Chapter 1

Neurotoxic implications of lead (Pb) exposure in *Drosophila* system and protein enrichment as a nutritional intervention strategy to alleviate oxidative stress and neurotoxicity
1.0 INTRODUCTION

Lead (Pb) is a ubiquitous, persistent and non-essential toxic heavy metal which can be detected in almost all areas of the environment and biological systems (Xu et al., 2008). The role of Pb as a neurological toxicant is well established and its exposure has been associated with reductions in cognitive function, hearing loss, hyperactivity, shortened concentration spans and poor school performance in children (Tong et al., 2000). Epidemiological evidence suggests Pb to be a high environmental risk factor for the development of attention-deficit hyperactivity disorder (ADHD) (Froehlich et al., 2009; Luo et al., 2014). Several surveys have also indicated higher blood Pb levels among children in cities in India (Kumar and Clark, 2009) and its correlation with increased neurobehavioral deficits/ADHD. A recent review has illustrated the scenario of Pb exposure in South Africa and concluded that not only children but a large numbers of people are at risk of Pb exposure and concomitant health, neuro-developmental and social effects (Mathee, 2014).

Since Pb has no known physiologically relevant role in the body, its toxicity comes from its ability to mimic other biologically important metals, most notably calcium, iron, and zinc which act as cofactors in many enzymatic reactions. Pb is able to bind to and interact with many of the same enzymes as these metals. However, due to its differing chemistry, does not properly function as a cofactor, thus interfering with the enzyme’s ability to catalyze its normal reaction(s). Pb exposure has been demonstrated to cause generation of excessive amount of ROS and perturbations of antioxidant defense systems in animals (Adonaylo and Oteiza, 1999; Bokara et al., 2008; Neal and Guilarte, 2010; Prasanthi et al., 2010) and in occupationally exposed workers (Gurer-Orhan et al., 2004). Substantial experimental evidence has shown that Pb is capable of interacting with nuclear proteins and DNA causing oxidative damage to biological macromolecules. Hence, there is general consensus that oxidative stress mechanisms play an important role in the adverse effects of Pb such as impaired learning ability in children.
Chronic exposure to environmentally relevant levels of Pb during early life is shown to alter morphology and neurogenesis in the hippocampus of young rats (Verina et al., 2007). Recent evidence in rats has convincingly demonstrated that pre- and neonatal exposure to Pb induces ultrastructural/molecular alterations in the hippocampus and alters postnatal cholinergic and aminergic systems (Baranowska-Bosiacka et al., 2012; Basha et al., 2012). Further, another recent study in rats has shown that pre- and neonatal exposure to Pb that results in Pb concentrations below the level currently thought to be safe (10 µg/dL) in offspring blood resulted in disruption of the pro/antioxidant balance as well as downregulation of mRNA and protein expression especially in the hippocampus (Baranowska-Bosiacka et al., 2012).

Epidemiological evidence have shown that malnutrition (MN), a worldwide problem affecting millions of unborn/young children during the most vulnerable stages of their brain development results in behavioral abnormalities, cognitive dysfunctions and impaired learning/memory (Morgane et al., 2002; Lister et al., 2005; Cardoso et al., 2013). Further, pre- and early postnatal MN produces behavioral impairments, memory deficits (Lukoyanov and Andrade, 2000; Almeida and De Araujo, 2001; Valadares and de Sousa Almeida, 2005) and deleterious effects on the population of GABA neurons in the dentate gyrus and cornu ammonis of the dorsal hippocampus (Diaz-Cintra et al., 2007). Further, it is well known that Pb exposure is often greater among children of low socioeconomic status (SES) (Toscano and Guilarte, 2005) and low SES is a powerful predictor of neurodevelopment (Tong et al., 2007). Besides, experimental and epidemiological data suggest that SES might also modify Pb neurotoxicity (Bellinger, 2008). Current therapeutic approaches in the treatment of childhood Pb intoxication are not effective in reversing learning deficits once they have occurred. Moreover, application of the conventional chelators in children is somewhat prohibited due to adverse health effects. Hence, researchers have focused on the use of selected nutrients such as methionine and choline, to prevent Pb-induced cognitive impairment (Fan et al., 2010). Low protein levels in the diet and the consequent decrease in essential amino acids are known to significantly alter the antioxidant system and the redox state in the
hippocampus (Bonatto et al., 2005; Feoli et al., 2006; Tatli et al., 2007). A recent study demonstrated that postnatal protein malnutrition (PMN) induces several neurochemical alterations leading to behavioral deficits in rats (Adebayo et al., 2014).

