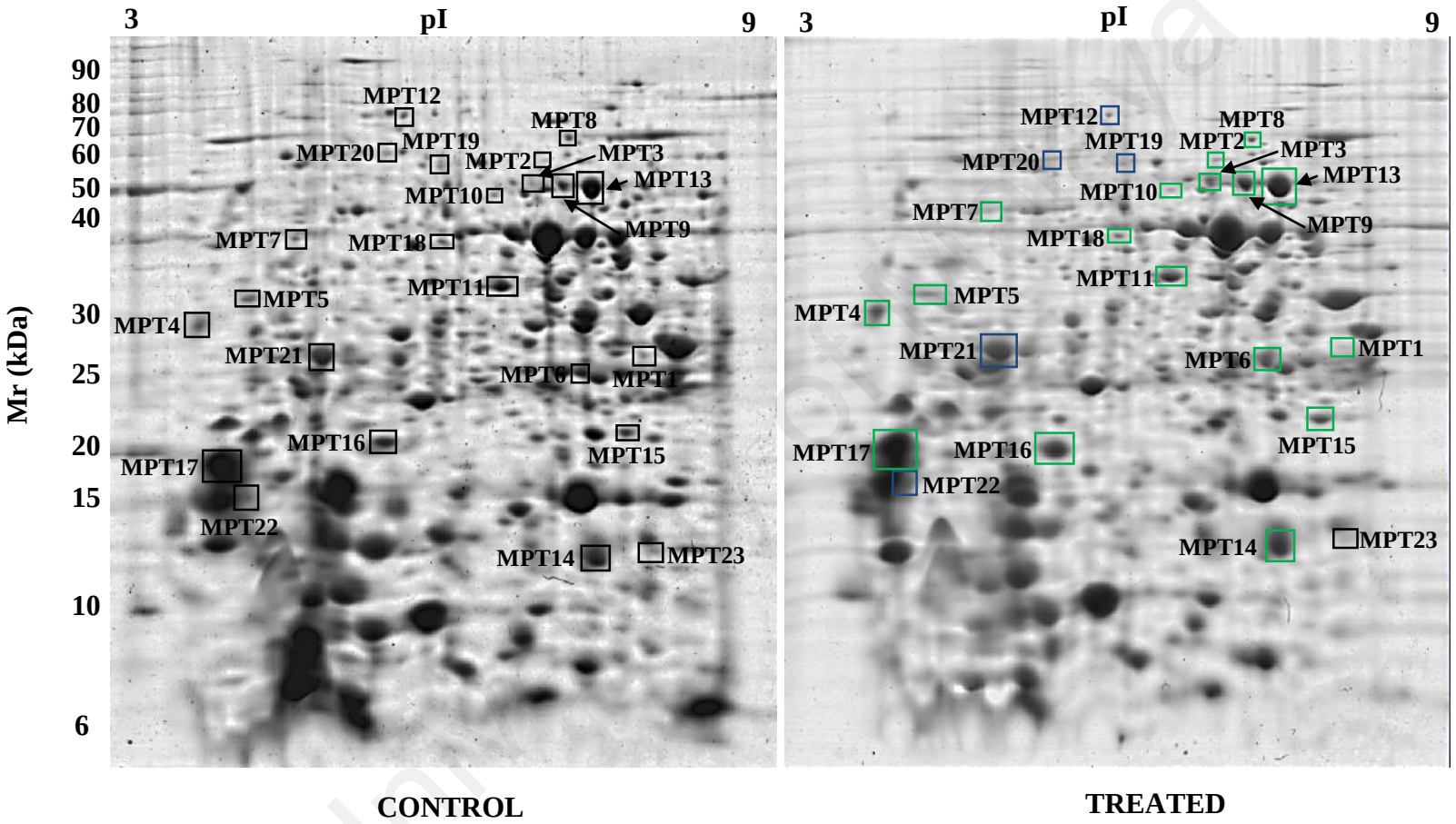


Figure 3.2: Protein spots with moderate fold change in treated larvae proteome. Spots boxed in green were in between 2 to 3 fold changes and spots boxed in blue were in between 3 to 4 fold changes. Estimated amount of protein loaded in control gel = 180.2955 µg, treated gel =193.9065 µg; Larvae samples were fed in 21.52% (v/v) H_2O_2 in treated samples)

Table 3.4: Summary of protein spots between 2-3 fold changes in abundance in treated proteome samples. (Note: MPT = moderate proteome treated) (Note: All spot intensity values in respective replicates were subjected to t-test and were of 90% confidence; Average spot intensities in replicates with 2-3 fold change were shaded.)

SPOT ID	Label	Mr	pI	Average spot intensity		Fold change of control samples
				Control	Treated	
8503	MPT1	27.0	7.9	332350.75	744426.81 ↑	2.24
6903	MPT2	59.9	6.0	100337.58	228788.07 ↑	2.28
5806	MPT3	54.8	6.0	643243.02	1469993.67 ↑	2.29
0502	MPT4	32.9	4.1	1856749.96	4459960.50 ↑	2.40
0605	MPT5	35.5	4.5	775818.53	1892672.83 ↑	2.44
6505	MPT6	25.6	6.8	1412236.46	3569123.83 ↑	2.53
2801	MPT7	50.1	5.0	401092.42	1026868.23 ↑	2.56
6902	MPT8	65.8	6.6	173128.91	443636.33 ↑	2.56
7801	MPT9	53.5	7.0	3402743.58	8804084.00 ↑	2.59
5801	MPT10	52.4	5.8	146313.59	382731.04 ↑	2.62

Table 3.4 Summary of protein spots between 2-3 fold changes in abundance in treated proteome samples. (cont'd)

SPOT ID	Label	Mr	pI	Average spot intensity		Fold change of control samples
				Control	Treated	
5601	MPT11	36.0	5.8	2010372.29	5269147.67 ↑	2.62
4901	MPT12	72.3	5.5	155872.27	415449.03 ↑	2.67
6803	MPT13	54.3	6.5	952986.23	2660050.17 ↑	2.79
7202	MPT14	11.9	7.0	4631567.55	13112680.00 ↑	2.83
8301	MPT15	19.7	7.5	926789.73	2696738.83 ↑	2.91
2304	MPT16	18.4	5.3	2906423.00	8503007.17 ↑	2.93
0307	MPT17	18.0	4.2	14134637.33	41382388.00 ↑	2.93
4707	MPT18	41.7	5.6	330210.54	985959.11 ↑	2.99

Table 3.5: Summary of protein spots between 3-4 fold changes in abundance in treated proteome samples. (Note: MPT = moderate proteome control) (Note: All spot intensity values in respective replicates were subjected to t-test and were of 90% confidence; Average spot intensities in replicates with 3-4 fold change were shaded.)

SPOT ID	Label	Mr	pI	Average spot intensity		Fold change of control samples
				Control	Treated	
4803	MPT19	58.8	5.6	264865.66	846104.75 ↑	3.19
3801	MPT20	61.1	5.3	2719612.00	951863.98 ↑	3.50
2502	MPT21	26.8	5.0	3336025.67	11889442.67 ↑	3.56
0208	MPT22	15.1	4.3	3793287.54	13845978.00 ↑	3.65
8205	MPT23	12.0	8.0	282288.96	1067930.40 ↑	3.78

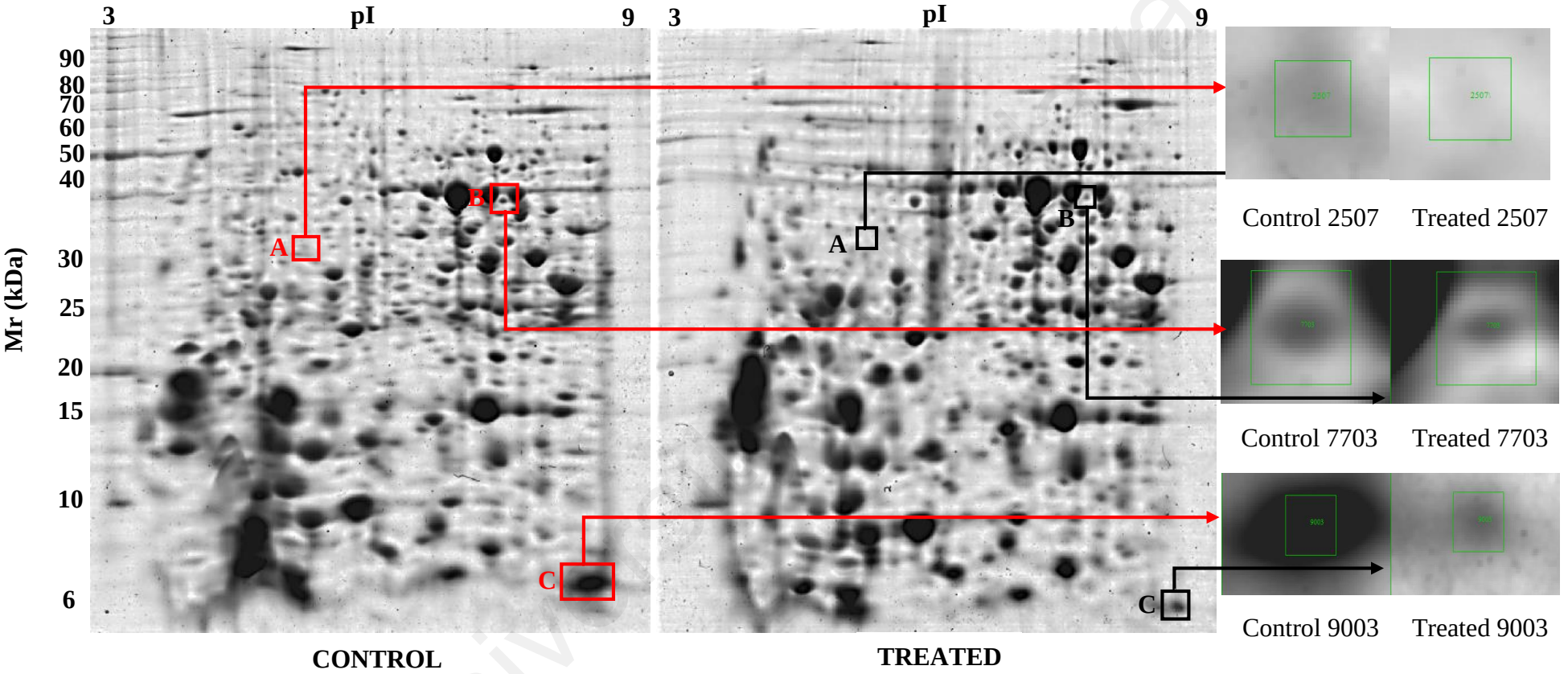


Figure 3.3: Spot position of proteome expression profile on more than 5 fold change in control samples of *Musca domestica* 3rd instar larvae. (Note: Spots more than 5 fold change are marked in red, whereas its lower fold counterpart is marked in black; Estimated amount of protein loaded in control gel = 180.2955 µg, treated gel =193.9065 µg; Larvae samples were fed in 21.52% (v/v) H_2O_2 in treated samples)

Table 3.6: Summary of proteome expression profile on more than 5 fold change in control samples of *Musca domestica* 3rd instar larvae. (Note: All spot intensity values in respective replicates were subjected to t-test and were of 90% confidence; Average spot intensities in replicates with more than 5 fold change were shaded.)

SPOT ID	Label	Mr	pI	Average spot intensity		Fold change of control samples
				Control	Treated	
2507	A	34.9	5.2	1046096.19 ↑	143442.90	7.29
7703	B	43.1	N/A	603953.61 ↑	78015.31	7.74
9003	C	6.1	N/A	19684792.00 ↑	2490603.29	7.90

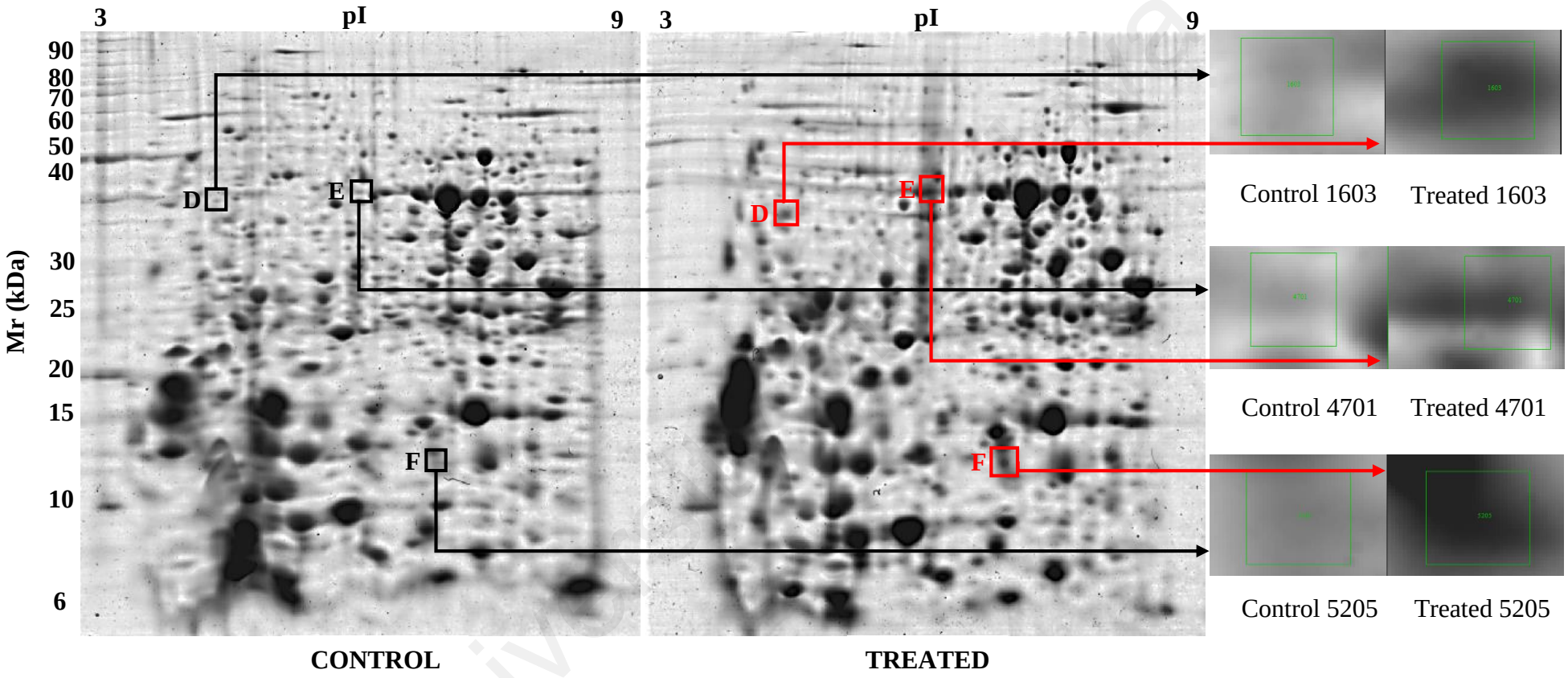


Figure 3.4: Spot position of proteome expression profile on more than 5 fold change in treated samples of *Musca domestica* 3rd instar larvae. (Note: Spots more than 5 fold change are marked in red, whereas its lower fold counterpart is marked in black; Estimated amount of protein loaded in control gel = 180.2955 µg, treated gel =193.9065 µg; Larvae samples were fed in 21.52% (v/v) H_2O_2 in treated samples)

Table 3.7: Summary of proteome expression profile on more than 5 fold change in treated samples of *Musca domestica* 3rd instar larvae. (Note: All spot intensity values in respective replicates were subjected to t-test and were of 90% confidence; Average spot intensities in replicates with more than 5 fold change were shaded.)

SPOT ID	Label	Mr	pI	Average spot intensity		Fold change of treated samples
				Control	Treated	
1603	D	40.2	4.7	80903.52	3162137.33 ↑	39.08
4701	E	44.6	5.6	300237.75	2694532.71 ↑	8.97
5205	F	11.5	5.9	906649.99	5281080.08 ↑	5.82

3.3.1.3 Presence of spots in proteome

By using PDQuest software the presence of the spots in each replicates were also probed. For proteome samples, 2 protein spots were detected to be present exclusively in control samples and 4 in treated samples (Figure 3.5, Table 3.8, Table 3.9).

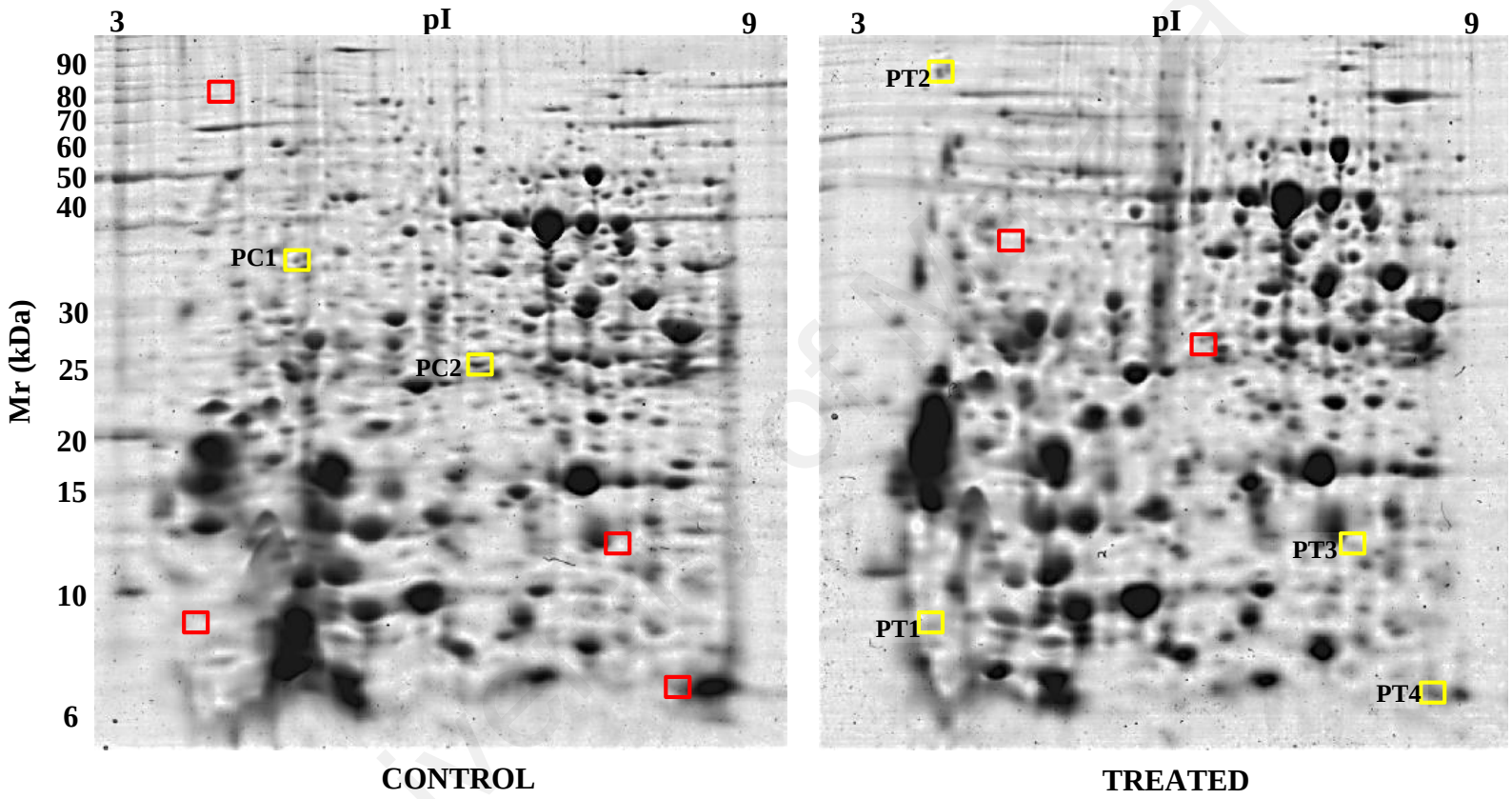


Figure 3.5: Presence of spots exclusively in control and treated gels of *Musca domestica* 3rd instar larvae proteome. (Note: Spots present are marked in yellow and labelled. Absent spots are marked in red in the same position at replicate of comparison and not labelled; Estimated amount of protein loaded in control gel = 180.2955 µg, treated gel =193.9065 µg; Larvae were fed in 21.52% (v/v) H_2O_2 in treated samples)

Table 3.8: Summary of 2D and 3D gel images in PDQuest from the proteome expression profile present in control samples of *Musca domestica* 3rd instar larvae. (Note: PC = present in proteome control samples)

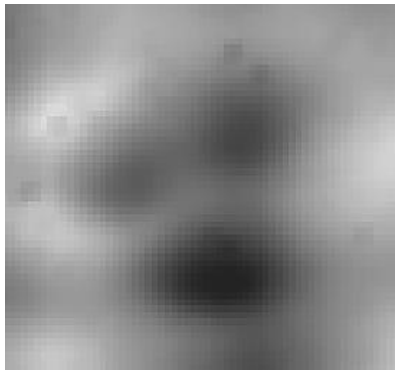
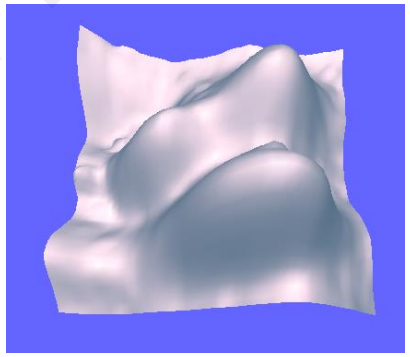
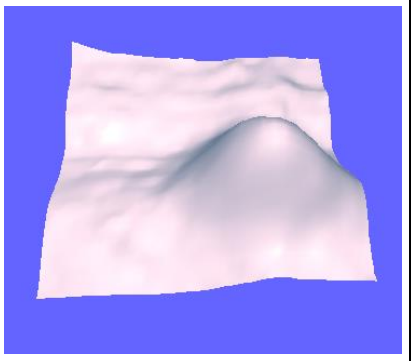
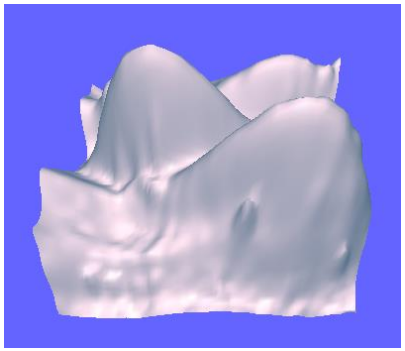
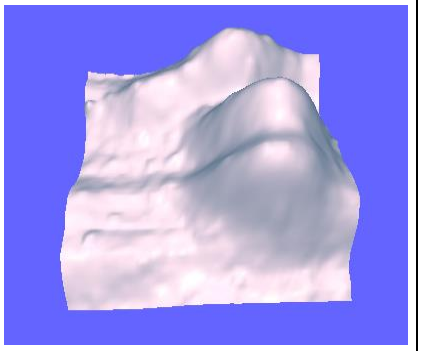
SPOT ID	Ref. Number	2D image		3D image	
		Control	Treated	Control	Treated
1607	PC1				
4405	PC2				

Table 3.9: Summary of 2D and 3D gel images in PDQuest from the proteome expression profile present in control samples of *Musca domestica* 3rd instar larvae. (Note: PT = present in proteome treated samples)

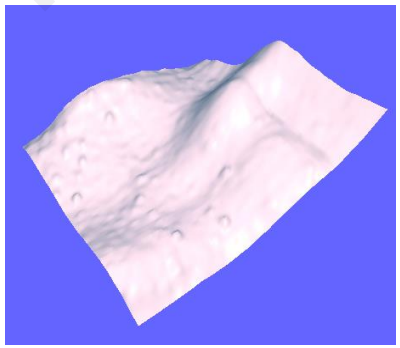
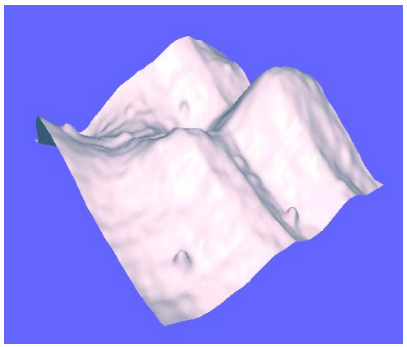
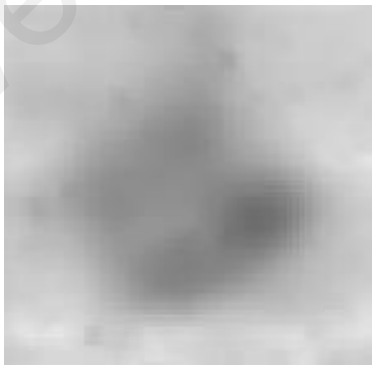
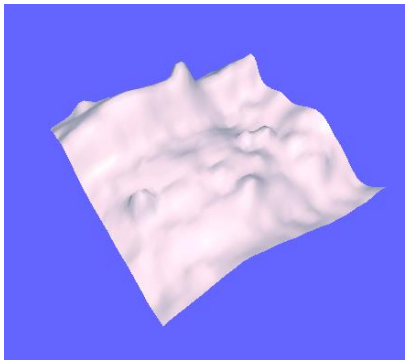
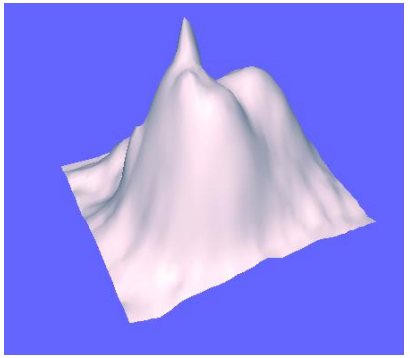
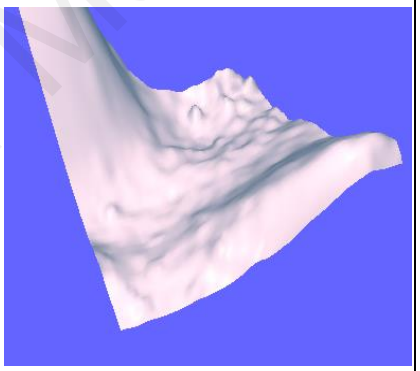
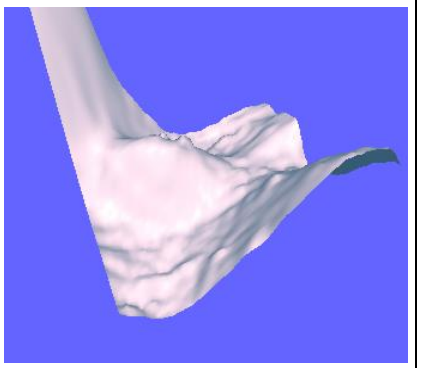
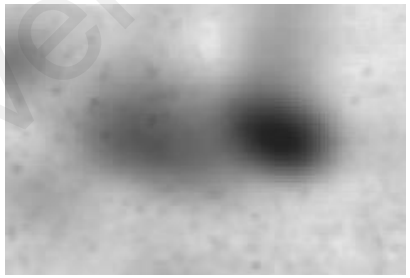
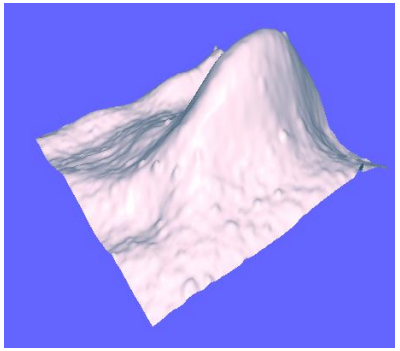
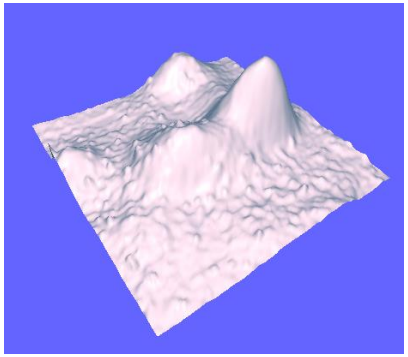
SPOT ID	Ref. Number	2D image		3D image	
		Control	Treated	Control	Treated
0105	PT1				
0901	PT2				

Table 3.9: Summary of 2D and 3D gel images from the proteome expression profile present in control samples of *Musca domestica* 3rd instar larvae. (cont'd)

SPOT ID	Ref. Number	2D image		3D image	
		Control	Treated	Control	Treated
7205	PT3				
9002	PT4				

3.3.2 Thiol proteome expression profile

3.3.2.1 Protein spots with moderate fold changes

Similar in the proteome samples, spots with fold change from 2 to 5 were considered as the protein with moderate fold change. 5 thiol protein spots have a moderate fold change in the control samples. 6 thiol protein spots were of moderate change in treated samples.

All 5 spots of control samples which have exhibited moderate fold change over treated samples were between 2 -3 times (Figure 3.6 and Table 3.10).

In treated samples, out of the 6 spots, 5 of them had shown fold change between 2 -3 times while one of them had exhibited fold change between 3- 4 times (Figure 3.7, Table 3.11 and Table 3.12).

3.3.2.2 Protein spots with high fold changes

Protein spots which have more than 5 fold of change is considered with high fold changes. A total of 2 protein spots each were in high fold change in control (Figure 3.8 and Table 3.13) and treated (Figure 3.9 and Table 3.14) samples each.

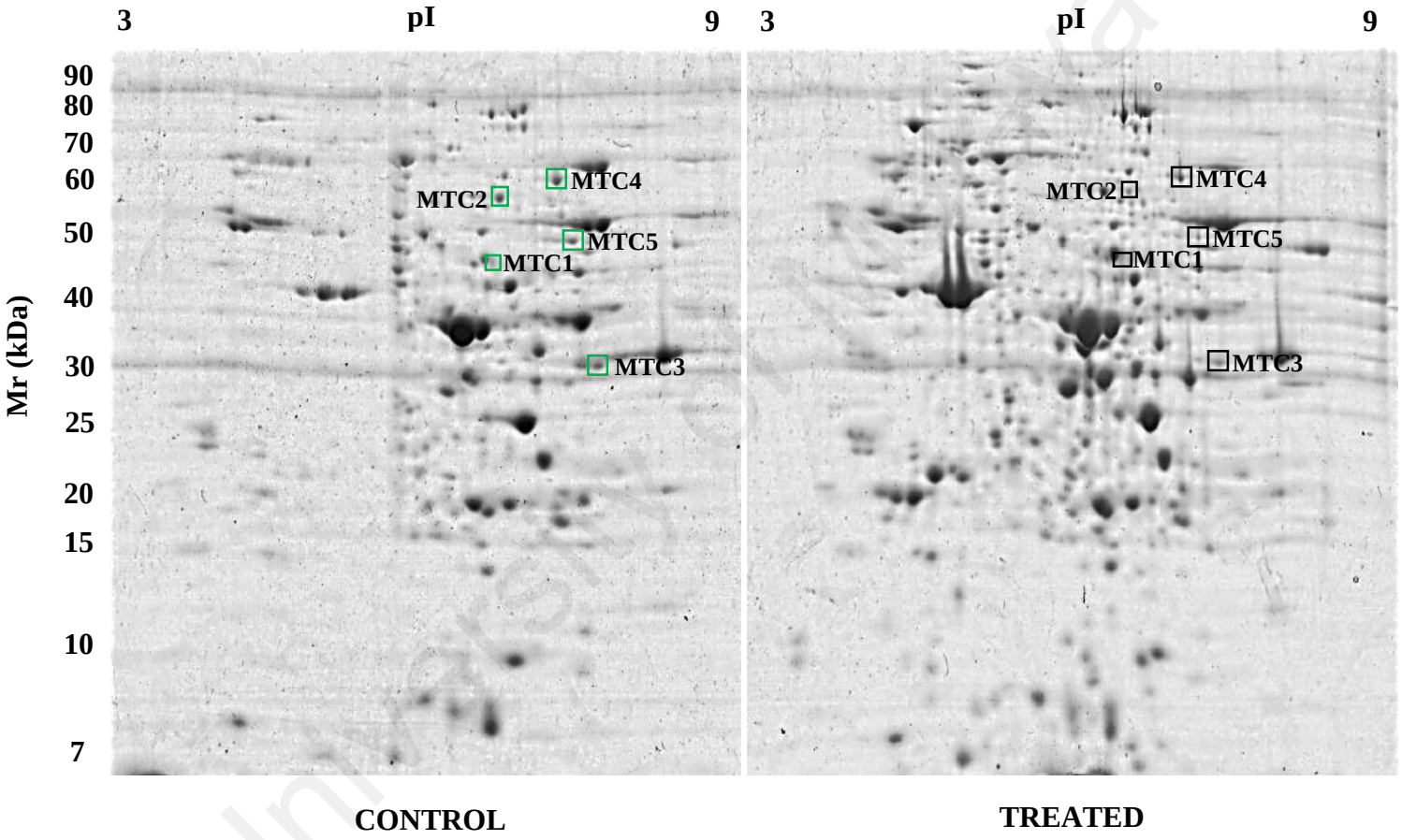


Figure 3.6: Spots with moderate fold change in control gels of *Musca domestica* 3rd instar larvae thiol proteome. (Note: Spots boxed in green have shown fold change between 2 - 3 times while the black boxed spots are their counterparts in treated samples. Estimated amount of protein loaded in control gel = 94.407µg, treated gel =94.659µg; Larvae samples were fed in 21.52% (v/v) H_2O_2 in treated samples)

Table 3.10: Summary of protein spots between 2-3 fold changes in abundance in control proteome samples. (Note: MTC = moderate thiol control) (Note: All spot intensity values in respective replicates were subjected to t-test and were of 90% confidence; Average spot intensities in replicates with 2 – 3 fold change were shaded.)

SPOT ID	Label	Mr	pI	Average spot intensity		Fold change of control samples
				Control	Treated	
4615	MTC1	50.4	6.1	201779.97 ↑	96495.39	2.09
6502	MTC2	34.8	6.9	1071365.23 ↑	491676.92	2.18
4801	MTC3	63.2	6.2	409228.08 ↑	146465.10	2.79
5803	MTC4	66.9	6.5	870563.48 ↑	297967.02	2.92
5704	MTC5	54.0	6.6	454699.33 ↑	153156.49	2.97

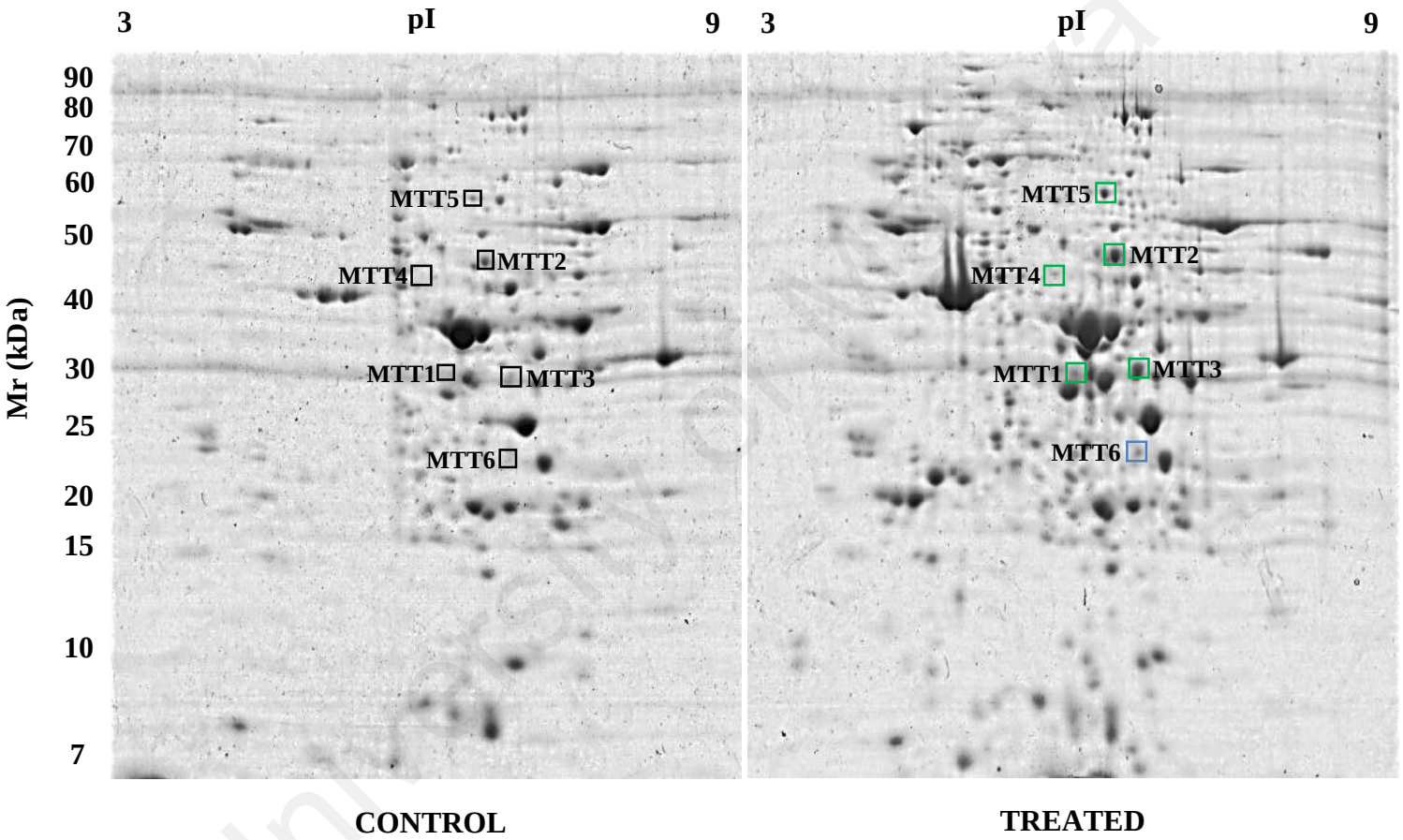


Figure 3.7: Spots with moderate fold change in treated gels of *Musca domestica* 3rd instar larvae thiol proteome. (Note: Spots boxed in green have shown fold change between 2 - 3 times while spots boxed in blue have 3 - 4 fold change. Estimated amount of protein loaded in control gel = 94.407µg, treated gel =94.659µg; Larvae samples were fed in 21.52% (v/v) H_2O_2 in treated samples)

Table 3.11: Summary of protein spots between 2-3 fold changes in treated proteome samples. (Note: MTT = moderate thiol treated) (Note: All spot intensity values in respective replicates were subjected to t-test and were of 90% confidence; Average spot intensities in replicates with 2 – 3 fold change were shaded.)

SPOT ID	Label	Mr	pI	Average spot intensity		Fold change of control samples
				Control	Treated	
3411	MTT1	51.2	6.1	404156.80	943081.69 ↑	2.33
3706	MTT2	33.6	6.2	522043.97	1219458.59 ↑	2.34
4504	MTT3	25.5	6.2	644845.93	1617695.96 ↑	2.51
2615	MTT4	33.5	5.9	51968.14	141463.10 ↑	2.72
3805	MTT5	48.8	5.8	136122.10	391188.02 ↑	2.87

Table 3.12: Summary of protein spot between 3-4 fold changes in treated proteome samples. (Note: MTC = moderate thiol treated) (Note: All spot intensity values in respective replicates were subjected to t-test and were of 90% confidence; Average spot intensities in replicates with 3 - 4 fold change were shaded.)