*Drosophila melanogaster* has been extensively used to understand the pathophysiology and genetics of several human neurodegenerative diseases (Botella et al., 2009; Feany, 2010). *Drosophila* has distinct advantages with respect to life cycle duration, laboratory expenses, genetic manipulability, efficiency of screening methods and conservation with higher organisms (Rand, 2010). In our laboratory, we have successfully employed the *Drosophila* system to obtain insights into the neuromodulatory propensity of spice bioactives and phytochemicals (Krishna and Muralidhara, 2015; Prasad and Muralidhara, 2012, 2014; Girish and Muralidhara, 2012; Hosamani and Muralidhara, 2009). Some researchers consider *Drosophila* an excellent animal model for studying the neurotoxicology of lead (Hirsch et al., 2003, 2012). Substantial evidence suggests that protein is the major dietary component affecting oxidative stress and longevity in flies (Bruce et al., 2013).

Understanding the interactions between protein deficiency and toxicity is highly critical since oxidative stress is a common denominator under such conditions. In recent times, the *Drosophila* system is extensively used to understand the interaction between nutrients and environmental toxicants. Currently, nutrition intervention strategies are being considered as therapeutic in the area of neuroprotection, since they may play a preventive or suppressive role (Virmani et al., 2013). Hence it may be important to investigate whether dietary protein (quantity and quality) has the potential to influence the adverse effects of Pb in animal models. It is in this context that we have employed *Drosophila* as a model to examine the ameliorative potential of casein-enriched diet under Pb exposure.

No attempts have been made to determine if dietary enrichment with protein such as casein can alleviate Pb-induced adverse effects in animal models. Hence, it was hypothesized that casein-enrichment is likely to modulate the susceptibility of flies to Pb exposure and may provide a dietary approach to
alleviate low-level Pb exposure in children in general and those subjected to protein deficiency conditions, in particular. With this objective, initially, toxicity profiles for Pb acetate was determined in the regular culture maintenance media (jaggery based) in two different age groups (young and adult), while gender difference was assessed in D. melanogaster. The Pb induced toxic response (oxidative stress, mitochondrial dysfunction and neurotoxicity) were compared with toxic response obtained employing a sucrose based synthetic medium. The results obtained have been presented in Section A.

In the second series of investigations, the propensity of selected proteins to attenuate Pb induced toxic response was studied in young flies. Comprehensive investigations were conducted to assess the potential of casein—enrichment as a nutrient intervention to alleviate Pb-mediated oxidative stress, neurotoxicity in young flies. The choice of young flies was based on their increased lethality response to Pb exposure and the fact that children are known to be more susceptible to Pb intoxication. The results obtained have been presented in Section B.

2.0 OBJECTIVE

The primary objective of this investigation was to utilize the wild strain of Drosophila melanogaster as a model system to obtain evidence on the neurotoxic implications of Pb and also examine its suitability as an in vivo model to understand if protein enrichment can significantly attenuate the neurotoxic impact of Pb.
3.0 EXPERIMENTAL DESIGN

SECTION – A

3.1 Recapitulation of lead (Pb) acetate-induced neurotoxic implications in *Drosophila melanogaster*

3.1.1 Preparation of Pb acetate (PbA)
A stock of 1000 mM lead acetate (PbA) solution was prepared in deionized water. Working standards were prepared in deionized water keeping a constant volume of 100 µL/ 2 mL media for each of the required concentration to be tested.