SPOT ID	Label	Mr	pI	Average spot intensity		Fold change of control samples
				Control	Treated	
4307	MTT6	63.7	6.0	103841.34	325662.52 ↑	3.14

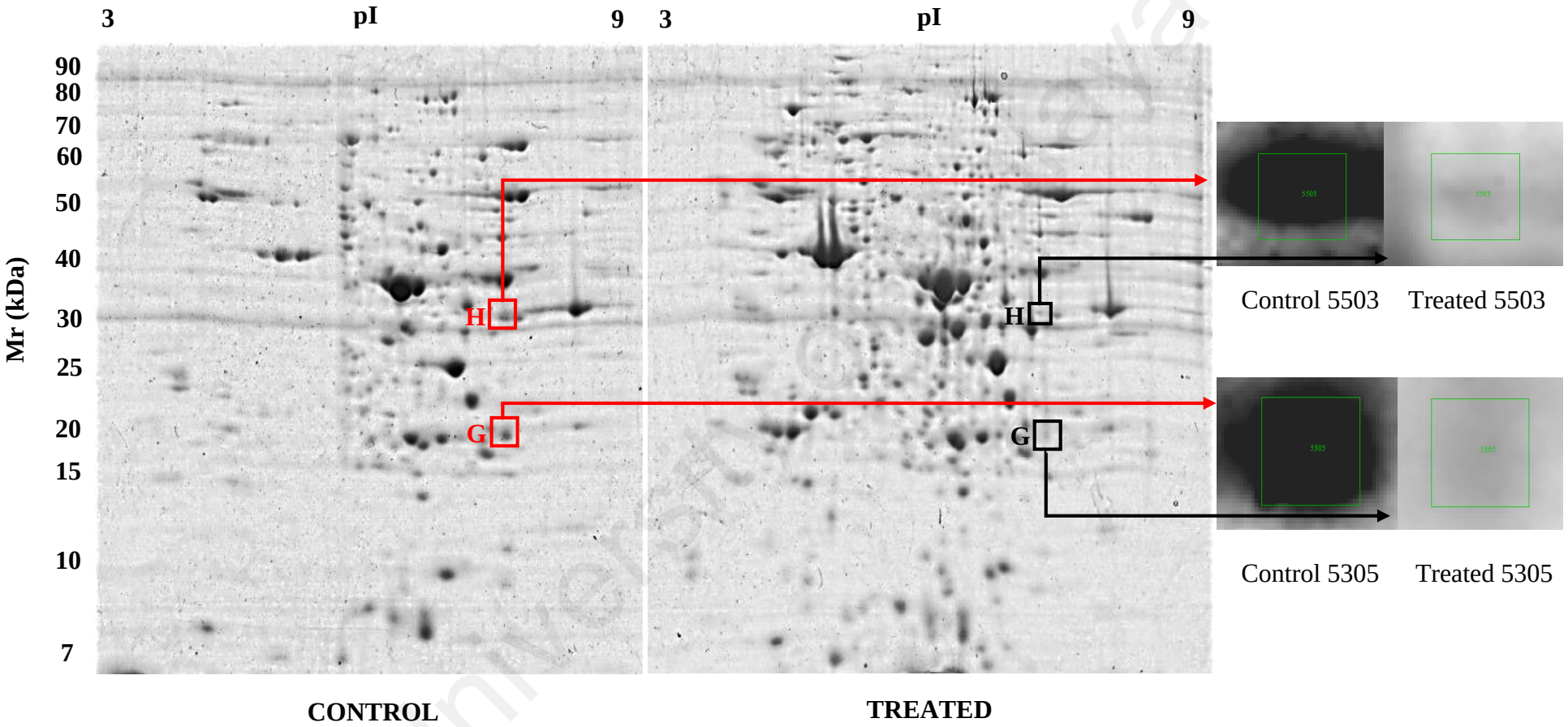


Figure 3.8: Spots with more than 5 fold of change in control gels of *Musca domestica* 3rd instar larvae thiol proteome. (Note: Spots more than 5 fold change are marked in red, whereas its lower fold counterpart is marked in black. Estimated amount of protein loaded in control gel = 94.407µg, treated gel =94.659µg; Larvae samples were fed in 21.52% (v/v) H_2O_2 in treated samples)

Table 3.13: Summary of thiol proteome expression profile on more than 5 fold change in control samples of *Musca domestica* 3rd instar larvae. (Note: All spot intensity values in respective replicates were subjected to t-test and were of 90% confidence; Average spot intensities in replicates more than 5 fold change were shaded.)

SPOT ID	Label	Mr	pI	Average spot intensity		Fold change of control samples
				Control	Treated	
5305	G	21.5	6.8	3317977.44 ↑	584182.65	5.67
5503	H	34.9	6.7	506433.95 ↑	62833.14	8.05

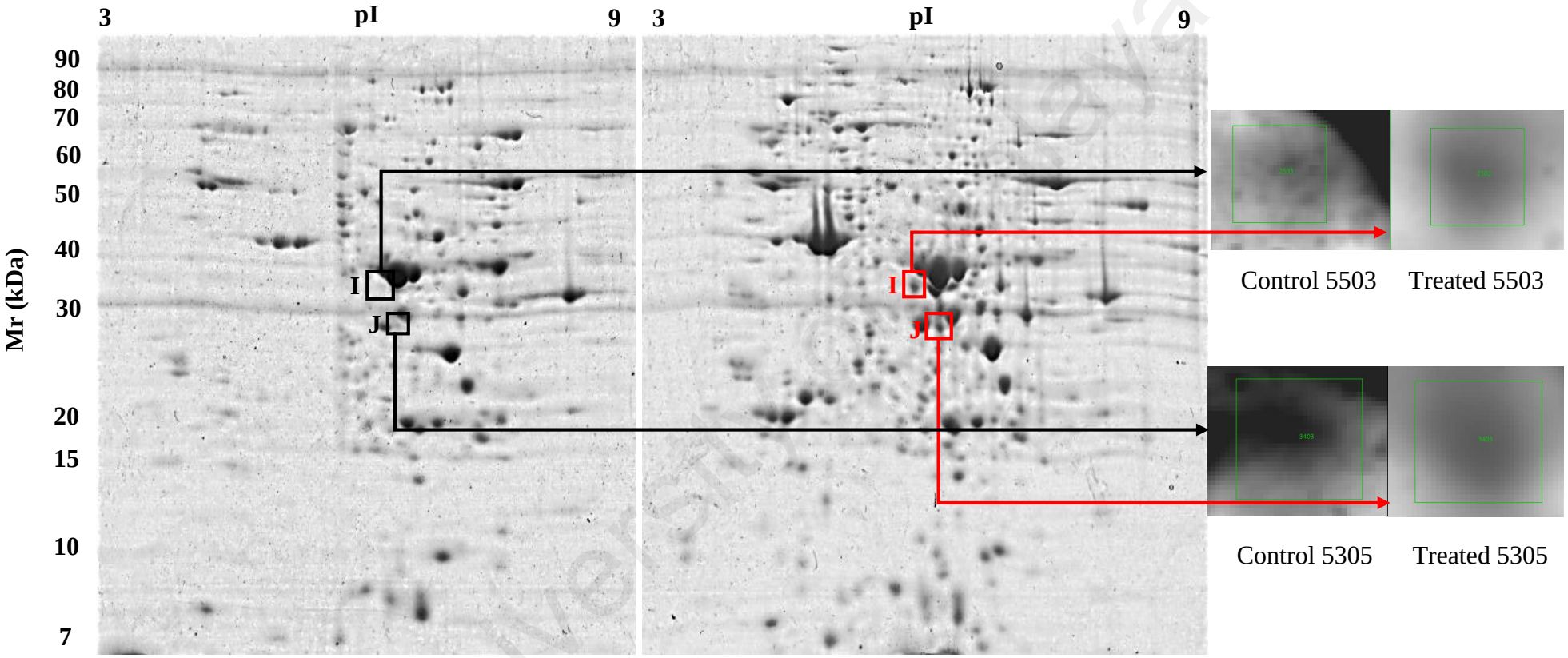


Figure 3.9: Spot with more than 5 fold of change in treated gels of *Musca domestica* 3rd instar larvae thiol proteome. (Note: Spots more than 5 fold change are marked in red, whereas its lower fold counterpart is marked in yellow. Estimated amount of protein loaded in control gel = 94.407µg, treated gel =94.659 µg; Larvae samples were fed in 21.52% (v/v) H_2O_2 in treated samples)

Table 3.14: Summary of thiol proteome expression profile on more than 5 fold change in treated samples of *Musca domestica* 3rd instar larvae. (Note: All spot intensity values in respective replicates were subjected to t-test and were of 90% confidence; Average spot intensities in replicates more than 5 fold change were shaded.)

SPOT ID	Label	Mr	pI	Average spot intensities		Fold change of treated samples
				Control	Treated	
2503	I	37.2	5.8	67202.12	589499.57 ↑	8.77
3403	J	31.8	6.0	104093.90	638692.95 ↑	6.14

3.3.2.3 Presence of spots in thiol proteome profile

The presence of spots in the thiol proteome of the larvae were examined as well. A total of 2 spots were found present only in treated samples (Figure 3.10 and Table 3.15).

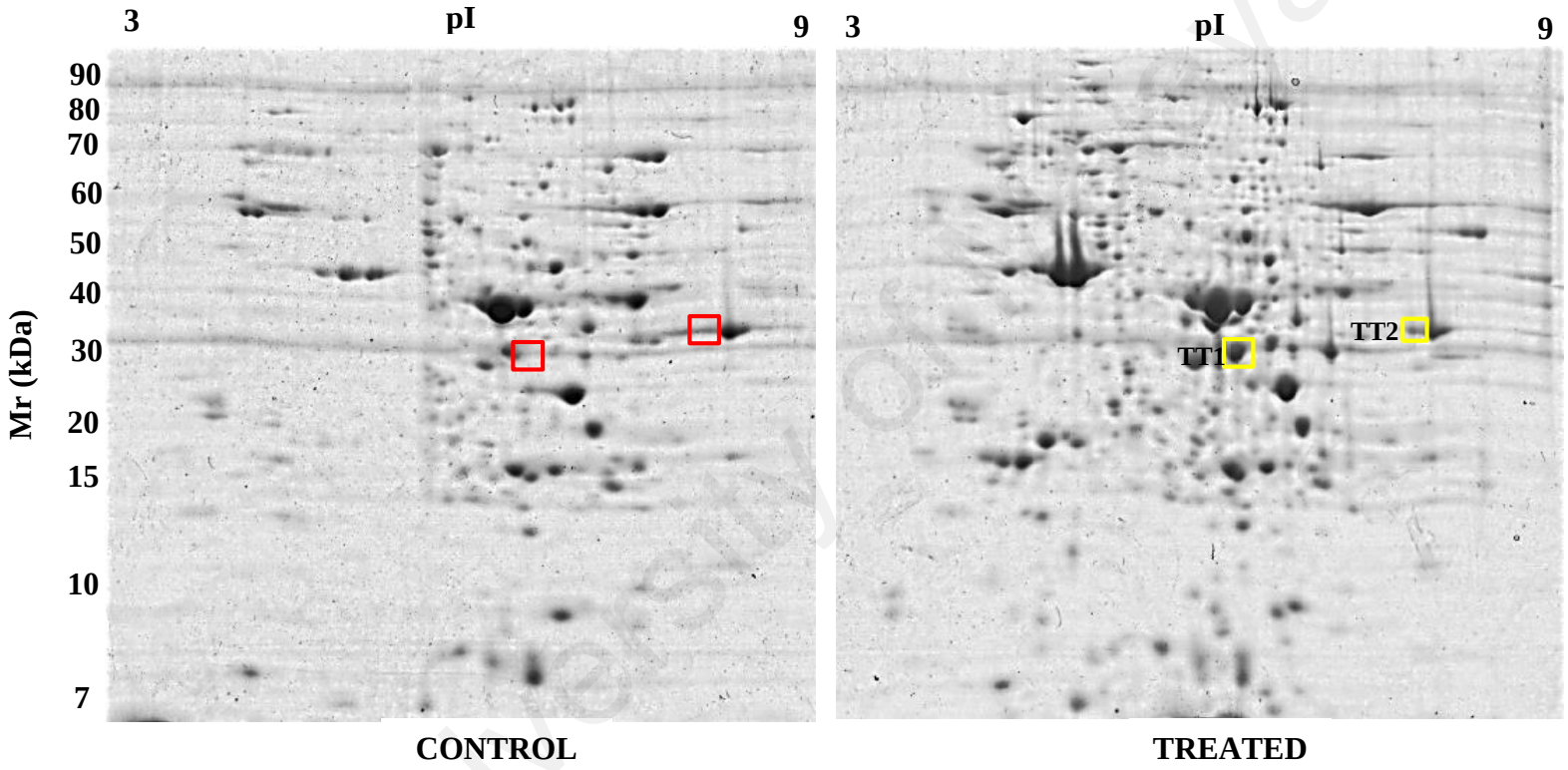


Figure 3.10: Spot that are present respectively in treated gels of *Musca domestica* 3rd instar larvae thiol proteome. (Note: Spots present are marked in yellow and labelled. Absent spots are marked in red in the same position at replicate of comparison and not labelled; Estimated amount of protein loaded in control gel = 94.407µg, treated gel =94.659 µg; Larvae samples were fed in 21.52% (v/v) H_2O_2 in treated samples)

Table 3.15: Summary of 2D and 3D gel spot images in PDQuest from the proteome expression profile present in treated samples of *Musca domestica* 3rd instar larvae. (Note: TT = present in thiol proteome treated samples)

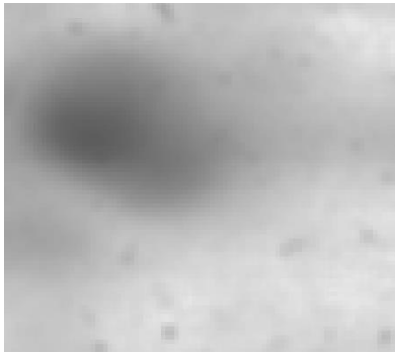
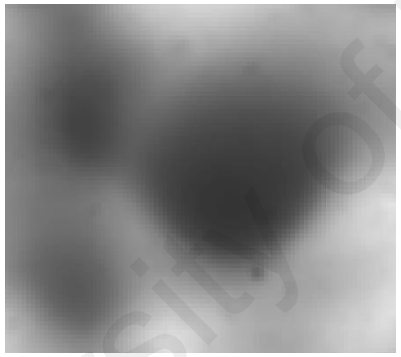
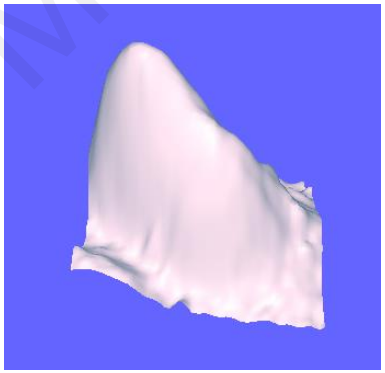
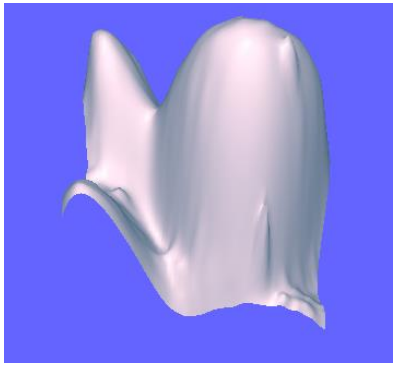
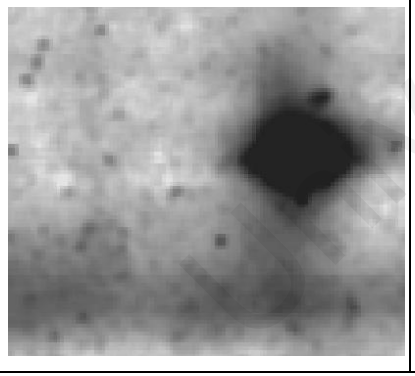
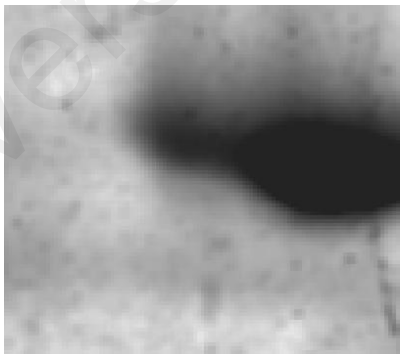
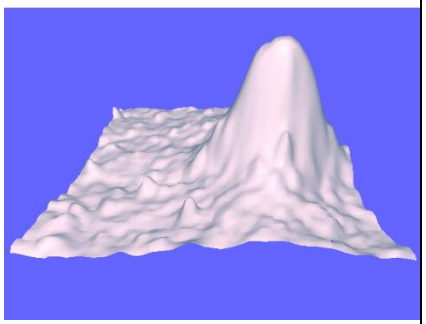
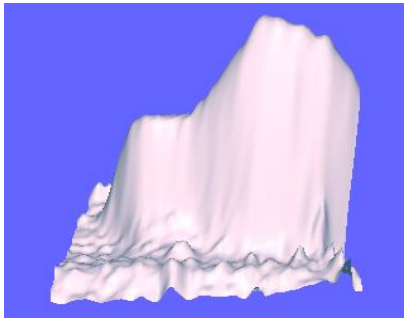
SPOT ID	Ref. Number	2D image		3D image	
		Control	Treated	Control	Treated
4405	TT1				
7402	TT2				

Table 3.16: Summary of presence of spots in both proteome and thiol proteome profile.

Spot label	Proteome		Thiol		Mr	pI
	Control	Treated	Control	Treated		
PC1	✓	-	-	-	38.7	5.0
PC2	✓	-	-	-	23.9	5.7
PC3	✓	-	-	-	13.5	5.9
PT1	-	✓	-	-	8.0	4.3
PT2	-	✓	-	-	74.4	4.4
PT3	-	✓	-	-	6.2	9.0
TT1	-	-	-	✓	32.9	6.0
TT2	-	-	-	✓	35.5	7.6

3.4 LC-MS/MS identification by peptide mass fingerprinting.

Table 3.17: Summary of returned database search results of LC-MS/MS identification.

Spot ID	2D GE analysis			Protein Name	Mr (Da; Predicted)	pI (Predicted)	Mr (Da)	pI	Database accession number	Database	Species	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity
	Profile	Replicate group	Fold change											
A	Proteome	Control	7.29	Actin	34900.0	5.20	42229.5	5.30	399943076	NCBI	*Musca domestica*	49.78	11.7	6.41e+004
				Actin	34900.0	5.20	42229.5	5.30	T1PL65	SwissProt	*Musca domestica*	49.78	11.7	6.41e+004
B	Proteome	Control	7.74	ATP: guanido phosphotransferase	43100.0	N/A	40399.0	5.91	3999399272	NCBI	*Musca domestica*	148.21	27.5	2.54e+005
				ATP: guanido phosphotransferase	43100.0	N/A	40399.0	5.91	L0ESP4	SwissProt	*Musca domestica*	148.21	27.5	2.54e+005
C	Proteome	Control	7.90	Ubiquitin (Fragment)	6100.0	N/A	8592.7	6.56	Q45TR8	NCBI	*Musca domestica*	99.06	64.4	8.54e+005
				Ubiquitin, partial	6100.0	N/A	8592.7	6.56	71040793	SwissProt	*Musca domestica*	99.06	64.4	8.54e+005
D	Proteome	Treated	39.08	Tropomyosin	40200.0	4.70	32742.9	4.74	39939626	NCBI	*Musca domestica*	90.43	25.2	1.01e+005
				Tropomyosin	40200.0	4.70	32742.9	4.74	T1PCF1	SwissProt	*Musca domestica*	90.43	25.2	1.01e+005
E	Proteome	Treated	8.97	Arginine kinase	44600.0	5.60	40339.0	5.91	430769005	NCBI	*Musca domestica*	419.39	62.6	7.21e+005
F	Proteome	Treated	5.82	PBP/GOBP family protein	11500.0	5.90	12579.5	7.92	399940676	NCBI	*Musca domestica*	135.37	47.1	5.89e+005
				PBP/GOBP family protein	11500.0	5.90	12579.5	7.92	T1PDX8	SwissProt	*Musca domestica*	135.37	47.1	5.89e+005

Table 3.17: Summary of returned database search results of LC-MS/MS identification. (cont'd)

Spot ID	2D GE analysis			Protein Name	Mr (Da; Predicted)	pI (Predicted)	Mr (Da)	pI	Database accession number	Database	Species	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity
	Profile	Replicate group	Fold change											
G	Thiol	Control	5.67	PREDICTED: alcohol dehydrogenase-like isoform X1	21500.0	6.80	28232.0	6.17	557782742	NCBI	*Musca domestica*	25.10	13.7	7.72e+004
				PREDICTED: alcohol dehydrogenase-like isoform X2	21500.0	6.80	28232.0	6.17	557782744	NCBI	*Musca domestica*	25.10	13.7	7.72e+004
				Short chain dehydrogenase	21500.0	6.80	24404.2	5.95	T1PI49	SwissProt	*Musca domestica*	15.87	3.6	5.48e+005
H	Thiol	Control	8.05	Lactate/malate dehydrogenase	34900.0	6.70	36158.0	7.91	399938050	NCBI	*Musca domestica*	80.40	16.2	2.17e+005
				L-lactate dehydrogenase	34900.0	6.70	36158.0	7.91	T1P9J0	NCBI	*Musca domestica*	80.40	16.2	2.17e+005
I	Thiol	Treated	8.77	Arginine kinase	37200.0	5.80	40399.0	5.91	430769005	NCBI	*Musca domestica*	235.29	40.4	1.58e+005
J	Thiol	Treated	6.14	Fructose-bisphosphate aldolase class-1	31800.0	6.00	39697.5	7.04	399943052	NCBI	*Musca domestica*	58.39	18.1	1.09e+005
				Fructose bisphosphate aldolase	31800.0	6.00	39697.5	7.04	399943052	SwissProt	*Musca domestica*	58.39	18.1	1.09e+005

CHAPTER 4: DISCUSSION

The growth of *Musca domestica* larvae and adults in our current lab conditions adheres to the condition stated: the cultivation of the housefly adult spanned from 2-3 weeks, the housefly larvae hatched overnight after it was oviposited in a suitable medium and reached full pupation at the 5th day after hatching. This facilitated our selection of peroxide treatment stage on the 3rd day larvae. All mortality and survival were recorded. Treated individuals were experiencing loss of wet weight and reduced in size. Worthwhile to note that larviform were observed and generally appeared across dead larvae. Emergence of adult housefly from pupation of surviving individual were not investigated. A turning point of mortality of larvae was observed when passing the 20% (v/v) mark in our feeding assay.

Probing of cellular levels malondialdehyde (MDA) as a chemical marker via spectrophotometer means effectively showed that under the treatment of 21.52%(v/v) of H_2O_2, 1.040±0.612 nmol/ g wet weight and 11.924±4.528 nmol/ g wet weight were observed in control and treated samples respectively. This was an approximately 7-10 fold of increase of MDA levels in 3rd instar larvae between control and treated samples. With the amount of change of MDA detected, it was evident that oxidative stress occurred inside housefly larvae during the treatment.

It is worth to mention that there are some limitations set in the experimental design. In our investigation, 7cm IPG strips and SDS gel systems were used, part of the selection criteria was relatively high (5 fold change). Under these boundaries of the experiment, it is conceivable that and in fact the expectation that the qualitative yield is on the low. Nonetheless, the experimental aim has been achieved as we aim to cover important proteins that represent the oxidative stress metabolism. Thiol proteome profile also exhibited a different proteome profile compared to the total cytosolic proteome profile.

Table 4.1: Summary of the LC-MS/MS identified proteins

Spot ID	Protein Name	Profile	Replicate group	Fold change
A	Actin	Proteome	Control	7.29
B	ATP:guanido phosphotransferase	Proteome	Control	7.74
C	Ubiquitin	Proteome	Control	7.90
D	Tropomyosin	Proteome	Treated	39.08
E	Arginine kinase	Proteome	Treated	8.97
F	PBP/GOBP family protein	Proteome	Treated	5.82
G	Alcohol dehydrogenase-like isoform	Thiol	Control	5.67
H	Lactate/malate dehydrogenase	Thiol	Control	8.05
I	Arginine kinase	Thiol	Treated	8.77
J	Fructose-bisphosphate aldolase	Thiol	Treated	6.14

Interestingly, identified proteins were neither involved in the known oxidative stress defence active protection (S-glutathionylation, thioredoxin and H_2O_2 scavenging proteins) nor any other cellular actions (transcription, signalling and post translational modifications) (Table 4.1). Instead, common proteins mostly including structural and metabolic were highly regulated possibly due to the acute treatment of the larvae. Moreover, the high fold change window set might allow detection of a different set of proteins. Whole larvae are homogenized and the proteome was observed instead of specific cells/tissues, hence we could have recovered more protein with general metabolic functions.

Protein spot A after subjected to LC-MS/MS based peptide fingerprinting returns actin, which has shown fold change of 7.29 times higher in proteome control samples. With molecular weight of 42kDa, actin is the monomer (G-actin) that polymerizes into filaments (F-actin) under physiological conditions to form cytoskeleton in cells (Huber *et al*., 2013). Actin has an ATP/ADP binding region where hydrolysing of ATP occurred during F-actin polymerization, which is vital for cell motility (Holmes, 2009).

Our investigation implied that in hydrogen peroxide-treated samples actin pool was in a low concentration. This had been with agreement on the extensive work *in vitro* had been done on P388D1 cell line (Hinshaw *et al*., 1986; Hinshaw *et al*., 1988; Hinshaw *et al*., 1991). p38-MAP kinase and HSP27 phosphorylation could be regulating the actin reorganization and F-actin stabilization in hydrogen peroxide treated specific human umbilical vascular endothelial cell line (Huot *et al*., 1997) and fibrioblast cells secreting wild type HSP27 (Huot, 1998), forming stress fibers. However large changes in both p38-MAP kinase and HSP27 were not detected in our investigation, at least not in our chosen large cutoff points (5 fold change). Reducing of levels of actin during peroxide challenge due to increase of actin polymerization could be occurring via S-thiolation of actin's Cys374 residue (Dalle-Donne et al., 2003) or formation of disulfide bonds during slow thiol oxidation, creating dimers which cross links F-actin (Tang *et al*, 1999), where both further strengthen the formation of actin filaments rather than in monomers.

Given the fact that actin participated in wide range of cellular processes such as cytokinesis (Dean *et al*., 2005), tube formation (Nie *et al*., 2014), cell signalling (Carpenter, 2000), cellular and nuclear integrity (Revenu *et al*., 2004; Falahzadeh et al., 2015), gene expression and transcription (Louvet and Percipalle, 2008) and cell-cell adhesion (Adams et al., 1996), actin polymerization could be a major physiological and cellular strategy for *Musca domestica* to resist against acute oxidative stress.

On the other hand, actin is often used as a housekeeping gene for quantification in molecular studies (Zhong *et al*., 2013), hence based on our results, we suggest that care and consideration must be taken in selecting and interpreting actin levels from oxidative stress research, particularly involving housefly larvae.

Meanwhile tropomyosin (Spot D) were found in unusually high abundance in hydrogen peroxide treated 3rd instar *Musca domestica* larvae (39.08 fold change). Tropomyosin is vital to stabilize actin filament and mediate actin binding proteins in muscular tissues (Cooper, 2002). High fold changes of tropomyosin during hydrogen peroxide treatment was observed in clams (Rodríguez-Ortega *et al*., 2003) and vascular smooth muscle cells (Partridge *et al*., 2005). Overexpression of tropomyosin during oxidative stress in housefly larvae could be accounted to calmodulin switching due to calcium ion influx, which in turn activates calcineurin that turns on muscle enhancer MEF2 (Lin *et al*., 1996; Wu *et al*., 2000), and enhances downstream TmI gene expression muscles in *Drosophila* (Lin and Stroti, 1997). Moreover, increase of tropomyosin expression could help endothelial cells stabilize F-actin (Gagat *et al*., 2014).

Taken together, a trend of opposing effects on actin (abundant in control samples) and tropomyosin (abundant in acute treatment samples) can be observed – suggesting that during oxidative stress *Musca domestica* larvae was active in maintaining the integrity of cytoskeletal network.

However, our untargeted investigation was incapable to discern the levels of possible tropomyosin isoforms and its localization in different tissues, muscle and non-muscle alike during peroxide treatment. Different isoforms of tropomyosin function differently on the actin cytoskeleton molecule, in terms of actin binding properties and effect on other actin-binding proteins (Gunning *et al*., 2005; Lin *et al*, 2008), contributed during alternative splicing (Basi *et al*., 1986). Future targeted investigations aiming on

resolving in vitro tropomyosin concentration and response should be isoform- and/or tissue- specific.

Arginine kinase was identified from sample E as the protein spot more than 5 fold abundance (8.97 fold and 8.77 fold respectively) in both treated proteome and thiol proteome profile via LCMS-based peptide mass fingerprinting.

Arginine kinase catalyzes the reversible reaction of transferring phosphorus group between MgATP and arginine acting as energy-storing phosphagen (Reaction 2)

$$\text{Phosphoarginine} + \text{MgADP}^- + \text{H}^+ \leftrightarrow \text{Arginine} + \text{MgATP}^{2-} \quad (2)$$

Phosphoarginine is one of the phosphagens which are smaller than ATP and able to diffuse to provide fast energy supply (Ellington, 2001). This enable cells to restore ATP levels during bursts of cellular events such as in nerves and muscles before catabolic events (Hird, 1986; Huennekens and Whiteley, 1960; McGilvery and Goldstein, 1979). L-arginine is shuttled to cytoplasm before converted into high-energy phosphoarginine for phoshorylating ADP to ATP in myofibrils in invertebrates (Schneider *et al*., 1989). Similar responses were also observed during treatment with hydrogen peroxide in protozoan *Trypanosoma cruzi*, where arginine kinase expression increased rapidly (Pereira *et al*., 2003; Miranda *et al*., 2006) and at the same time, due to the rapid phosphorylation of ADP, phosphoarginine levels were observed to decrease in nematode (Platzer *et al*. 1999) and prawns (Abe *et al*. 2006).

In thiol proteome two dimensional gel profile, a conserved Cys271 residue at the active site of arginine kinase were selected via our thiol purification protocol during hydrogen peroxide treatment. This residue acts as a salt bridge clamp on the guanidinium of the substrate arginine by forming a thiolate (Zhou *et al*., 1998). This cysteine residue was not only conserved but apparently highly protected against oxidative thiol modification, and highly expressed during peroxide treatment, albeit we are not sure

whether this is caused by active protection (S-glutathionylation/thioredoxin) or constant activity of the enzyme.

At the same time, peptide mass fingerprinting results also returned in control proteome profile that a protein (Spot B) from ATP: guanidino phosphotranferase family was identified to be more than 5 times in abundance in control replicates, with similar molecular weight compared with E and I (both arginine kinases in Mr = ~ 43100kDa) albeit in different position. ATP: guanidino phosphotransferases is a protein family, where arginine kinase belongs to, alongside with glycocyamine kinase, hypotaurocyamine kinase, lombricine kinase, opheline kinase, taurocyamine kinase, and thalessemine kinase in invertebrates (Jarilla and Agatsuma, 2010).

With arginine kinase the only plausible candidate of all the proteins in *Musca domestica* (based on genome database searches), we postulate this arginine kinase could possibly a different isoform that expressed in control samples and deactivates rapidly in the event of acute peroxide challenge. Arginine kinase exist in mitochondrial and cytoplasmic isoforms from a single gene (Munneke and Collier, 1988; Uda *et al*., 2006) and based on our results, during oxidative stress the isoforms of arginine kinase from different locations of the cell might act antagonistically to meet the cellular energy requirements. Taken together, during oxidative stress, arginine kinase through varied levels of possible isoforms and its high resistance, could be vital in *Musca domestica* larvae's energy metabolism response, especially in terms of providing alternative cellular energy source and ATP buffering.

Ubiquitin is the only entry that returned from LC-MS/MS identification of Spot C. A 76-residue polypeptide, ubiquitin is highly conserved and present in all eukaryotes (Goldstein *et al*., 1975). Expressed in bulk (Ryu *et al*., 2007) and have a rather long half-

life in the cell (Shabek and Ciechanover, 2010), this polypeptide is firstly known for ubiquitinylating substrate proteins for 26S proteasome damage.

From our investigation, ubiquitin abundance has been reduced in the event of the oxidative stress, suggesting a disruption or a shift of homeostasis of ubiquitin. Reduction of monomeric ubiquitin were observed in deletion of deubiquitinating enzymes (DUBs) encoding genes in yeast (Swaminathan *et al*., 1999). DUBs are enzymes catalyzing the process of breaking down free and ubiquitinated substrates, free polyubiquitin chain (Amerik and Hochstrasser, 2004), and synthesis of free ubiquitin (Redman and Rechsteiner, 1989). Interestingly, DUB's catalytic consists of highly conserved regions of histidine and cysteine boxes (Reyes-Turcu *et al*., 2006), in which cysteine residues is highly susceptible for peroxide attack. Therefore, it is conceivable that oxidative damage occurred in DUBs during larvae acute peroxide treatment, which make larval cells unable to recycle free ubiquitin monomer from deubiquitination.

PBP/GOBP protein family (pheromone binding protein/general odorant binding protein) was identified from Spot F. Shown an increase of 5.82 times than control samples, this family of proteins are located in the aqueous fluid around olfactory sensory dendrites to bind and transport hydrophobic odorants (Vogt and Riddiford, 1981; Vogt *et al*., 1991b; Pelosi *et al*., 2006). PBP, binds specifically towards pheromone (Du and Prestwich, 1995; Feixas *et al*., 1995) compared to GOBP which associate with general-odorant sensitive neurons (Vogt *et al*., 1991a). Interestingly, both insect PBP and GOBP are characterized by three pairs of disulfide bonds with the same cysteine residue pairings (Scaloni *et al*., 1999), which limits the folding and versatility in terms of functions (Pelosi *et al*., 2014). Though known to bind pheromonal compounds, fatty acids and long-chain alcohols (Campanacci *et al*., 2001), it is still intriguing that how this protein family underexpressed during hydrogen peroxide treatment.

Present in abundance of 5.67 times in control samples, spot G belongs to one of the member of short-chain alcohol dehydrogenase family. Sequence analysis research of alcohol dehydrogenase from *Drosophila melanogaster* and *Aedes aegypti* suggested that the selection via our thiol protein purification methods, possibly on its Cys-218 (which is in proximity to NAD^+ binding site and does not have any catalytic properties) (Chen *et al*., 1990) and two other solvent facing cysteine residues (Mayoral *et al*., 2013). It is a ubiquitous NAD(P)(H)-dependent enzyme belongs to large short-chain dehydrogenase/reductase family with over 46000 related members (Persson *et al*., 2009). Other than sharing a highly similar nucleotide binding region, the proteins in this family have very low pairwise sequence identity (15%-30%) (Filling *et al*., 2002), hence making this group of enzyme extremely diverse (Kallberg *et al*., 2002). In *Drosophila* sp. alone, other than common reactions of long chain and ethanol oxidation in aldehyde, some SDR gene products are found to be capable to oxidize farnesol and geraniol (Mayoral *et al*., 2013). Thus it is still far from conclusive that which exact short chain dehydrogenase involved in the current peroxide treatment of larvae of *Musca domestica*, and what possible catalytic activity undertaken which leads to its "sacrifice" during hydrogen peroxide attack.

Spot H are found to be in abundance for 8.05 fold change in control samples is a protein that belongs to lactate/malate dehydrogenase family. Lactate/malate dehydrogenases interconvert lactate and pyruvate/oxaloacetate and malate utilizing NAD(P)/NAD(P)(H) (Holbrook *et al*., 1975). A highly conserved cysteine residue, Cys-165 located at the proximity of co-substrate binding site (Taylor *et al*., 1973) in lactate dehydrogenase and Cys-330 residues (Hara *et al*., 2006) in the malate dehydrogenase allow this group of protein to be purified in our thiol purification methods.

For lactate dehydrogenase, the decrease of its levels during peroxide attack might be due to the extracellular leakage of plasma membrane caused by damage of the plasma

membrane, which is a common parameter during cell death (Bagchi *et al*., 1995), hence this might be a physiological leakage rather than a metabolic regulation on our observation. Malate dehydrogenase in the mean time had been found downregulated and reduced in activity in oxidatively stressed organisms (George *et al*., 2012; Lv *et al*., 2013; Padmaja and Raju, 2005; Sharma *et al*., 2007)

Given the fact that, lactate dehydrogenases are vital to anaerobic metabolism (Holbrook *et al*., 1975) while malate dehydrogenases are critical in tricarboxylic acid cycle, glyoxylate bypass, amino acid synthesis and gluconeogenesis (Goward and Nicholls, 1994), lactate/malate dehydrogenase Cys residues could be susceptible against oxidative thiol modifications. Loss of this protein might indicate that arginine kinase could had taken over the role as energy reservoir during peroxide attack in *Musca domestica* larvae.

Fructose bisphosphate aldolase, upon peroxidant challenge, exhibited 6.14 times of fold change in treated samples. Sequence analysis by Brenner-Holzach (1979) showed that there are no exposed thiol groups of fructose bisphosphate aldolase in *Drosophila melanogaster*. We postulate that for fructose bisphosphate aldolase of *Musca domestica*, which contains 6 putative cysteine residues in the Uniprot repository to have sufficient exposed thiol groups to be selected from our purification methods.

Overexpression of fructose bisphosphate aldolase during oxidative stress were discovered in oral pathogen *Fusobacterium nucleatum* (oxygen-induced oxidative stress) (Steeves *et al*., 2011), and *Agrostis capillaris* roots (Hego *et al*., 2014).

Due to importance in the central metabolism which regulate a great amount of pathways downstream (e.g. energy production, amino acid synthesis, fatty acid synthesis, pentose phosphate pathway), and possible deactivation of preceding enzyme glyceraldehyde 3-phosphate dehydrogenase in glycolytic pathway by thiol modification

(Ralser *et al*., 2007), upregulation of fructose bisphosphate aldolase could be another key protective mechanism on glycolytic pathway against acute peroxide challenge in *Musca domestica* larvae.

CHAPTER 5: CONCLUSION

We have cultivated 3rd instar larvae of *Musca domestica* and determined LC_{50} of hydrogen peroxide treatment (21.52% (v/v)). Acute treatment of hydrogen peroxide of the concentration was later performed via feeding for 24 hours, both the proteome and the thiol protein purified protein were obtained and subjected to two dimensional gel electrophoresis. Differential analysis had mapped out in proteome samples with moderate fold change (2-5 times), high fold change (>5 times) and absence of spots.

Protein spots with high fold changes were identified using LC-MS/MS based peptide mass fingerprinting and proteins related with important biological functions were identified including cytoskeleton formation (actin and tropomyosin), protein degradation (ubiquitin), odorant binding and sensory (PBP/GOBP family protein), energy metabolism (arginine kinase), anaerobic metabolism/gluconeogenesis/TCA cycle (Lactate/malate dehydrogenase), and glycolysis/gluconeogenesis (Fructose bisphosphate aldolase). Arginine kinase and fructose bisphosphate aldolase are high in abundance in thiol proteome profile, suggesting their high level of protection under acute hydrogen peroxide induced oxidative stress.

Since the proteins involved in high fold changes have been detected, we suggest that in future more research should be focused on the protein with moderate fold changes for more holistic understanding in *Musca domestica*'s 3rd instar larvae oxidative stress responses. Exact isoform and localization of the protein and concentration of its substrate *in vitro* can be further determined to justify the direction of metabolic shift upon oxidative stress alongside with proteomic analyses.

References

2-D Quant Kit Product Specification Sheet. (2009). GE Healthcare, Uppsala, Sweden.

Abe, H., Hirai, S., and Okada, S. (2007). Metabolic responses and arginine kinase expression under hypoxic stress of the kuruma prawn *Marsupenaeus japonicus*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, **146**(1), 40-46.

Acevedo, G.R., Zapater, M., and Toloza, A.C. (2009). Insecticide resistance of house fly,*Musca domestica* (L.) from Argentina. *Parasitology Research*, **105,** 489-493**.**

Activated Thiol–Sepharose® 4B lyophilized powder | Sigma Aldrich (n.d). In Sigma Aldrich's website. Retrieved from http://www.sigmaaldrich.com/catalog/product/sigma/t8512?lang=en&region=MY

Adams, C. L., Nelson, W. J., and Smith, S. J. (1996). Quantitative analysis of cadherin catenin-actin reorganization during development of cell-cell adhesion. *The Journal of Cell Biology*, **135**(6), 1899–1911.

Akhgari, M., Abdollahi, M., Kebryaeezadeh, A., Hosseini, R., and Sabzevari, O. (2003). Biochemical evidence for free radical-induced lipid peroxidation as a mechanism for subchronic toxicity of malathion in blood and liver of rats. *Human and Experimental Toxicology*, **22**, 205–11.

Alessio, H. M. (2000). Chapter 5 - Lipid peroxidation in healthy and diseased models: Influence of different types of exercise. In: Hanninen, O, Packer, L., and Sen, C. K. (Eds.) *Handbook of Oxidants and Antioxidants in Exercise* (pp. 115–198). Amsterdam: Elsevier.

Allen, R.G., Farmer, K.J., and Sohal, R.S. (1983). Effect of catalase inactivation on levels of inorganic peroxides, superoxide dismutase, glutathione, oxygen consumption and life span in adult houseflies (*Musca domestica*). *Biochemical Journal,* **216(**2), 503–506.

Allen, R.G., Oberley, L.W., Elwell, J.H., and Sohal, R.S. (1991). Developmental patterns in the antioxidant defenses of the housefly, *Musca domestica*. *Journal of Cellular Physiology.* **146(**2), 270–276.

Alonso, A., Sasin, J., Bottini, N., Friedberg, I., Friedberg, I., Osterman, A., Godzik, A., Hunter, T., Dixon, J., and Mustelin, T. (2004). Protein tyrosine phosphatases in the human genome. *Cell*, **117**, 699–711.

Amerik, A.Y., and Hochstrasser, M. (2004). Mechanism and function of deubiquitinating enzymes. *Biochimica et Biophysica Acta,* **1695**, 189–207.

Azevedo, D., Tacnet, F., Delaunay, A., Rodrigues–Pousada, C., and Toledano, M.B. (2003). Two redox centers within Yap1 for H_2O_2 and thiol-reactive chemicals signaling. *Free Radical Biology and Medicine,* **35**, 889–900.

Bagchi, D., Bagchi, M., Hassoun, E. A., and Stohs, S. J. (1995). In vitro and in vivo generation of reactive oxygen species, DNA damage and lactate dehydrogenase leakage by selected pesticides. *Toxicology*, **104**(1), 129–140.

Barford. D. (2004). The role of cysteine residues as redox-sensitive regulatory switches. *Current Opinion in Structural Biology,* **14**, 679–686.