3.1.2 Pb exposure and lethality response among young and adult flies
In the first set of experiments, both young and adult male flies (n= 50/replicate; 3 replicates per group) maintained on media containing wheat cream agar (Jaggery-based) diet were exposed to Pb at varying concentrations (1, 5, 10 and 20 mM) in the media (2 mL) for 7 days to assess their susceptibility pattern in terms of lethality. Flies were monitored regularly for the incidence of mortality.

3.1.3 Pb-induced locomotor phenotype
In a second set of experiments, young and adult flies were exposed to Pb at three concentrations (1, 5 and 10 mM) in the diet for 5 days to determine hyperactivity phenotype following standardized assay methods (as described in Materials and Methods).

3.1.4 Pb-Induction of oxidative stress response
In a separate series of experiments, flies were exposed (for 5 days) to three concentrations of PbA (1, 5 and 10 mM) for assessing the early induction of oxidative stress and alterations in biochemical markers. At the end of 5 days, head and body regions were separated (under mild ether anesthesia) and were pooled from each group and processed separately to obtain cytosolic and mitochondrial fractions (as described in Materials and Methods).
The following biochemical measurements were made in the cytosolic fractions of both head/body regions (as described in Materials and Methods):

**Assessment of oxidative damage:**

**Markers of oxidative stress:** Reactive oxygen species (ROS) and hydroperoxide (HP) levels.

**Redox status:** Glutathione (GSH) and total thiols (TSH) levels.

**Nitrite levels and protein oxidation:** Nitric oxide (NO) and protein carbonyl levels.

**Antioxidant enzyme activities:** Antioxidant enzymes activities (viz., catalase, SOD, TRR and GST).

**Mitochondrial function:** Activities of functional enzyme viz., complex I-III and MTT reduction.

**Neurochemical markers:** The activity levels of acetylcholinesterase (AChE) enzyme and the dopamine (DA) levels.

### 3.2 Neurotoxic effects of Pb: Synthetic media

The objective of this series of experiments was to determine whether synthetic media (Sucrose-based) has any impact on Pb-induced locomotor phenotype, mortality response and oxidative stress induction.

#### 3.2.1 Susceptibility pattern among young and adult male flies

In the first set of experiments, both young and adult male flies (n = 50/replicate; 3 replicates per group), were fed on media containing sucrose-agar diet (Good and Tatar, 2001) with PbA (5, 10 and 20 mM) in the medium (2 mL) for 7 days in order to assess their susceptibility pattern in terms of lethality. Flies were monitored regularly for the incidence of mortality.

#### 3.2.2 Susceptibility pattern among young male and female flies

In the second set of experiments, both young male and female flies (n = 50/replicate; 3 replicates per group), were fed on media containing sucrose-agar diet (Good and Tatar, 2001) with PbA (5, 10 and 20 mM) in the medium (2 mL) for 7 days in order to assess their gender differences with respect to Pb-induced lethality.
3.2.3 Locomotor phenotype

Young male flies were exposed to Pb at three concentrations (1, 5 and 10 mM) in the sucrose-based diet for 5 days to determine hyperactivity phenotype following standardized assay methods (as described in Materials and Methods).

3.2.4 Pb-induced oxidative stress, mitochondrial dysfunction and neurotoxicity (5 day exposure)

Various biochemical assays were carried out in young flies after 5 day exposure to Pb (1, 5 and 10mM). These comprised of - Assessment of oxidative damage, alterations in redox status, antioxidant enzyme activities, mitochondrial function, and perturbations in the neurochemical markers. The biochemical assays are described in the previous experimental design (Section 3.1).