Barin, A., Arabkhazaeli, F., Rahbari, S., and Madani, S. A. (2010) The housefly, *Musca domestica*, as a possible mechanical vector of Newcastle disease virus in the laboratory and field. *Medical and Veterinary Entomology.* **24**, 88–90

Basi, G. S., Boardman, M., and Storti, R. V. (1984). Alternative splicing of a *Drosophila* tropomyosin gene generates muscle tropomyosin isoforms with different carboxy-terminal ends. *Molecular and Cellular Biology*, **4**(12), 2828 –2836.

Betteridge D.J. (2000). What is oxidative stress? *Metabolism,* **49**(2 Suppl. 1), 3–8.

Biswas, S., Chida, A. S., and Rahman, I. (2006). Redox modifications of protein–thiols: Emerging roles in cell signalling, *Biochemical Pharmacology*, **71**, 551– 564.

Bong, L.J. and Zairi, J. (2010). Temporal fluctuations of insecticides resistance in *Musca domestica* Linn. (Diptera: Muscidae) in Malaysia. *Tropical Biomedicine,* **27**(2),317–325.

Boulesteix, G., Le Dantec, P., Chevalier, B., Dieng, M., Niang, B. and Diatta, B. (2005). Role of *Musca domestica* in the transmission of multiresistant bacteria in the centres of intensive care setting in a sub-Saharan Africa. *Annales Francaises d'Anesthésie et de Réanimation*. **24**, 361–365.

Brandes, N., Rinck, A., Leichert, L.I., and Jakob, U. (2007). Nitrosative stress treatment of *E. coli* targets distinct set of thiol containing proteins. *Molecular Microbiology*, **66**, 901–914.

Brandes, N., Schmitt. S., and Jakob, U. (2009) Thiol-based redox switches in eukaryotic proteins. *Antioxidants and Redox Signalling*, **11**(5), 997–1014.

Brenner-Holzach, O. (1979). Fructose 1,6-diphosphate aldolase of *Drosophila melanogaster*: Comparative sequence analyses of the cysteine-containing peptides. *Archives of Biochemistry and Biophysics*, **194**, 328–335.

Brigelius-Flohe, R., and Maiorino, M. (2012). Glutathione peroxidases. *Biochimica et Biophysica Acta*, **1830**, 3289–3303.

Burmeister, C., Lüersen, K., Heinick, A., Hussein, A., Domagalski, M., Walter, R. D., and Liebau, E. (2008). Oxidative stress in *Caenorhabditis elegans*: protective effects of the Omega class glutathione transferase (GSTO-1). *The FASEB Journal*, 22(2), 343-354.

Cadenas, E., Boveris, A., Ragan, C.I., and Stoppani, A.O.M. (1977). Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol cytochrome c reductase from beef heart mitochondria. *Archives of Biochemistry and Biophysics*, **180**, 248–257.

Cadenas, E., and Davies, K. J. (2000). Mitochondrial free radical generation oxidative stress and aging. *Free Radical Biology and Medicine*, **29**, 222–230.

Caldas, T. D., El Yaagoubi, A., Kohiyama, M., and Richarme, G. (1998). Purification of Elongation Factors EF-Tu and EF-G from *Escherichia coli* by Covalent Chromatography on Thiol–Sepharose. *Protein Expression and Purification*, **14**(1), 65–70.

Campanacci, V., Krieger, J., Bette, S., Sturgis, J. N., Lartigue, A., Cambillau, C., Breer, H., and Tegoni, M. (2001). Revisiting the specificity of *Mamestra brassicae* and *Antheraea polyphemus* pheromone-binding proteins with a fluorescence binding assay. *Journal of Biological Chemistry*, **276**(23), 20078–20084.

Cao, X. M., Song, F. L., Zhao, T. Y., Dong, Y. D., Sun, CH. X., and Lui, B. L. (2006). Survey of deltamethrin resistance in house flies (*Musca domestica*) from urban garbage dumps in northern China. *Environmental Entomology*, **35**(1), 1–9.

Carpenter, C. L. (2000). Actin cytoskeleton and cell signaling. *Critical Care Medicine*, **28**(4), N94–N99.

Carmel–Harel, O, Stearman, R., Gasch, A.P., Botstein, D., Brown, P.O., and Storz, G. (2001). Role of thioredoxin reductase in the Yap1p-dependent response to oxidative stress in *Saccharomyces cerevisiae*. *Molecular Microbiology*, **39**, 595 –605.

Cetin, H., Erler, F., and Yanikoglu, A. (2009). Survey of insect growth regulator (IGR) resistance in house flies (*Musca domestica* L.) from southwestern Turkey. *Journal of Vector Ecology*. **34** (2), 329–337.

Cetin, H., Erler, F., and Yanikoglu, A. (2006). Larvicidal activity of novaluron, a chitin synthesis inhibitor, against the housefly, *Musca domestica*. *Journal of Insect Science*, **6**, 50.

Chance, B. (1952). The State of Catalase in the Respiring Bacterial Cell. *Science*, **116**(3008), 202–3.

Chance, B., Sies, H., and Boveris, A. (1979). Hydroperoxide metabolism in mammalian organs. *Physiological Reviews*, **59**, 527–605.

Chen, G.D., and Pramanik, B.N. (2009). Application of LC/MS to proteomics studies: current status and future prospects. *Drug Discovery Today*, **14**(9/10), 465–471.

Chen, Z., Lu, L., Shirley, M., Lee, W. R., and Chang, S. H. (1990). Site-directed mutagenesis of glycine-14 and two "critical" cysteinyl residues in *Drosophila* alcohol dehydrogenase. *Biochemistry*, **29**(5), 1112–1118.

Chu, F.F., Doroshow, J.H., and Esworthy, R.S. (1993). Expression characterization, and tissue-distribution of a new cellular selenium-dependent glutathione peroxidase, GSHPx-GI. Journal of Biological Chemistry, **268**, 2571–2576.

Cooper, J. A. (2002). Actin dynamics: tropomyosin provides stability. *Current Biology*, **12**(15), R523–R525.

Crespo, D. C., Lecuona, R. E., and Hogsette, J. A. (1998). Biological control: An important component in integrated management of *Musca domestica* (Diptera: Muscidae) in caged layer poultry houses in Buenos Aires. *Biological Control*, **13**, 16–24.

Dabas, A., Nagpure, N.S., Kumar, R., Kushwaha, B., Kumar, P., and Lakra, W.S. (2012). Assessment of tissue-specific effect of cadmium on antioxidant defense system and lipid peroxidation in freshwater murrel, *Channa punctatus*. *Fish Physiology and Biochemistry*, **38**(2), 469–82.

Dahlem, G. (2003). House Fly (*Musca Domestica*). In V. Resh and R. Carde (Eds.), *Encyclopedia on Insects, Vol. 1, 1 Edition* (pp. 532–534). San Diego, CA: Academic Press.

Dalle-Donne, I., Giustarini, D., Rossi, R., Colombo, R., & Milzani, A. (2003). Reversible S-glutathionylation of Cys 374 regulates actin filament formation by inducing structural changes in the actin molecule. *Free Radical Biology and Medicine*, **34**(1), 23–32.

Dalle-Donne, I., Rossi, R., Colombo, G., Giustarini, D., and Milzani, A. (2009). Protein S-glutathionylation: a regulatory device from bacteria to humans. *Trends in Biochemical Sciences*, **34**(2), 85–96.

Dean, S. O., Rogers, S. L., Stuurman, N., Vale, R. D., and Spudich, J. A. (2005). Distinct pathways control recruitment and maintenance of myosin II at the cleavage furrow during cytokinesis. *Proceedings of the National Academy of Sciences of the United States of America*, **102**(38), 13473–13478.

Dear, T. N., Campbell, K., and Rabbitts, T.H. (1991). Molecular cloning of putative odorant-binding and odorant metabolizing proteins. *Biochemistry*, **30**, 10376 –10382.

Delaunay, A., Isnard, A.D., and Toledano, M.B. (2000). H_2O_2 sensing through oxidation of the Yap1 transcription factor. *EMBO Journal*, **19**, 5157–5166.

Demasi, M., Piassa Filho, G. M., Castro, L. M., Ferreira, J. C., Rioli, V., and Ferro, E. S. (2008). Oligomerization of the cysteinyl-rich oligopeptidase EP24. 15 is triggered by S-glutathionylation. *Free Radical Biology and Medicine*, **44**(6), 1180–1190.

Demidchik, V. (2015). Mechanisms of oxidative stress in plants: From classical chemistry to cell biology. *Environmental and Experimental Botany*, **109**, 212 –228.

den Hartog, G. J. M., Haenen, G.R.M.M., Vegt, E., van der Vijgh, W. J. F., and Bast, A. (2003). Superoxide dismutase: the balance between prevention and induction of oxidative damage. *Chemico-Biological Interactions*, **145**, 33–39.

Dorts, J., Silvestrea, F., Huynh, T. T., Tybergheina, A. E., Nguyen, T. P. and Kestemonta, P. (2009). Oxidative stress, protein carbonylation and heat shock proteins in the black tiger shrimp, *Penaeus monodon*, following exposure to endosulfan and deltamethrin. *Environmental Toxicology and Pharmacology*. **28**, 302–310.

Drahota, Z., Chowdhury, S.K., Floryk, D., Mracek, T., Wilhelm, J., Rauchova, H., Lenaz, G. and Houstek, J. (2002). Glycerophosphate-dependent hydrogen peroxide production by brown adipose tissue mitochondria and its activation by ferricyanide. *Journal of Bioenergtics and Biomember*, **34**, 105–113.

Du, G., and Prestwich, G. D. (1995). Protein structure encodes the ligand binding specificity in pheromone binding proteins. *Biochemistry*, **34**(27), 8726–8732.

Eaton, D.L. and Bammler, T.K. (1999). Concise review of the glutathione S transferases and their significance to toxicology. *Toxicological Sciences*, **49**, 156–64.

El-Demerdash, F.M. (2011). Lipid peroxidation, oxidative stress and acteylcholineesterase in rat brain exposed to organophosphate and pyrethroid insecticides. *Food and Chemical Toxicology*. **49**, 1346–1352.

Ellington, W. R. (2001) Evolution and physiological roles of phosphagen systems. *Annual Review of Physiology*, **63**(1), 289–325.

Enoksson, M., Fernandes, A.P., Prast, S., Lillig, C.H., Holmgren, A., and Orrenius, S. (2005). Overexpression of glutaredoxin 2 attenuates apoptosis by preventing cytochrome c release. *Biochemical and Biophysical Research Communication*, **327**, 774–9.

Esterbauer, H., and Cheeseman, K. H. (1990). Determination of aldehydic lipid peroxidation products. *Methods of Enzymology*, **186**, 407–421.

Falahzadeh, K., Banaei-Esfahani, A., and Shahhoseini, M. (2015). The potential roles of actin in the nucleus. *Cell Journal (Yakhteh)*, **17**(1), 7.

Fahey, R.C., Hunt, J.S., and Windham, G. C. (1977). On the cysteine and cysteine content of proteins. Differences between intracellular and extracellular proteins. *Journal of Molecular Evolution*, **10**(2), 155–60.

Fang, J., and Beattie, D.S. (2003). External alternative NADH dehydrogenase of *Saccharomyces cerevisiae*: a potential source of superoxide. *Free Radical Biology and Medicine*, **34**, 478–488.

Feixas, J., Prestwich, G. D., and Guerrero, A. (1995). Ligand specificity of pheromone binding proteins of the processionary moth. *European Journal of Biochemistry*, **234**(2), 521–526.

Feyereisen R. (1999). Insect P450 enzymes. *Annu Rev Entomol*, **44**, 507–533.

ffrench-Constant R. H., Daborn P.J., and Le Goff G. (2004). The genetics and genomics of insecticide resistance. *Trends in Genetics*. **20**(3), 163–170.

Filling, C., Berndt, K. D., Benach, J., Knapp, S., Prozorovski, T., Nordling, E., Ladenstein, R., Jörnvall, H., and Oppermann, U. (2002). Critical residues for structure and catalysis in short-chain dehydrogenases/reductases. *Journal of Biological Chemistry*, **277**(28), 25677–25684.

Finney, D. J. (1947). Probit analysis; a statistical treatment of the sigmoid response curve. Oxford, England: Macmillan.

Fly control in Livestock and Poultry Production. (2010). Retrieved March 29, 2012, from http://www.flycontrol.novartis.com/

Forman, H.J., and Kennedy, J. (1976). Dihydroorotate-dependent superoxide production in rat brain and liver. A function of the primary dehydrogenase. *Archives of Biochemistry and Biophysics*, **173**, 219–224.

Forstrom, J.W., Zakowski, J.J., Tappel, A.L. (1978). Identification of the catalytic site of rat liver glutathione peroxidase as selenocysteine. *Biochemistry*, **17**(13), 2639–44.

Fortunato, J. J., Agostinho, F. R., Réus, G. Z., Petronilho, F. C., Dal-Pizzol, F., and Quevedo, J. (2006). Lipid peroxidative damage on malathion exposure in rats. *Neurotoxicity Research*, **9**(1), 23–28.

Fournier, D. (2001). Mutations of acetylcholinesterase which confer insecticide resistance in insect populations. *Chemico-Biological Interactions*, **157–158**, 257–261.

Fridovich, I. (1995). Superoxide radical and superoxide dismutases. *Annual Review of Biochemistry*, **64**, 97–112.

Gagat, M., Grzanka, D., Izdebska, M., Sroka, W. D., Marszałł, M. P., and Grzanka, A. (2014). Tropomyosin-1 protects endothelial cell–cell junctions against cigarette smoke extract through F-actin stabilization in EA. hy926 cell line. *Acta Histochemica*, **116**(4), 606–618.

Gallogly, M. M., & Mieyal, J. J. (2007). Mechanisms of reversible protein glutathionylation in redox signaling and oxidative stress. *Current Opinion in Pharmacology*, **7**(4), 381–391.

Geden, C. J., Arends J. J., Axtell R. C., Barnard D. R., Gaydon D. M., Hickle L. A., Hogstette J. A., Jones W. F., Mullens B. A., Nolan Jr. M. P., Petersen J. J., and Sheppard D. C. (1994, April 12-14). Economic significance of poultry. *In G*eden C. J. and Hogsette J. A. (eds.), Research and extension needs for integrated pest management for arthropods of veterinary importance: Proceedings of a Workshop in Lincoln, Nebraska (pp. 1-328)., Gainesville, Florida: Center for Medical, Agricultural, and Veterinary Entomology USDA -ARS.

George, N., Peeyush Kumar, T., Antony, S., Jayanarayanan, S., and Paulose, C. S. (2012). Effect of vitamin D3 in reducing metabolic and oxidative stress in the liver of streptozotocin-induced diabetic rats. *British Journal of Nutrition*, **108**(08), 1410–1418.

Georgiou G., and Masip L. Biochemistry. (2003). An overoxidation journey with a return ticket. *Science*, **300**, 592–4.

Gerard-Monnier, D., Erdelmeier, I., Regnard, K., Moze-Henry, N., Yadan, J. C., and Chaudiere, J. (1998) Reactions of 1-methyl-2-phenylindole with malondialdehyde and 4-hydroalkenals. Analytical applications to a colorimetric assay of lipid peroxidation. *Chemical Research in Toxicology*, **11**(10), 1176–83.

Ghezzi, P. (2005). Oxidoreduction of protein thiols in redox regulation. *Biochemical Society Transactions*, **33**(6), 1378–1381.

Ghyselinck, N. B., and Dufaure, J. P. (1990). A mouse cDNA sequence for epididymal androgen regulated proteins related to glutathione peroxidase, *Nucleic Acids Research*, **18,** 7144.

Gilbert, H. F. (1984). Redox control of enzyme activities by thiol/disulfide exchange. *Methods in Enzymology*, **107**, 330–351.

Giray, B., Gürbay, A., and Hincal, F. (2001). Cypermethrin-induced oxidative stress in rat brain and liver is prevented by vitamin E or allopurinol. *Toxicology Letters*, **118**, 139–46.

Godovac-Zimmermann J. (2000). Editorial – The age of proteomics arrives. *Proteome*. 1-2.

Goldstein, G., Scheid, M., Hammerling, U., Schlesinger, D.H., Niall, H.D., and Boyse, E.A. (1975). Isolation of a polypeptide that has lymphocyte differentiating properties and is probably represented universally in living cells. *Proceedings of the National Academy of Sciences of the United States of America*, **72** (1), 11 –15.

Goward, C. R., and Nicholls, D. J. (1994). Malate dehydrogenase: a model for structure, evolution, and catalysis. *Protein Science*, **3**(10), 1883–1888.

Graczyk, T.K., Knight, R., and Tamang, L. (2005). Mechanical transmission of human protozoan parasites by insects. *Clinical Microbiology Reviews*, **18**, 128–132.

Graczyk, T.K., Knight, R., Gilman, R.H., and Cranfield, M.R. (2001). The role of non biting flies in the epidemiology of human infectious diseases. *Microbes and Infection*, **3**, 231–235.

Graf, P.C., Martinez-Yamout, M., Van Haerents, S., Lilie, H., Dyson, J.H., and Jakob, U. (2004). Activation of the redox-regulated chaperone Hsp33 by domain unfolding, *Journal of Biological Chemistry*, **279** (19), 20529–20538.

Greenberg, B., Kowalski, J.A., and Klowden, M.J. (1970). Factors affecting the transmission of *Salmonella* by flies: natural resistance to colonization and bacterial interference. *Infection and Immunity*, **2**, 800–809.

Griendling, K.K., Sorescu, D., and Ushio-Fukai, M. (2000). NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ. Res.* **86**, 494–501.

Groitl, B., and Jakob, U. (2014). Thiol-based redox switches. *Biochimica Et Biophysica Acta-Proteins and Proteomics*. **1844**(8), 1335–1343.

Grubel, P., Hoffman, J.S., Chong, F.K., Burstein, N.A., Mepani, C., and Cave, D. (1997). Vector potential of houseflies (*Musca domestica*) for *Helicobacter pylori*. *Journal of Clinical Microbiology*, **35**, 1300–1303.

Guidot, D.M., McCord J. M., Wright R. M., and Repine J. E. (1993). Absence of electron transport (Rho 0 state) restores growth of a manganese-superoxide dismutase-deficient *Saccharomyces cerevisiae* in hyperoxia. Evidence for electron transport as a major source of superoxide generation in vivo. *The Journal of Biological Chemistry*, **268**, 26699–26703.

Gunning, P. W., Schevzov, G., Kee, A. J., and Hardeman, E. C. (2005). Tropomyosin isoforms: divining rods for actin cytoskeleton function. *Trends in Cell Biology*, **15**(6), 333–341.

Gupta, A., Nigam, D., Gupta, A., Shukla, G. S., and Agarwal, A. K. (1999). Effect of pyrethroid-based liquid mosquito repellent inhalation on the blood-brain barrier function and oxidative damage in selected organs of developing rats. *Journal of Applied Toxicology*, 1999, **19**, 67–72

Halliwell, B., and Gutteridge, J.M.C. (2006). *Free Radicals in Biology and Medicine*, Ed 4. Oxford: Clarendon Press.

Hamann, M., Zhang, T., Hendrich, S., and Thomas, J. A. (2001). Quantitation of protein sulfinic and sulfonic acid, irreversibly oxidized protein cysteine sites in cellular proteins. *Methods in Enzymology*, **348**, 146–156.

Hara, S., Motohashi, K., Arisaka, F., Romano, P. G., Hosoya-Matsuda, N., Kikuchi, N., Fusada, N., and Hisabori, T. (2006). Thioredoxin-h1 reduces and reactivates the oxidized cytosolic malate dehydrogenase dimer in higher plants. *Journal of Biological Chemistry*, **281**(43), 32065–32071.

Hauptmann, N., Grimsby, J., Shih, J.C., and Cadenas, E. (1996). The metabolism of tyramine by monoamine oxidase A/B causes oxidative damage to mitochondrial DNA. *Archives of Biochemistry and Biophysics*, **335**, 295–304.

Hego, E., Bes, C. M., Bedon, F., Palagi, P. M., Chaumeil, P., Barré, A., Claverol, S., Dupuy, J.-W., Bonneu, M., Lalanne, C., Plomion, C., and Mench, M. (2014) Differential accumulation of soluble proteins in roots of metallicolous and nonmetallicolous populations of *Agrostis capillaris* L. exposed to Cu. *Proteomics*, **14**, 1746–1758.

Hemingway, J. (2000). The molecular basis of two contrasting metabolic mechanisms of insecticide resistance. *Insect Biochemistry and Molecular Biology*, **30**, 1009 –1015.

Hewitt, C. (1914). *The House Fly: Musca Domestica, Linnaeus: Its Structure, Habits, Development, Relation to Disease and Control.* Cambridge: University Press.