SECTION – B

3.3 Protein-enrichment as a strategy to alleviate Pb neurotoxicity

With an objective of determining whether protein enrichment can alleviate/attenuate Pb-induced neurotoxic response, experiments were designed in young flies and the mortality response was determined following 7day co-exposure paradigm. For this study, three dietary proteins namely casein, whey protein isolate and soy protein isolate were selected and incorporated into the synthetic medium at 1 and 2%. Casein conferred protection against Pb-induced lethality. Hence, casein (1 and 2%) was employed to assess its protective effect on locomotor phenotype and to study the biochemical alterations in head and body regions.

3.4 Casein-enrichment as a nutrient intervention to abrogate Pb-induced neurotoxic implications

In this set of experiments, casein (1 and 2%) enriched medium was employed as a nutrient intervention to attenuate Pb–induced neurotoxic implications in young male flies. Prior to the modulatory studies, the influence of casein–enrichment on
the various endogenous levels of oxidative markers were determined in both head and body regions of the flies.

3.4.1 Influence of casein-enriched medium on endogenous levels of oxidative markers

Young male flies (n=50/replicate; 3 replicates per group) were exposed to Pb (5mM) with or without casein (1 and 2 %) for 5d. Terminally, the efficacy of casein to modulate the endogenous levels of oxidative markers was determined in the cytosolic and mitochondrial fractions of both head and body regions of flies.

3.4.2 Modulatory effect of casein-enrichment against Pb-induced neurotoxicity

In a co-exposure paradigm, the modulatory effect of casein (1 and 2%) on Pb-induced (10mM) lethality was assessed. In a second set of experiments, flies were exposed to Pb (5 mM) with or without casein (1 and 2%) for 5d and locomotor phenotype were assayed on days 5. Biochemical markers listed below were determined in the cytosolic fractions of both head and body regions at the end of 3 days (as described in Materials and Methods).

Oxidative stress markers: ROS, HP, MDA, TSH, GSH, antioxidant enzymes activities (viz., catalase, SOD, TRR and GST) protein carbonyl levels; were determined in cytosolic fractions of both head and body regions.

Mitochondrial function: Complex I-III and MTT reduction.

Neurochemical markers: Acetylcholinesterase (AChE) activity and dopamine (DA) content.
4.0 RESULTS

SECTION -A

4.1 Recapitulation of lead (Pb) acetate induced neurotoxic implications in *Drosophila melanogaster*

Exposure of Pb in regular culture media

For this study, experiments were designed by exposing young and adult flies to Pb acetate in the regular media (detailed in materials and methods section-Table 1) which is employed to maintain the fly cultures.

4.1.1 Lethality response among young and adult flies

Exposure of young and adult flies to Pb acetate in regular culture media (Jaggery based) on 7 consecutive days resulted in a concentration dependent lethality (Fig 1.1A). In general, young flies were more susceptible to Pb compared to adult flies. Pb at lower concentrations (1 and 5mM) failed to cause any mortality among either young or adult flies. However, young flies exhibited a higher degree of mortality to Pb exposure (10mM -12%; 20mM -70%) In comparison, adult flies were more resistant, since no mortality ensued even at a concentration of 10mM. However, Pb at the highest concentration (20mM) caused 44% lethality) among adult flies (Fig 1.1A).

4.1.2 Locomotor phenotype

The locomotor phenotype -hyperactivity response was measured in terms of flight speed on day 5 following exposure to varying concentrations of Pb. Both young and adult flies exhibited a significant degree of hyperactivity at all concentrations (Fig 1.1B) and the fastest speed was evident at the mid concentration of 5mM (nearly 90% increase over the control) in both young and adult male flies (Fig 1.1B).
4.1.3 Induction of global oxidative stress