Hinshaw, D. B., Armstrong, B. C., Burger, J. M., Beals, T. F., and Hyslop, P. A. (1988). ATP and microfilaments in cellular oxidant injury. *The American Journal of Pathology*, 132(3), 479–488.

Hinshaw, D. B., Burger, J. M., Beals, T. F., Armstrong, B. C., and Hyslop, P. A. (1991). Actin polymerization in cellular oxidant injury. *Archives of Biochemistry and Biophysics*, **288**(2), 311–316.

Hinshaw, D. B., Sklar, L. A., Bohl, B., Schraufstatter, I. U., Hyslop, P. A., Rossi, M. W., Spragg, R.G., and Cochrane, C. G. (1986). Cytoskeletal and morphologic impact of cellular oxidant injury. *The American Journal of Pathology*, **123**(3), 454–464.

Hird, F. J. (1986). The importance of arginine in evolution. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, **85**(2), 285–288.

Hofmann, B., Hecht, H.J., and Flohé, L. (2002). Peroxiredoxins. *Biological Chemistry*, **383**(3–4), 347–64.

Holbrook, J. J., Liljas, A., Steindel, S. J., and Rossmann, M. G. (1975). Lactate dehydrogenase. In P. D. Boyer (Ed.), *The Enzymes* (pp. 191–292). New York, NY: Academic Press.

Holmes, K. C. (2009). Structural biology: actin in a twist. *Nature*, **457**(7228), 389–390.

Hu, W., Tedesco, S., McDonagh, B., Barcena, J.A., Keane, K., and Sheehan, D. (2010). Selection of thiol- and disulfide-containing proteins of Escherichia coli on activated thiol-Sepharose. *Analytical Biochemistry*, **398**, 245-253.

Huber, F., Schnauss, J., Rönicke, S., Rauch, P., Müller, K., Fütterer, C., and Käs, J. (2013). Emergent complexity of the cytoskeleton: from single filaments to tissue. *Advances in Physics*, **62**(1), 1–112.

Huennekens, F. M., and Whiteley, H. R. (1960). Chapter 4: Phosphoric acid anhydrides and other energy-rich compounds. In M. Florkin (Ed.) *Comparative Biochemistry V1: A Comprehensive Treatise*, pp. 107–164.

Hülya, B. (2005). Reactive oxygen species. *Critical Care Medicine*, **33**(12), S498 –S501.

Huot, J., Houle, F., Marceau, F., and Landry, J. (1997). Oxidative stress-induced actin reorganization mediated by the p38 mitogen-activated protein kinase/heat shock protein 27 pathway in vascular endothelial cells. *Circulation Research*, **80**(3), 383–392.

Huot, J., Houle, F., Rousseau, S., Deschesnes, R. G., Shah, G. M., and Landry, J. (1998). SAPK2/p38-dependent F-actin reorganization regulates early membrane blebbing during stress-induced apoptosis. *The Journal of Cell Biology*, **143**(5), 1361–1373.

Inoue, Y., Matsuda, T., Sugiyama, K., Izawa, S., and Kimura, A. (1999). Genetic analysis of glutathione peroxidase in oxidative stress response of *Saccharomyces cerevisiae*. *Journal of Biological Chemistry*, **274**, 27002–27009.

Jahngen-Hodge, J., Obin, M.S., Gong, X., Shang, F., Nowell Jr., T.R., Gong J., Abasi, H., Blumberg, J., and Taylor, A. (1997). Regulation of Ubiquitin conjugating Enzymes by Glutathione Following Oxidative Stress. *Journal of Biological Chemistry*, **272**(45), 28218–28226.

Jakob, U., Eser, M., and Bardwell, J.C. (2000). Redox switch of Hsp33 has a novel zinc binding motif, *Journal of Biological Chemistry*, **275**(49), 38302–38310.

Jakob, U., Muse, W., Eser M., and Bardwell J.C. (1999). Chaperone activity with a redox switch. *Cell*, **96**, 341–352.

Jarilla, B. R., and Agatsuma, T. (2010) Phosphagen kinases of parasites: unexplored chemotherapeutic targets. *The Korean Journal of Parasitology*, **48**(4), 281–284.

Jastroch, M., Divakaruni, A. S., Mookerjee, S., Treberg, J. R., and Brand, M. D. (2010). Mitochondrial proton and electron leaks. *Essays in Biochemistry*, **47**, 53–67.

Jönsson, T.J., Murray, M.S., Johnson, L.C., Poole, L.B., and Lowther, W.T. (2005). Structural basis for the retroreduction of inactivated peroxiredoxins by human sulfiredoxin. *Biochemistry*, **44**, 8634–8642.

Kale, M., Rathore, N., John, S., and Bhatnagar, D. (1999). Lipid peroxidative damage on pyrethroid exposure and alterations in antioxidant status in rat erythrocytes: a possible involvement of reactive oxygen species. *Toxicology Letters*, **105**, 197 –205.

Kaufman, P. E., Gerry, A. C., Rutz, D. A., and Scott J. G. (2006). Monitoring susceptibility of houseflies (*Musca domestica* L.) in the United States to imidacloprid. *Journal of Agricultural and Urban Entomology*. **23**(4), 195–200.

Kallberg, Y., Oppermann, U., Jörnvall, H., and Persson, B. (2002). Short-chain dehydrogenases/reductases (SDRs). *European Journal of Biochemistry*, **269**(18), 4409–4417.

Keiding, J. (1976). Resistance to fenitrothion in Danish houseflies, *Musca domestica. Journal of Hygiene, Epidemiology, Microbiology, and Immunology*, **1**(2), 171 –85.

Keiding, J. (1986). *The House Fly - Biology and Control.* Geneva: World Health Organization.

Keilin, D., and Hartree, E. F. (1938). On the Mechanism of the Decomposition of Hydrogen Peroxide by Catalase. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **124**(837), 397–405.

Kelley, E. E., Khoo, N. K. H., Hundley, N. J., Malik, U. Z., Freeman, B. A., and Tarpey M. M. (2010). Hydrogen peroxide is the major oxidant product of xanthine oxidase, *Free Radical Biology and Medicine*, **48**(4), 493–498.

Ketterer, B. (1982). The role of nonenzymatic reactions of glutathione in xenobiotic mechanism. *Drug Metabolism Reviews*, **13**, 161–187.

Ketterer, B. (1998). Glutathione S-transferases and prevention of cellular free radical damage. *Free Radical Research*, **28**, 647 – 68.

Kettle, D.S. (1990). Muscidae (houseflies, stableflies). In D. S. Kettle (Ed.), *Medical and Veterinary Entomology* (pp. 223–240). Wallingford: CAB International.

Kice, J. L. (1981). Mechanisms and reactivity in reactions of organic oxyacids of sulphur and their anhydrides. *Advances in Physical Organic Chemistry*, **17**, 165 –181.

Kim, K., Kim, I.H., Lee, K.Y., Rhee, S.G., and Stadtman, E.R. (1988). The isolation and purification of a specific "protector" protein which inhibits enzyme inactivation by a thiol/Fe(III)/O_2 mixed-function oxidation system. *Journal of Biological Chemistry*, **263**, 4704–4711.

Klose, J. (1975). Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals. *Humangenetik*, **26**, 231–243.

Kočišová, A., Petrovský, M., Toporčák, J., and Novák, P. (2004). The potential of some insect growth regulators in housefly (*Musca domestica*) control. *Biologia*, **59**, 661–668.

Koner, B. C., Banerjee, B. D., and Ray, A. (1998). Organochlorine pesticide-induced oxidative stress and immune suppression in rats. *Indian Journal of Experimental Biology*, **36**(4), 395–398.

Kristensen, M. (2005). Glutathione S-transferase and insecticide resistance in laboratory strains and field populations of *Musca domestica. Journal of Economic Entomology*, **98**(4), 1341–1348.

Kristensen, M. and Jespersen, J.B. (2003). Larvicide resistance in *Musca domestica* (Diptera: Muscidae) populations in Denmark and establishment of resistant laboratory strains. *Journal of Economic Entomology*, **96**(4), 1300–1306.

Kristensen, M., Spencer, A.G., and Jespersen, J.B. (2001). The status and development of insecticide resistance in Danish populations of the housefly *Musca domestica* L. *Pest Management Science*, **57**(1), 82–89.

Kryukov, G. V., Castellano, S., Novoselov, S. V., Lobanov, A. V., Zehtab, O., Guigó, R., and Gladyshev, V. N. (2003). Characterization of mammalian selenoproteomes. *Science*, **300**, 1439–1443.

Kuge, S. and Jones, N. (1994). YAP1 dependent activation of TRX2 is essential for the response of *Saccharomyces cerevisiae* to oxidative stress by hydroperoxides. *EMBO Journal*, **13**, 655–664.

Kushnareva, Y., Murphy, A.N., and Andreyev, A. (2002). Complex I mediated reactive oxygen species generation: modulation by cytochrome c and $NAD(P)^+$ oxidation-reduction state. *Biochemical Journal*, **368**, 545–553.

Latchoumycandane, C., Chitra, K. C., and Mathur, P. P. (2002). The effect of methoxychlor on the epididymal antioxidant system of adult rats. *Reproductive Toxicology*, **16**(2), 161–172.

Learmount, J., Chapman, P., and Macnicoll, A. (2002). Impact of an Insecticide Resistance Strategy for House Fly (Diptera: Muscidae) Control in Intensive Animal Units in the United Kingdom. *Journal of Economic Entomology*, **95**(6), 1245–1250.

Leichert, L.I., and Jakob, U. (2004). Protein thiol modifications visualized in vivo. *PLoS Biology*., **2**, e333.

Leiers, B., Kampkötter, A., Grevelding, C.G., Link, C.D., Johnson, T.E., and Henkle, Dührsen K. (2003). A stress-responsive glutathione S-transferase confers resistance to oxidative stress in *Caenorhabditis elegans*. *Free Radical Biology and Medicine*, **34**(11), 1405–15.

Lenaz, G. (2001). The mitochondrial production of reactive oxygen species: mechanisms and implications in human pathology. *IUBMB Life*, **52**(3-5), 159 –164.

Levine, O.S., and Levine, M.M. (1991). Houseflies (*Musca domestica*) as mechanical vectors of Shigellosis. *Reviews of Clinical Infectious Diseases*, **13**, 688–696.

Li, T., Zhao, X.P., Wang, L.Y., Gao, S., Zhao, J., Fan, Y.C., and Wang, K. (2013) Glutathione S-transferase P1 correlated with oxidative stress in hepatocellular carcinoma. *International Journal of Medical Sciences*, **10**(6), 683–690.

Lin, J. J. C., Eppinga, R. D., Warren, K. S., and McCrae, K. R. (2008). Human tropomyosin isoforms in the regulation of cytoskeleton functions. In Tropomyosin (pp. 201–222). New York, NY: Springer.

Lin, M. H., Nguyen, H. T., Dybala, C., and Storti, R. V. (1996). Myocyte-specific enhancer factor 2 acts cooperatively with a muscle activator region to regulate *Drosophila* tropomyosin gene muscle expression. *Proceedings of the National Academy of Sciences*, **93**(10), 4623–4628.

Lin, S. C., and Storti, R. V. (1997). Developmental regulation of the *Drosophila* tropomyosin I (TmI) gene is controlled by a muscle activator enhancer region that contains multiple cis‐elements and binding sites for multiple proteins. *Developmental Genetics*, **20**(4), 297–306.

Liu, F., Tang, T., Sun, L., and Priya, T. A. J. (2012) Transcriptomic analysis of the housefly (*Musca domestica*) larva using massively parallel pyrosequencing. *Molecular Biology Reports*, **39**, 1927 –1934.

Loew, O. (1900). A new enzyme of general occurrence in organisms. *Science*, **11**(279), 701–702.

Loschen, G., Azzi, A., Richter C., and Flohé, L. (1974). Superoxide radicals as precursors of mitochondrial hydrogen peroxide. *FEBS Letters*, **42**(1), 68–72.

Louvet, E., and Percipalle, P. (2008). Transcriptional control of gene expression by actin and myosin. *International Review of Cell and Molecular Biology*, **272**, 107–147.

Lukaszewicz-Hussain, A. (2010). Role of oxidative stress in organophosphate insecticide toxicity–Short review. Pesticide Biochemistry and Physiology, **98**(2), 145–150.

Lukaszewicz-Hussain, A. and Moniuszko-Jakoniuk, J. (2003). Organophosphate insecticide chlorfenvinphos affects enzymatic and nonenzymatic antioxidants in erythrocytes and serum of rats. *Polish Journal of Environmental Studies,* **12**, 417–423.

Lukaszewicz-Hussain, A. and Moniuszko-Jakoniuk, J. (1997) Procesy glikolityczne w wa˛trobie szczurawzatruciu chlorfenwinfosem. *Medycyna Pracy,* **48**, 580–583.

Lukaszewicz-Hussain, A. and Moniuszko-Jakoniuk, J. (1999). Activities of superoxide dismutase and catalase in erythrocytes and concentration of malondialdehyde in serum of rats intoxicated with chlorfenvinfos in low doses, *Polish Journal of Environmental Studies*, **8,** 234–236.

Luo, D., Smith, S. W., and Anderson, B. D. (2005). Kinetics and mechanism of the reaction of cysteine and hydrogen peroxide in aqueous solution. *Journal of Pharmaceutical Sciences*, **94**(2), 304–316.

Lv, Y., Liu, P., Xiang, C., and Yang, H. (2013). Oxidative stress and hypoxia observed in the kidneys of mice after a 13-week oral administration of melamine and cyanuric acid combination. *Research in Veterinary Science*, **95**(3), 1100–1106.

Magwere, T., West, M., Riyahi, K., Murphy, M.P., Smith, R.A.J., and Partridge, L. (2005). The effects of exogenous antioxidants on lifespan and oxidative stress resistance in *Drosophila melanogaster*. *Mechanisms of Ageing and Development*, **127**, 356-370.

Malik, A., Singh, N., and Santosh, S. (2007). Housefly (*Musca domestica*): A review of control strategies for a challenging pest, *Journal of Environmental Science and Health, Part B*, **42**, 453–469.

Mallick, P., Boutz, D. R., Eisenberg, D., and Yeates, T. O. (2002). Genomic evidence that the intracellular proteins of archaeal microbes contain disulfide bonds. *Proceedings of the National Academy of Sciences*, **99**(15), 9679–9684.

Marçon, P. C. R. G., Thomas, G. D., Siegfried, B. D., Campbell, J. B., and Skoda, S. R. (2003). Resistance Status of House Flies (Diptera: Muscidae) from Southeastern Nebraska Beef Cattle Feedlots to Selected Insecticides. *Journal of Economic Entomology,* **96**(3), 1016–1020.

Marino, S.M., and Gladyshev, V.N. (2010). Cysteine function governs its conservation and degeneration and restricts its utilization on protein surfaces. *Journal of Molecular Biology,* **404**, 902–916.

Marshall, S. (2006). *Insects: Their Natural History and Diversity.* Buffalo, New York: Firefly Books Ltd.

Mayoral, J. G., Leonard, K. T., Nouzova, M., Noriega, F. G., Defelipe, L. A., and Turjanski, A. G. (2013). Functional analysis of a mosquito short-chain dehydrogenase cluster. *Archives of Insect Biochemistry and Physiology*, **82**(2), 96–115.

McCord, J.M., and Fridovich, I. (1969). Superoxide dismutase: an enzymic function for erythrocuprein. *The Journal of Biological Chemistry*. **224**(22), 6049–6055.

McGilvery, R. W., and Goldstein, G. (1979). Biochemistry: a functional approach. WB Saunders Co.

Meier, B., Radeke, H.H., Selle, S., Younes, M., Sies, H., Resch, K., and Habermehl, G.G. (1989). Human fibroblasts release reactive oxygen species in response to interleukin-1 or tumour necrosis factor-alpha. *Biochemical Journal.* **263**, 539 –545.

Milatovic, D., Gupta, R. C., and Aschner, M. (2006). Anticholinesterase toxicity, oxidative stress. *Scientific World Journal*, **6**, 295–310.

Mills, G.C. (1957). Hemoglobin catabolism. Glutathione peroxidase, an erythrocyte enzyme which protects hemoglobin from oxidative breakdown. *Journal of Biological Chemistry*, **266**, 20752–20760

Mills, G. C. (1959). The Purification and Properties of Glutathione Peroxidase of Erythrocytes. *Journal of Biological Chemistry*, **234**, 502–506.

Monteiro, D. A., Rantin, F. T., and Kalinin, A. L. (2009). The effects of selenium on oxidative stress biomarkers in the freshwater characid fish matrinxã, *Brycon cephalus* (Günther, 1869) exposed to organophosphate insecticide Folisuper 600 BR® (methyl parathion). *Comparative Biochemistry and Physiology (Part C),* **149,** 40–49.

Munneke, L. R., and Collier, G. E. (1988). Cytoplasmic and mitochondrial arginine kinases in *Drosophila*: Evidence for a single gene. *Biochemical Genetics*, **26**(1 –2), 131–141.

Nagy, P., and Ashby, M. T. (2007). Reactive sulfur species: kinetics and mechanisms of the oxidation of cysteine by hypohalous acid to give cysteine sulfenic acid. *Journal of the American Chemical Society*, **129**(45), 14082–14091.

Nazni, W.A., Ursula, M.P., Lee, H.L., and Sa'diyah, I. (1999). Susceptibility of *Musca domestica* L. (Diptera: Muscidae) from various breeding sites to commonly used insecticides. *Journal of Vector Ecology,* **24**(1), 54–60.

NCBI Protein. Retrieved from http://www.ncbi.nlm.nih.gov/protein

Nicholls, P. (2012). Classical catalase: Ancient and modern. *Archives of Biochemistry and Biophysics*. **525**, 95–101.

Nie, J., Mahato, S., and Zelhof, A. C. (2014). The Actomyosin Machinery Is Required for *Drosophila* Retinal Lumen Formation. *PLoS Genetics*, **10**(9), e1004608.

O'Farrell, P.H. (1975). High-resolution two-dimensional electrophoresis of proteins. *Journal of Biological Chemistry*, **250**, 4007–4021.

Oshino, N., Jamieson, D., and Chance, B. (1975). The properties of hydrogen peroxide production under hyperoxic and hypoxic conditions of perfused rat liver. *Biochemical Journal*, **146**, 53–65.

Padmaja, S., and Raju, T. N. (2005). Protective effect of curcumin during selenium induced toxicity on dehydrogenases in hepatic tissue. *Indian Journal of Physiology and Pharmacology*, **49**(1), 111–114.

Partridge, C. R., Williams, E. S., Barhoumi, R., Tadesse, M. G., Johnson, C. D., Lu, K. P., Meininger, G. A., Wilson, E., and Ramos, K. S. (2005). Novel genomic targets in oxidant-induced vascular injury. *Journal of Molecular and Cellular Cardiology*, **38**(6), 983–996.

Pelosi, P., Iovinella, I., Felicioli, A., and Dani, F. R. (2014). Soluble proteins of chemical communication: an overview across arthropods. *Frontiers in Physiology*, **5**, 320.

Pelosi, P., Zhou, J. J., Ban, L. P., and Calvello, M. (2006). Soluble proteins in insect chemical communication. *Cellular and Molecular Life Sciences CMLS*, **63**(14), 1658–1676.

Pereira, C. A., Alonso, G. D., Ivaldi, S., Silber, A. M., Alves, M. J. M., Torres, H. N., and Flawiá, M. M. (2003). Arginine kinase overexpression improves *Trypanosoma cruzi* survival capability. *FEBS Letters*, **554**(1), 201–205.

Perry, T., Batterham, P., and Daborn, P.J. (2011). The biology of insecticidal activity and resistance. *Insect Biochemistry and Molecular Biology*, **41**, 411–422.

Persson, B., Kallberg, Y., Bray, J. E., Bruford, E., Dellaporta, S. L., Favia, A. D., Jörnvall, H., Kavanagh, K.L., Kedishvili, N., Kisiela, M., Maser, E., Mindnich, R., Orchard, S., Penning, T.M., Thornton, J.M., Adamski, J., and Oppermann, U. (2009). The SDR (short-chain dehydrogenase/reductase and related enzymes) nomenclature initiative. *Chemico-biological Interactions*, **178**(1), 94–98.

Peskin, A. V., Low, F. M., Paton, L.N., Maghzal, G. J., Hampton, M. B., and Winterbourn, C.C. (2007). The high reactivity of peroxiredoxin 2 with H2O2 is not reflected in its reaction with other oxidants and thiol reagents. *The Journal of Biological Chemistry*, **282**(16), 11885–11892.

Pinto, M. C., and Prado, A. P. D. (2001). Resistance of *Musca domestica* L. populations to cyromazine (insect growth regulator) in Brazil. *Memórias do Instituto Oswaldo Cruz*, **96**(5), 729–732.