Data on the oxidative stress response determined as ROS generation and hydroperoxide (HP) levels in both head and body regions of young and adult flies following exposure to Pb acetate (1, 5 and 10 mM) are presented in Fig 1.2A-D. Pb caused significant elevations in ROS levels both in head and body regions (Fig 1.2A). In the head region of young flies significant increase in ROS levels (1mM-32%; 5mM-36%) was evident compared to head region of adult flies (1mM-25%; 5mM-23%). Likewise, significant increase in ROS levels in the body region (5mM-45%; 10mM-79%) of young flies was also evident, while, Pb exposure had no effect on ROS levels in the body region of adult flies (Fig 1.2B). Further, in the head region of young flies, significant decrease in HP levels (5mM-28%; 10mM-15%) was observed. Interestingly, HP levels were elevated in head region of the adult flies (15% at all concentrations) (Fig 1.2C). However, the HP levels in the body region of young flies were marginally decreased (5mM-14%), while, there was no change in the body region of adult flies (Fig 1.2D).

4.1.4 Pb-induced alterations in Redox status

Glutathione (GSH) levels in the head region of young flies were marginally elevated (10mM-22%) (Fig 1.3A). However, Pb had no significant no effect on the levels in the head region of adult flies (Fig 1.3A). Interestingly, significant increase in the GSH levels in the body region (10mM-17-21%) of both young and adult flies was observed (Fig 1.3B). Similarly, marginal increase in total thiol levels in the head region (10mM-10%) of young flies was evident, whereas, no change was noted in the head region of adult flies (Fig 1.3C). Interestingly, significant decreases in the total thiol levels in body region (10mM-18-21%) of both young and adult flies were observed (Fig 1.3D).

4.1.5 Effect of Pb on nitrite levels and protein oxidation

Nitric oxide levels were significantly enhanced in the head region of both young (5mM-22%; 10mM-22%) and adult (5mM-49%; 10mM-45%) flies (Fig 1.3E). Similarly, in young flies, the protein carbonyl levels in the body region were significantly increased (Fig 1.3F).
4.1.6 Effect on antioxidant enzyme activities

Data on the effect of Pb exposure on the activity levels of antioxidant enzymes is presented in Fig. 1.4. Although the activity of catalase was consistently elevated in head region of both young and adult flies (Fig 1.4A), differential response was evident in the body region. While, significant increase in the CAT activity in the body region of young flies was observed, the levels were diminished in body region of adult flies (Fig 1.4B). In contrast, the activity of SOD was diminished in the body region of both the young (5mM- 25%; 10mM- 25%) and adult flies (5mM- 19%; 10mM- 33%) (Fig 1.4C).

4.1.7 Effect on thioredoxin reductase and glutathione-S-transferase activity

Pb exposure caused significant elevation in the activity of thioredoxin reductase (TRR) (a major cellular protein disulfide reductase) in both the head region of young/ adult flies. While the increase in TRR activity in the head region among young flies was relatively low (5mM: 22%; 10mM: 22%), it was concentration dependent among adult flies (1mM- 29%; 5mM: 63% and 10mM: 41%) (Fig 1.5A). Further, the activity levels of TRR in the body region of young flies were also elevated (5mM: 15% and 10mM: 26%) (Fig 1.5B). Interestingly, in the head region of both young and adult flies, glutathione-s-transferase (GST) activity levels were unaltered (Fig 1.5C). However, in the body region of both young (5mM: 22% and 10mM: 28%) and adult flies (1mM- 24%; 5mM: 24% and 10mM: 38%) the GST activity levels were elevated (Fig 1.5D).

4.1.8 Effect on mitochondrial function

Pb exposure caused a differential response in the activity levels of mitochondrial complex I-III (Fig 1.6A&B). The activity levels were increased in the head region of both young (5mM: 30%; 10mM: 19%) and adult flies (5mM: 25%; 10mM: 19%) (Fig 1.6A). While the activity levels were consistently elevated in the body region of young flies (1mM- 30%5mM- 43%; 10mM-40%) (Fig 1.6B), increased complex activity was evident in adult flies only at the mid concentration (5mM- 33%) (Fig 1.6B). However, Pb exposure caused significant diminution in MTT reduction in head (5mM- 32%; 10mM-32%) (Fig 1.6C) and body region of young
flies (5mM 24%; 10mM-19%) (Fig 1.6D). In contrast, Pb exposure had no effect on the MTT reduction in head and body region of adult flies (Fig 1.6C, D).