Platzer, E. G., Wang, W., Thompson, S. N., and Borchardt, D. B. (1999). Arginine kinase and phosphoarginine, a functional phosphagen, in the rhabditoid nematode *Steinernema carpocapsae. The Journal of Parasitology,* **85**(4), 603 –607.

Poole, L.B. (2015). The basics of thiols and cysteines in redox biology and chemistry. *Free Radical Biology and Medicine,* **80**, 148–157.

Poole, L.B., Karplus, P.A., and Claiborne, A. (2004). Protein sulfenic acids in redox signaling, *Annuals Reviews of Pharmacology and Toxicology,* **44**, 325–347.

Rahimi, R., and Abdollahi, M. (2007) A review on the mechanisms involved in hyperglycemia induced by organophosphorus pesticides. *Pesticide Biochemistry and Physiology,* **88**, 115–121.

Ralser, M., Wamelink, M. M., Kowald, A., Gerisch, B., Heeren, G., Struys, E.A., Klipp, E., Jakobs, C., Breitenbach, M., Lehrach, H., and Krobitsch, S. (2007). Dynamic rerouting of the carbohydrate flux is key to counteracting oxidative stress. *Journal of Biology*, **6**(4), 10.

Ranjbar, A., Solhi, H., Mashayekhi, F. J., Susanabdi, A., Rezaie, A., and Abdollahi, M. (2005). Oxidative stress in acute human poisoning with organophosphorus insecticides; a case control study. *Environmental Toxicology and Pharmacology*, **20**, 88–91.

Ranson, H., Claudinos, C., Ortelli, F., Abgrall, C., Hemingway, J., Sharakova, M.V., Unger, M.F., Collins, F.H., and Feyereisen, R. (2002) Evolution of supergene families associated with insecticide resistance. *Science,* **298**, 179–181.

ReadyPrep™ Protein Extraction Kit (Cytoplasmic/Nuclear) Instruction Manual. Bio-Rad Laboratories, Inc. California, USA.

Rebrin, I., and Sohal, R. (2006). Comparison between the effects of aging and hyperoxia on glutathione redox state and protein mixed disulfides in *Drosophila melanogaster*. *Mechanisms of Ageing and Development*. **127**, 869–874.

Redman, K.L., and Rechsteiner, M. (1989). Identification of the long ubiquitin extension as ribosomal protein S27a. *Nature,* **338**, 438–440.

Reyes-Turcu, F.E., Horton, J. R., Mullally, J.E., Heroux, A., Cheng, X., and Wilkinson, K. D. (2006). The Ubiquitin Binding Domain ZnF UBP Recognizes the C Terminal Diglycine Motif of Unanchored Ubiquitin. *Cell*, **124**(6), 1197–1208.

Revenu, C., Athman, R., Robine, S., and Louvard, D. (2004). The co-workers of actin filaments: from cell structures to signals. *Nature Reviews Molecular Cell Biology,* **5**(8), 635–646.

Robinson, W. (2005). *Urban Insects and Arachnids: A Handbook of Urban Entomology*. Cambridge, UK: Cambridge University Press.

Rodríguez-Ortega, M. J., Grøsvik, B. E., Rodríguez-Ariza, A., Goksøyr, A., and López -Barea, J. (2003). Changes in protein expression profiles in bivalve molluscs (*Chamaelea gallina*) exposed to four model environmental pollutants. *Proteomics*, **3**(8), 1535–1543.

Rotruck, J.T., Pope, A.L., Ganther, H.E., Swanson, A.B., Hafeman, D.G., and Hoekstra, W.G. (1973). Selenium: Biochemical role as a component of glutathione peroxidase. *Science,* **179**(4073), 588–590.

Ryu, K.Y., Maehr, R., Gilchrist, C.A., Long, M.A., Bouley, D.M., Mueller, B., Ploegh, H.L., and Kopito, R.R. (2007). The mouse polyubiquitin gene UbC is essential for fetal liver development, cell-cycle progression and stress tolerance. *EMBO Journal*, **26**, 2693–2706.

Saccà, G. (1947). Sull' estenza di mosche domestiche resistenti al DDT. *Rivista di Parassitologia*, **8**,127–28.

Salmeen, A., Andersen, J. N., Myers, M. P., Meng, T. C., Hinks, J. A., Tonks, N. K., and Barford, D. (2003). Redox regulation of protein tyrosine phosphatase 1B involves a sulphenyl-amide intermediate. *Nature*, **423**(6941), 769–773.

Scaloni, A., Monti, M., Angeli, S., and Pelosi, P. (1999). Structural analysis and disulfide-bridge pairing of two odorant-binding proteins from *Bombyx mori*. *Biochemical and Biophysical Research Communications*, **266**(2), 386–391.

Schafer, F. Q., and Buettner, G. R. (2001). Redox environment of the cell as viewed through the redox state of the glutathione disulphide/glutathione couple. *Free Radical Biology and Medicine*, **30**(11), 1191–1212.

Schneider, A., Wiesner, R. J., and Grieshaber, M. K. (1989). On the role of arginine kinase in insect flight muscle. *Insect Biochemistry*, **19**(5), 471–480.

Scott, J. G., Liu, N., Kristensen, M., and Clark, A.G. (2009) A case for sequencing the genome of *Musca domestica* (Diptera: Muscidae). *Journal of Medical Entomology*, **46**(2), 175–182.

Scott, J. G., Warren, W. C., Beukeborn, L. W., Bopp, D., Clark, A. G., Giers, S. D., Hedger, M., Jones, A. K., Kasai, S., Leichter, C. A., Li, M., Meisel, R. P., Minx, P., Murphy, T. D., Nelson, D. R., Reid, W. R., Rinkevich, F. D., Robertson, H. M., Sackton, T. B., Sattelle, D. B., Thibaud-Nissen, F., Tomlinson, C., van de Zande, L., Walden, K. KO., Wilson, R. K., and Liu, N. (2014) Genome of the house fly, *Musca domestica* L., a global vector of diseases with adaptations to a septic environment. *Genome Biology*, **15**(466).

Scott, J.G., and Georghiou, G.P. (1985) Rapid development of high-level permethrin resistance in a field-collected strain of housefly (Diptera: Muscidae) under laboratory selection. *Journal of Economic Entomology*, **78**, 316–319.

Scott, J.G., Alefantis, T.G., Kaufman, P.E, and Rutz, D.A. (2000). Insecticide resistance in house flies from caged-layer poultry facilities. *Pest Management Science,* **56**, 147–153.

Seres, T., Ravichandran, V., Moriguchi, T., Rokutan, K., Thomas, J. A., and Johnston, R. B. (1996). Protein S-thiolation and dethiolation during respiratory burst in human monocytes. *Journal of Immunology*, **156**, 1973–1980.

Shabek, N., and Ciechanover, A. (2010). Degradation of ubiquitin: the fate of the cellular reaper. *Cell Cycle*, **9**, 523–530.

Shang, F., and Taylor, A. (1995). Oxidative stress and recovery from oxidative stress are associated with altered ubiquitin conjugating and proteolytic activities in bovine lens epithelial cells. *Biochemical Journal*, **307**, 297–303.

Sharma, A. B., Sun, J., Howard, L. L., Williams, A. G., and Mallet, R. T. (2007). Oxidative stress reversibly inactivates myocardial enzymes during cardiac arrest. *American Journal of Physiology-Heart and Circulatory Physiology*, **292**(1), H198–H206.

Shofer, S. L., Willis, J. A., and Tjeerdema, R. S. (1996). Sublethal effects of pentachlorophenol and hypoxia on rates of arginine kinase flux in red abalone (*Haliotis rufescens*) as measured by 31 P magnetization saturation transfer NMR. *Marine Environmental Research*, **42(**1), 363–367.

Sies, H., and Chance, B. (1970). The steady state level of catalase compound I in isolated haemoglobin-free perfused rat liver. *FEBS Letters*, **11**(3), 173–176.

Sies, H. (1991). Oxidative stress: Oxidants and antioxidants. *Experimental Physiology*, **82**, 291–295.

Simmons, T.W., Jamail, I. S., and Lockshin, R.A. (1989). Selenium-independent glutathione peroxidase activity associated with glutathione S-transferase from the housefly, *Musca domestica*. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, **94**(2), 323–32.

Simmons, T.W., Jamail, I. S., and Lockshin, R.A. (1987). The effect of selenium deficiency on peroxidative injury in the house fly, *Musca domestica*: A role for glutathione peroxidase. *FEBS Letters*, **218**(2), 251–254.

Sohal, R. S., and Weindruch, R. (1996). Oxidative stress, caloric restriction, and aging. *Science*, **273,** 59–63.

Song, J. J., Rhee, J. G., Suntharalingam, M., Walsh, S. A., Spitz, D. R., and Lee, Y.J. (2002). Role of glutaredoxin in metabolic oxidative stress. Glutaredoxin as a sensor of oxidative stress mediated by H_2O_2. *Journal of Biological Chemistry*, **277**, 566–75.

Srinivasan, R., Jambulingam, P., Gunasekaran, K., and Boopathidoss, P. (2008). Tolerance of housefly, *Musca domestica* L. (Diptera: Muscidae) to dichlorvos (76% EC) an insecticide used for fly control in the tsunami-hit coastal villages of southern India. *Acta Tropica*, **105**,187–190.

Steeves, C. H., Potrykus, J., Barnett, D. A., and Bearne, L. B. (2011). Oxidative stress response in the opportunistic oral pathogen *Fusobacterium nucleatum*. *Proteomics*, **11**, 2027–2037.

Stein, L. D., Harn, D. A., and David, J. R. (1990). A cloned ATP: guanidino kinase in the trematode *Schistosoma mansoni* has a novel duplicated structure. *The Journal of Biological Chemistry*, **265**(12), 6582–6588.

Strange, R.C., Jones, P.W., and Fryer, A.A. (2000). Glutathione S-transferase: genetics and role in toxicology. *Toxicology Letters*, **15**, 357–63.

Sturtz, L. A., Diekert, K., Jensen, L. T., Lill, R., and Culotta, V. C. (2001). A fraction of yeast Cu, Zn-superoxide dismutase and its metallochaperone, CCS, localize to the intermembrane space of mitochondria: a physiological role for SOD1 in guarding against mitochondrial oxidative damage. *Journal of Biological Chemistry*, **276**, 38084–38089.

Sukontason, K. L., Boonchu, N., Sukontason, K., and Choochote, W. (2004). Effects of eucalyptol on housefly (Diptera: Muscidae) and blow fly (Diptera:

Calliphoridae). *Revista do Instituto de Medicina Tropical de São Paulo*, **46**(2), 97–101.

Swaminathan, S., Amerik, A.Y., and Hochstrasser, M. (1999). The Doa4 deubiquitinating enzyme is required for ubiquitin homeostasis in yeast. *Molecular Biology of the Cell*, **10**, 2583–2594.

Swan, L., and Papp. C. (1972). *The Common Insects of North America.* New York, N.Y.: Harper and Row, Publishers, Inc.

Szalanski, A. L., Owens, C. B., Mckay, T., and Steelman, C. D. (2004). Detection of *Camplyobacter* and Escherichia coli O157:H7 from filth flies by polymerase chain reaction. *Medical and Veterinary Entomology*, **18**, 241–246.

Takahashi, K., Avissar, N., Whitin, J., and Cohen, H. (1987). Purification and characterization of human plasma glutathione peroxidase: a selenoglycoprotein distinct from the known cellular enzyme. *Archives of Biochemistry and Biophysics*, **256**, 677–686.

Tang, J. X., Janmey, P. A., Stossel, T. P., and Ito, T. (1999). Thiol oxidation of actin produces dimers that enhance the elasticity of the F-actin network. *Biophysical Journal*, **76**(4), 2208–2215.

Taylor, S. S., Oxley, S. S., Allison, W. S., and Kaplan, N. O. (1973). Amino acid sequence of dogfish M4 lactate dehydrogenase. *Proceedings of the National Academy of Sciences*, **70**(6), 1790–1793.

Tedesco, S., Doyleb, H., Blascoc, J., Redmond, G., and Sheehan, D. (2010) Oxidative stress and toxicity of gold nanoparticles in *Mytilus edulis. Aquatic Toxicology*, **100**, 178–186.

Thompson, F.C. (2004). Biosystematic Database of World Diptera. Retrieved from www.diptera.org

Trachoma. (n.d.). In World Health Organzation's website. Retrieved from http://www.who.int/topics/trachoma/en/

Turrens, J.F., Alexandre, A., and Lehninger, A.L. (1985). Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. *Archives of Biochemistry and Biophysics*, **237**, 408–414.

Turrens, J.F., and Boveris, A. (1980). Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *Biochemical Journal*, **191**, 421 –427.

Uda, K., Fujimoto, N., Akiyama, Y., Mizuta, K., Tanaka, K., Ellington, W. R., and Suzuki, T. (2006). Evolution of the arginine kinase gene family. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*, **1**(2), 209–218.

Ugbogu, O.C., Nwachukwu, N.C., and Ogbuagu, M.N. (2006) Isolation of *Salmonella* and *Shigella* species from houseflies (*Musca domestica* L.) in Utru, Nigeria. *African Journal of Biotechnology*, **5**, 1090–1091.

UniProtKB/Swiss-Prot. Retrieved at 30th January 2015 at www.uniprot.org

Ursini, F., Maiorino, M., and Gregolin, C. (1985). The selenoenzyme phospholipid hydroperoxide glutathione peroxidase. *Biochimica et Biophysica Acta*, **839**, 62 –70.

Ursini, F., Maiorino, M., Brigelius-Flohe, R., Aumann, K. D., Roveri, A., Schomburg, D., and Flohe, L. (1995). Diversity of glutathione peroxidases. *Methods of Enzymology*, **252**, 38–53.

van Montfort, R.L., Congreve, M., Tisi, D., Carr, R., and Jhoti, H. (2003). Oxidation state of the active-site cysteine in protein tyrosine phosphatase 1B. *Nature*, **423**, 773–777.

Veal, E. A., Toone, W. M., Jones, N., and Morgan, B. A. (2002). Distinct roles for glutathione S-transferases in the oxidative stress response in *Schizosaccharomyces pombe*. *Journal of Biological Chemistry*, **277**(38), 35523 –35531.

Velu, C. S., Niture, S. K., Doneanu, C. E., Pattabiraman, N., and Srivenugopal, K. S. (2007). Human p53 is inhibited by glutathionylation of cysteines present in the proximal DNA-binding domain during oxidative stress. *Biochemistry*, **46**(26), 7765–7780.

Vogt, R. G., and Riddiford, L. M. (1981). Pheromone binding and inactivation by moth antennae. *Nature*, **293**, 161–163.

Vogt, R. G., Prestwich, G. D., and Lerner, M. R. (1991a). Odorant-binding-protein subfamilies associate with distinct classes of olfactory receptor neurons in insects. *Journal of Neurobiology*, **22**(1), 74–84.

Vogt, R. G., Rybczynski, R., and Lerner, M. R. (1991b). Molecular cloning and sequencing of general odorant-binding proteins GOBP1 and GOBP2 from the tobacco hawk moth *Manduca sexta*: comparisons with other insect OBPs and their signal peptides. *The Journal of Neuroscience*, **11**(10), 2972–2984.

Wasinger, V. C., Cordwell, S. J., Cerpa-Poljak, A., Yan, J. X., Gooley, A. A., Wilkins, M. R., Duncan, M. W., Harris, R., Williams, K. L., and Humphery-Smith, I. (1995). Progress with gene-product mapping of the Mollicutes: Mycoplasma genitalium. *Electrophoresis*, **16**(1), 1090–1094.

Watson, W. H., Yang, X., Choi, Y. E., Jones, D. P., and Kehrer, J.P. (2004). Thioredoxin and its role in toxicology. *Toxicological Sciences*, **78**, 3–14.

Winter, J., Linke1, K., Jatzek, A., and Jakob, U. (2005). Severe oxidative stress causes inactivation of DnaK and activation of the redox-regulated chaperone Hsp33, *Molecular Cell*, **17**(3), 381–392.

Woo, H.A., Jeong, W., Chang, T.S., Park, K.J., Park, S.J., Yang, J.S., and Rhee, S. G. (2005). Reduction of cysteine sulfinic acid by sulfiredoxin is specific to 2-cys peroxiredoxins. *The Journal of Biological Chemistry*, **280**, 3125–3128.

Wu, H., Naya, F. J., McKinsey, T. A., Mercer, B., Shelton, J. M., Chin, E. R., Simard, A. R., Michel, R. N., Bassel-Duby, R., Olson, E. N., and Williams, R. S. (2000). MEF2 responds to multiple calcium-regulated signals in the control of skeletal muscle fiber type. *The EMBO Journal*, **19**(9), 1963–1973.

Yan, L.J., Levine, R.L., and Sohal, R. (2000). Effects of aging and hyperoxia on oxidative damage to cytochrome c in the housefly, *Musca domestica. Free Radical Biology and Medicine*, **29**(1), 90–97.

Yin, Z., Ivanov, V.N., Habelhah, H., Tew, K., and Ronai, Z. (2000). Glutathione S transferase elicits protection against H_2O_2-induced cell death via coordinated regulation of stress kinases. *Cancer Research*, **60**, 4053–4057.

Zangar, R. C., Davydov, D. R., and Verma, S. (2004). Mechanisms that regulate production of reactive oxygen species by cytochrome P450. *Toxicology and Applied Pharmacology*, **199**(3), 316–331.

Zheng, M., Åslund, F., and Storz, G. (1998). Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science*, **279**(5357), 1718–1722.

Zhou, G., Somasundaram, T., Blanc, E., Parthasarathy, G., Ellington, W. R., and Chapman, M. S. (1998). Transition state structure of arginine kinase: implications for catalysis of bimolecular reactions. *Proceedings of the NationalAcademy of Sciences,* **95**(15), 8449–8454.

Zhong, M., Wang, X., Wen, J., Cai, J., Wu, C., and Aly, S. M. (2013). Selection of reference genes for quantitative gene expression studies in the house fly (*Musca domestica* L.) using reverse transcription quantitative real-time PCR. *Acta biochimica et biophysica Sinica*, **45**(12), 1069–1073.

Zhu, F., Li, T., Zhang, L., and Liu, N. (2008). Co-up-regulation of three P450 genes in response to permethrin exposure in permethrin resistant house flies, *Musca domestica*. *BMC Physiology.* **8**(18).

Zurek, L., Denning, S.S., Schal, C., and Watson, D.W. (2001). Vector competence of *Musca domestica* (Diptera: Muscidae) for *Yersinia pseudotuberculosis*. *Journal of Medical Entomology*, **38**, 333–335.

APPENDICES

Appendix A: Buffer solution preparation

Binding buffer solution (0.1M Tris-HCl pH 7.5, 0.5M NaCl, 1mM EDTA)

To make 1L of binding buffer solution, 141.1g of Tris-base was dissolved into 1L of distilled water. 29.22g of NaCl and 0.2922g of EDTA was mixed with the 1L of the Tris solution and the pH was adjusted with 1M HCl.

Homogenizing buffer

25mM NaH_2PO_4 buffer, pH 7.4 was prepared by dissolving 0.390g of NaH_2PO_4 in 1L of distilled water and the pH was adjusted with 1M NaOH. 50ml of NaH_2PO_4 was dissolved with 0.5ml of protease inhibitor (Sigma-Aldrich), 0.019g of EDTA, 0.008g of DTT and traces of phenylthiourea.

Appendix B: Solutions for 2-dimensional gel electrophoresis

Solubilizing buffer (8M urea, 4% CHAPS, 65mM DTT, 3M thiourea)

To prepare the solubilizing buffer, 0.48g of urea was dissolved in approximately 0.45ml of deionized water with 0.01g of DTT and 0.23g of thiourea. 0.04g of CHAPS was added later, the mixture topped to 1ml and a trace of bromphenol blue was added. The mixture was mixed thoroughly by inverting.

Rehydration solution (8M urea, 2% CHAPS, 0.2%(w/v) DTT, 2% carrier ampholytes(pH 3-10))

To prepare the solubilizing buffer, 0.48g of urea was dissolved in approximately 0.45ml of deionized water with 0.002g of DTT and 20µl of carrier ampholytes (pH 3-10). 0.02g of CHAPS was added later, the mixture topped to 1ml and a trace of bromphenol blue was added. The mixture was mixed thoroughly by inverting.

Equilibration buffer 1 (1.5M Tris-HCl buffer, 6M Urea, 4.7M glycerol, 2% (w/v) SDS, 16.2mM DTT)

1ml of pH 8.8, 3.6036g of urea, 3.5ml of glycerol, and 0.2g of SDS was added and topped up into 10ml of solution with deionized water in a 10ml centrifuge tube.