**4.1.9 Effect on cholinergic function and dopamine levels**

In general, Pb exposure resulted in a significant increase in the cholinergic function in both young and adult flies (Fig 1.7). The AChE levels were significantly elevated in head region of both young (5mM- 28%; 10mM- 34%) and adult flies (5mM- 28%; 10mM- 34%) (Fig 1.7A). However, in the body region, the increase in AChE levels were marginal in young (10mM-16%) and adult flies (Fig 1.7B). Concomitantly, the activity levels of BChE in head region of both young (5mM- 21%; 10mM- 33%) and adult flies (10mM- 34%) were also elevated (Fig 1.7C). However, only in the body region of young flies, the activity levels were increased (5mM- 12%; 10mM- 17%) (Fig 1.7D). Interestingly, a significant increase in dopamine levels was evident in head region of young flies (1mM- 17%; 5mM- 33%; 10mM- 50%) compared to head region of adult flies (5mM- 14%; 10mM- 20%) (Fig 1.7E). However, in the body region of young and adult flies, marginal decrease in dopamine levels was evident (Fig 1.7F).

**4.2 Neurotoxic effects of Pb acetate: Synthetic media**

For this study, experiments were designed by exposing young and adult flies to Pb acetate in a synthetic media (detailed in materials and methods section-Table 2) which is based on sucrose as the carbohydrate source.

**4.2.1 Pb-induced lethality response and hyperactivity phenotype**

As observed in the first set of experiments, Pb exposure caused differential lethality response among young and adult flies. Young flies were more susceptible to Pb compared to adult flies. Pb at lower concentration (5mM) failed to cause any mortality among flies of both age groups. However, young flies exhibited a higher degree of mortality to Pb exposure (10mM -40%; 20mM- 90%) (Fig 1.8A). In comparison, a lower incidence of lethality was evident among adult flies (10mM- 25%; and 20mM- 65%). As seen in the earlier study, males were more susceptible compared to females among the younger age group (Fig 1.8B).
Hyperactivity was measured in terms of flight speed on day 5 following Pb exposure. Flies (young) exhibited a significant degree of hyperactivity at all concentrations (Fig 1.8C) and the fastest speed was evident at the mid concentration of 5mM (nearly 90% increase over the control).

4.2.2 Effect of Pb on markers of oxidative stress

Data on the effect of Pb exposure on levels of oxidative markers determined in both head and body regions is presented in Tables 1.1 & 1.2. Pb caused significant elevations in ROS levels both in head and body regions. Concomitantly, marginal decreased in HP levels head and body region (5mM: 57%; 10mM: 64%) was evident. However, significant decrease in MDA levels (62%) at both concentrations and decreased protein carbonyl levels in body region (5mM: 22%; 10mM: 26%) was also observed (Table 1.1). While GSH levels were also elevated in head (5mM: 22%; 10mM: 17%), there was no significant effect in the body region. However, the total thiol were diminished marginally in head region (10mM: 12%), while markedly decrease was evident in the body region (5mM: 33%; 10mM: 50%) (Table 1.2).

4.2.3 Effect of Pb on antioxidant enzymes

The activity of CAT was consistently elevated irrespective of the Pb concentration, both in head (21-23%), and body (17-30%) regions (Table 1.3). In contrast, the activity of SOD was diminished (17%) in the body region. Further, the activity levels of TRR, was elevated in both the head (5mM- 70% and 10mM-34%) and body regions (5mM- 34%; 10mM- 23%). However, the activity of GST was consistently elevated in both the head (5mM-17% and 10mM- 14%) and body (5mM- 53% and 10mM- 62% regions (Table 1.3).