25mg of DTT was added into and mixed thoroughly.

Equilibration buffer 2 (1.5M Tris-HCl buffer, 6M Urea, 4.7M glycerol, 2% (w/v) SDS, 0.243mM 2-iodoacetamide)

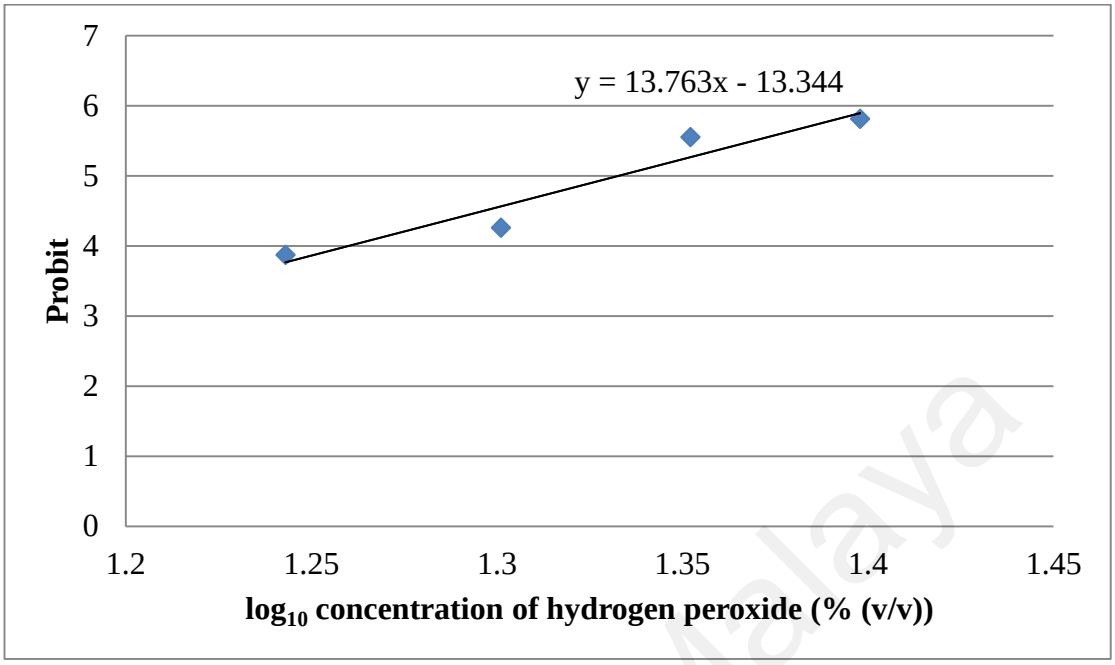
1ml of pH 8.8, 3.6036g of urea, 3.5ml of glycerol, and 0.2g of SDS was added and topped up into 10ml of solution with deionized water in a 10ml centrifuge tube. 0.45g of 2-iodoacetamide was added into and mixed thoroughly.

SDS gel staining

To prepare 1L of 5% Coomassie Brilliant Blue R-250 stain, 1g of CBB was dissolved in 20ml of distilled water. The mixture was sonicated around 5 minutes until the CBB was fully dissolved. 11.8ml of concentrated phosphoric acid was added into the CBB solution and mixed thoroughly. 100g of ammonium sulphate was dissolved in 600-700ml of distilled water and poured into 1L volumetric flask through a glass funnel. The volume was topped up to 1L.

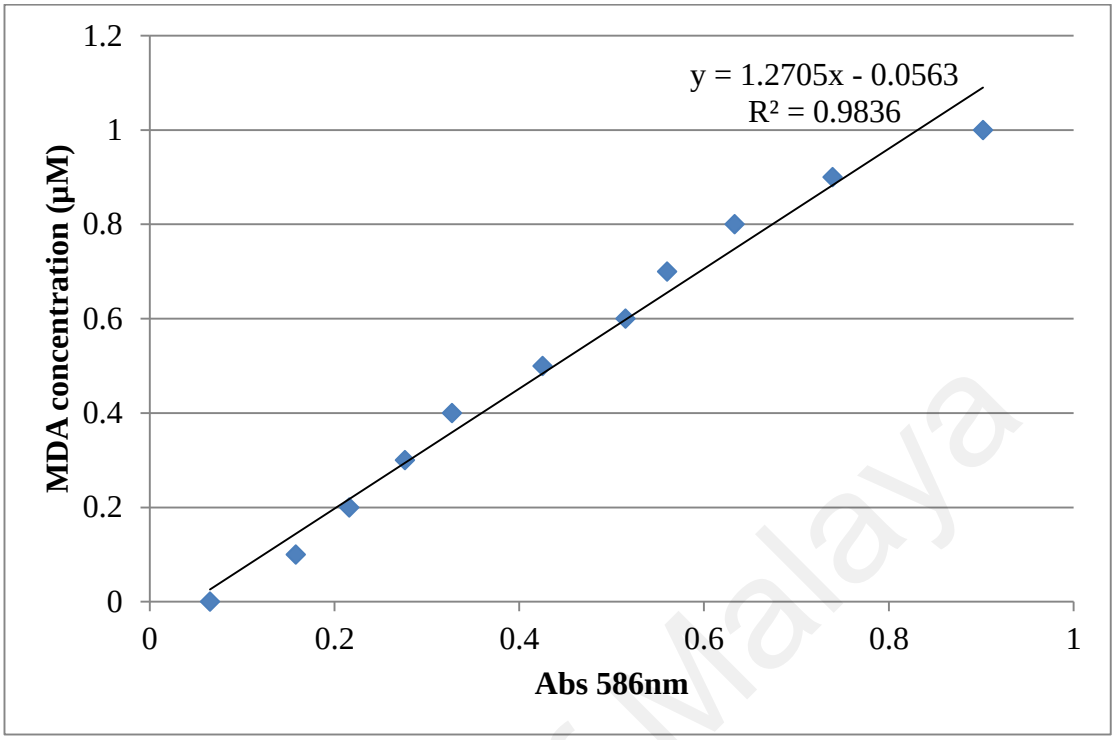
For gel destaining, CBB stain was prepared by mixed 80ml of 5% CBB solution to 20ml of methanol.

Appendix C: Probit analysis for LC_{50} determination



Graph: The probit graph plotted with probit values against log10 concentration of hydrogen peroxide (% (v/v))

Appendix D: Standard curve of malondialdehyde



Graph: Malondialdehyde standard curve, plotted with concentrations of MDA (µM) against Abs 586nm.

Appendix E: 3D images of spots with high fold change.

Table: 3D image of control spots more than 5x in fold change in proteome expression profile of *Musca domestica* 3rd instar larvae.

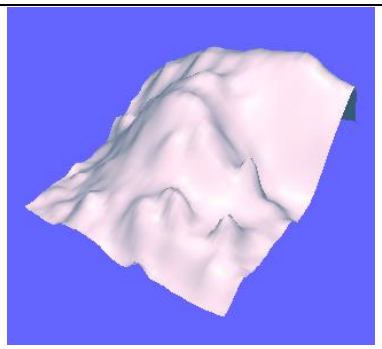
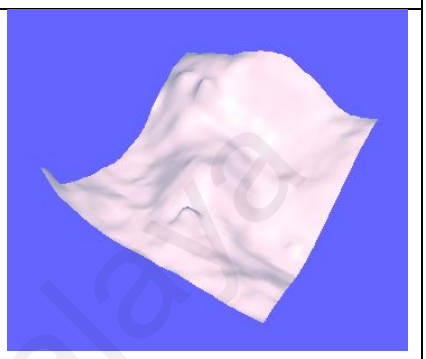
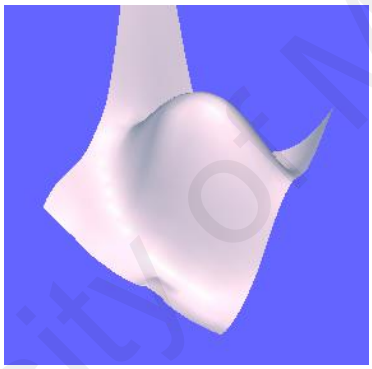
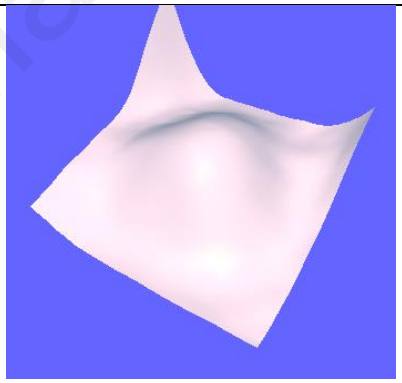
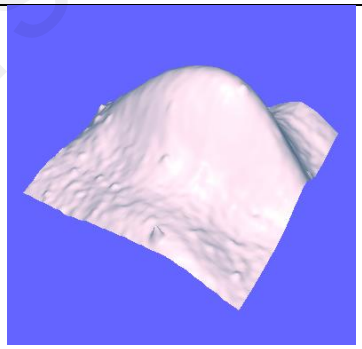
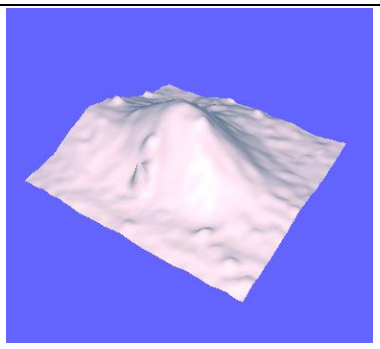
Spot ID	Label	Control	Treated
2507	A		
7703	B		
9003	C		

Table: 3D image of treated spots more than 5x in fold change in proteome expression profile of *Musca domestica* 3rd instar larvae.

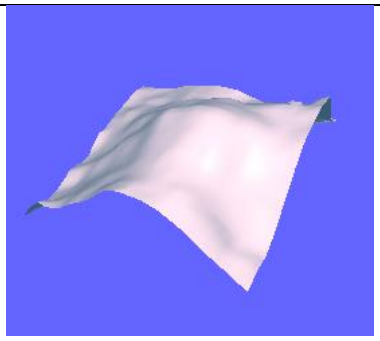
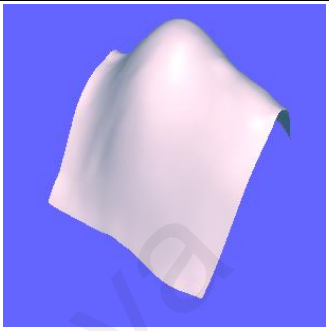
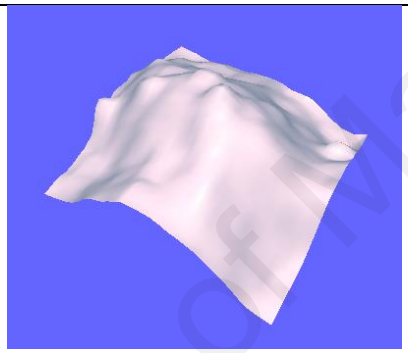
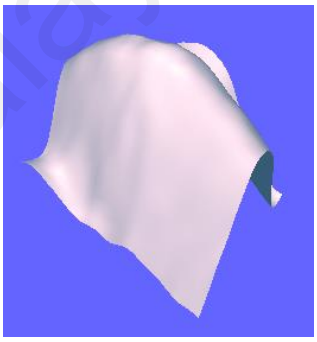
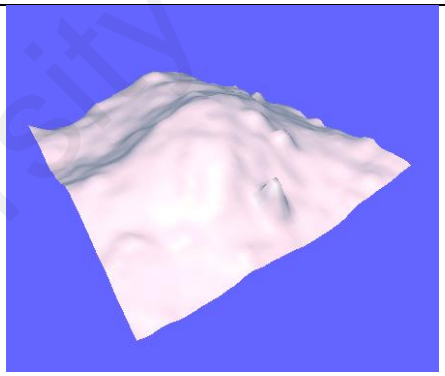
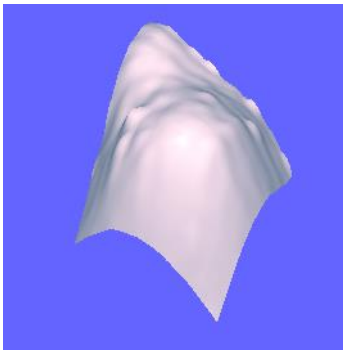
Spot ID	Label	Control	Treated
1603	D		
4701	E		
5205	F		

Table: 3D image of control spots more than 5x in fold change in thiol proteome expression profile of *Musca domestica* 3rd instar larvae.

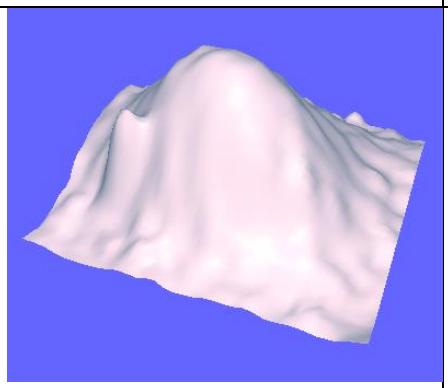
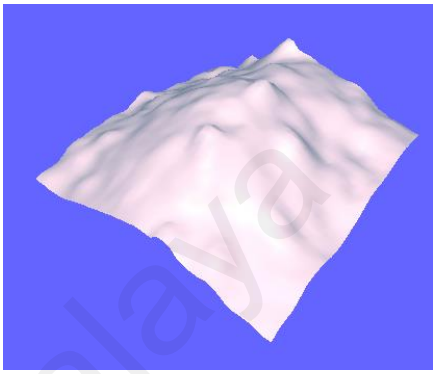
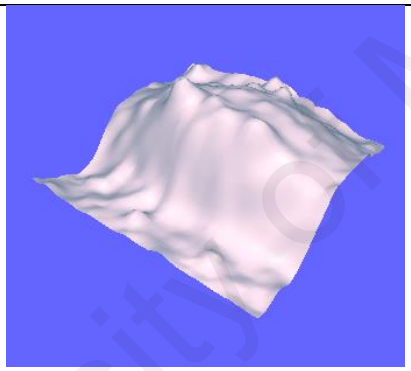
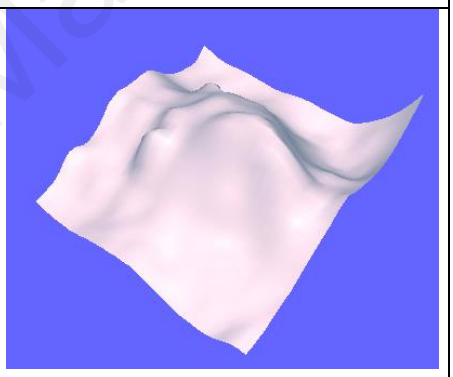
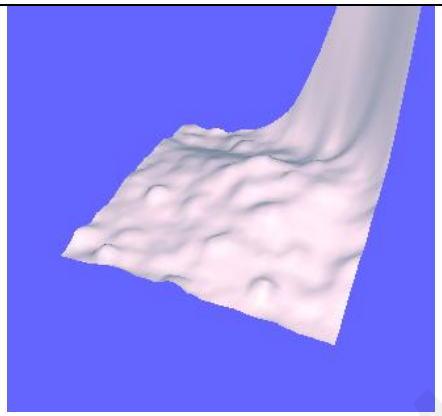
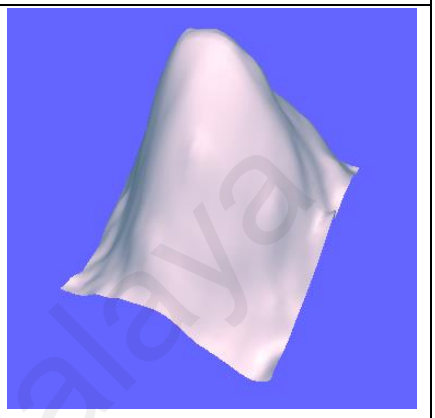
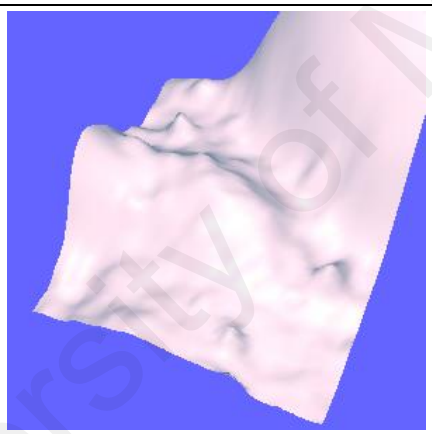
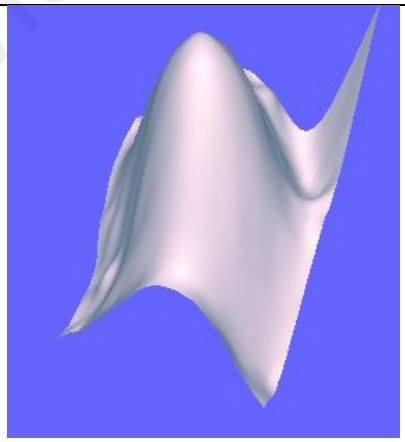
Spot ID	Label	Control	Treated
5305	G		
5503	H		

Table: Treated spots more than 5x in fold change in thiol proteome expression profile of *Musca domestica* 3rd instar larvae.

Spot ID	Label	Control	Treated
2503	I		
3403	J		

Appendix F: Summary of the LC-MS/MS analysis.

ZipTip® C18 resin (Merck Millipore) desalting protocols

After in gel digestion, the sample prior to injection, the digested peptide was desalted to improve mass spectrometry detection and purify the peptides.

Table: Solutions for ZipTip® C18 resin desalting procedure.

Solution	Composition
Hydration Solution	50:50, ACN:H_2O, 0.1% TFA
Wash Solution	0.1% TFA in H_2O
Peptide Elution Solution	60:40, ACN:H_2O, 0.1% TFA
Reconstitution Solution For Dried Samples	5:95, ACN:H_2O, 0.1% TFA

Dried samples was reconstituted with 13 µl of reconstitution solution; vortex and centrifuge. The pH of the sample was checked and readjusted to equal or less than 3 with 10% TFA.

C18 resin was used for peptide desalting. It was important to pipet slowly to avoid introducing air into the packing material and to maximize binding throughout the process. The peptide elution solution was chilled in ice.

The resin was hydrated by aspirating and discarding 10 µl of hydration solution twice and followed by aspirating and discarding 10 µl of wash solution, twice. 10 µl of sample was slowly aspirated and expelled the liquid back into the tube. This process was repeated 5-6 times.

After that 10 µl H_2O was drawn and expelled and this step was repeated 4-5 times. 1.3 µl aliquot of peptide elution solution was aspirated and expelled twice.

Before injection, the sample was transferred by the ZipTip and dried with SpeedVac^{TM} (Thermo Scientific) and resuspended in 4µl 0.1% formic acid in water.

Liquid chromatography parameters

The column used was C18, 160nl enrichment column and 75µmx150mm analytical column (Agilent). Flow rate employed was 4µl/min from Agilent 1200 Series Capillary pump and 0.3µl/min from Agilent 1200 Series Nano Pump. Sample injection volume was 2µl. The solvent gradient was summarized at table below.

Table: Solvent gradient used during LC-MS/MS analysis

Time (min)	Solvent A(%)	Solvent B(%)
Initial	97	3
30	50	50
32	5	95
37	5	95
38	97	3

(Note: Stop time was at 47 minutes;Solvent A was 90% acetonitrile in water and solution B was 0.1% formic acid.)

Mass spectrometry parameters

Positive ionization was used with the Vcap of 1800V and the fragmentor voltage was set to 175V. Drying gas temperature was 325ºC and the flow was 5.0 L/min.

In the data acquisition department, the spectra acquired via two modes with the MS scan range of 110-3000 m/z, while in the MS/MS the scan range 50-3000 m/z was set.

Precursor selection

For the precursor selection I, the absorbance threshold was 200. Precursor selection II was performed to select fragments of charge states of 2, 3, or more than 3. Precursor ion with the m/z of 922.009798 (charge state=1) and 121.050873 (charge state=1) are excluded as they were reference ions.

Data analysis

Data was processed with aforementioned Agilent Spectrum Mill MS Proteomics Workbench software packages. The scan range for MH^+ ion was set from 600 to 4000 Da. Database search was done on SwissProt and NCBI. Filter by protein score was set by more than 11, and the peptide search filter was set to be more than 6. %SPI filter was toggled to be more than 60%.

Table: Additional protein rules of autovalidation of database search.

Rule	Precursor Charge	Score Threshold	% SPI Threshold	Fw-Rev Score Threshold	Rank 1-2 Score Threshold
1	2	6	60	2	2
2	1	6	70	2	2
3	3	8	70	2	2
4	4	8	70	2	2
5	5	12	70	2	2
6	2	6	90	1	1