4.2.4 Effect of Pb on mitochondrial function

Pb exposure caused a differential response in the activity levels of mitochondrial complex I-III (Table 1.4). The activity levels were increased in head (5mM: 27%; 10mM: 18%), while robust enhancement was evident in body region (5mM- 45%; 10mM-82%). However, Pb exposure caused significant diminution in MTT reduction in both head and body regions (Table 1.4).
4.2.5 Effect on AChE activity and dopamine levels

As in the previous study, Pb exposure resulted in a significant increase in the activity of AChE levels in head (5mM- 23%; 10mM- 16%) (Fig 1.9A) and BChE levels in head (5mM- 14%; 10mM- 14%) (Fig 1.9B). Further, a significant increase in dopamine levels was evident in head (5mM- 36%; 10mM- 16%) (Fig 1.9C).

SECTION-B

4.3 Protein enrichment as a strategy to alleviate neurotoxicity

4.3.1 Exposure of Pb acetate in protein –enriched medium

In this study, three different proteins, casein (Fig 1.10A), whey protein isolate (Fig 1.10B), soy protein isolate (Fig 1.10C) were assessed for their protective effect against Pb-induced (10mM) lethality in a 7d co-exposure paradigm. Interestingly, casein at the end of 7d provided 50% at CSN 1% and 100% at CSN 2%) (Fig 1.10A).

4.4 Casein-enrichment as a nutrient intervention to abrogate Pb-induced neurotoxic implications

4.4.1 Effect of casein on endogenous levels of oxidative markers and antioxidant enzymes in head and body regions of flies

In general young flies maintained on casein-enriched diet (1-2%) for 5d exhibited no hyperactivity. Data on oxidative markers (Table 1.5), glutathione and protein carbonyls (Table 1.6) and activity levels of antioxidant enzymes determined in both head and body regions are represented in (Table 1.7). With casein (2%), a marginal increase in ROS levels was observed in head region, while in the body region ROS levels were elevated at both levels. However, at casein (1%), significant increase in hydroperoxide levels were evident in both head (23%) and body (19%) regions were evident. Interestingly, at casein 2%, malondialdehyde levels were significantly decreased in body region (30%) (Table 1.5). Interestingly, protein carbonyl levels were significantly decreased at casein 2% in
body region. However, casein had no effect on the GSH levels at either of the dietary levels (Table 1.6). The activity of CAT was significantly increased in both head (29-14%) and body region (21-27%). Further, the activity levels of SOD were enhanced (15-22%) in body region. Interestingly, the activity levels of TRR were markedly enhanced in both head and body region (Table 1.7).

4.4.2 Casein enrichment offsets Pb-induced lethality and hyperactivity

For this study, flies maintained on casein enriched diet (1 and 2%) were exposed to Pb (10mM) for 7 days. Casein enrichment markedly offset Pb-induced lethality. At 1%, casein provided 50% protection, while complete protection was evidenced at 2% (Fig 1.11A). Further, Pb (5 mM) treated flies exhibited increased locomotor activity and dietary casein also caused significant increase (33%) in speed. However, flies exposed to Pb in casein enriched medium exhibited a significant reduction in the speed at both concentrations (Fig 1.11B).

4.4.3 Casein enrichment alleviates Pb-induced oxidative markers

Casein enrichment significantly modulated the extent of Pb-induced biochemical perturbations measured as oxidative markers, GSH levels and nitric oxide levels in both head and body regions (Table 2.8). GSH levels among Pb treated flies were elevated in the head (15%), while no such effects were evident in the body region. However, with casein enrichment (2%) the elevated head levels, were marginally diminished and there was no effect in the body region. A differential protective response of casein was observed against Pb-induced elevation in ROS levels in the head region. While Pb significantly enhanced the ROS levels, the levels were further enhanced in both head and body regions of flies provided casein enrichment. However hydroperoxide levels were further enhanced in head (CSN1%-52%; CSN2%-1fold) and body region (CSN2%-51%). Similarly, Pb-induced decrease in MDA levels were further increased by CSN enrichment. Although NO levels were diminished with Pb exposure, they were marginally elevated with casein enrichment (Table 2.8).
4.4.4 Effect of casein; Pb-induced alterations in antioxidant enzymes

Pb exposure caused a robust elevation in the activity of catalase in both head and body regions (Fig 1.12A&B), while CSN enrichment marginally enhanced activity levels in the body (Fig 1.12B). In contrast, Pb exposure caused a significant reduction (14%) in the activity of SOD in body region and the enzyme activity was normalized with CSN (Fig 1.12C). Further, Pb exposure resulted in elevated activity levels of TRR in both head and body regions and the activity levels were further enhanced with CSN enrichment in head (Fig 1.13A) and body regions (Fig 1.13B). Further, Pb caused a significant increase in GST CSN enrichment had differential effects in both regions (Fig 1.13C&D).

4.4.5 Modulatory effect on the activity of AChE enzyme and DA levels

Pb caused an elevation in the activity of AChE in both regions (head: 23%; body 18%), and casein enrichment had no measurable effect (Fig 1.14A&B). Further, Pb exposure caused a significant increase in dopamine levels in both head and body (head: 26%; body: 18%). Casein (1%) further increased DA levels, while at 2%, DA levels were normalized in head (Fig 1.14C). Casein enrichment had no effect on the DA levels in body region (Fig 1.14D).

4.4.6 Casein enrichment modulates mitochondrial functions

Pb exposure resulted in elevated complex I-III activity in both head and body regions and casein enriched diet restored the activity levels to normal in the head region (Fig 1.15A) and to near normal levels in the body regions (Fig 1.15B). Further, Pb exposure caused a marked diminution in MTT reduction in both head and body: only 2% casein provided some reversal of this effect (Fig 1.15C&D).
Fig 1.1

Lethality response (A) and hyperactivity phenotype (flying speed) (B) among *Drosophila melanogaster* (Young/adult) exposed to varying concentrations of lead (Pb) acetate in culture media.

![Graph A: Percent mortality](image1)

![Graph B: Speed (cm/sec)](image2)

Values are mean ± SE. (n-6 vials of 25 flies/vial); Pooled data from 3 independent experiments.

Flies were exposed to Pb in the regular culture media (Jaggery based).

Data analyzed by one way analysis of variance (ANOVA) followed by post hoc Tukey's test.

* significant against control at p < 0.05 within the groups.

'a' Significantly different at p < 0.05, compared to 10mM young/male flies.

'b' Significantly different at p < 0.05, compared to 20mM young/male flies.

A- Percent mortality; B-Flying speed (Hyperactivity)
Fig 1.2

Oxidative stress response measured as reactive oxygen species formation and hydroperoxide levels in cytosol of head/body regions of young/adult *Drosophila melanogaster* exposed to lead (Pb) acetate

Values are mean ± SE. (n = 50 flies per replicate (three replicates each).

Data analyzed by one way analysis of variance (ANOVA) followed by Tukey’s test.
* significant against control at p < 0.05.
Young and adult male flies were exposed to Pb acetate (1-10mM) in the media for 5 days.

A, B- Reactive oxygen species; C, D- Hydroperoxides
Fig 1.3

Effect of lead (Pb) acetate on redox status (GSH and total thiols) and nitric oxide levels in cytosol of head and body regions of young and adult *Drosophila melanogaster*

Values are mean ± SE. (n = 50 flies per replicate (three replicates each).

Data analyzed by one way analysis of variance (ANOVA) followed by Tukey’s test.
* significant against control at p < 0.05.
Young and adult male flies were exposed to Pb acetate (1-10mM) in the media for 5 days.

A, B- Glutathione; C, D- Total thiols, E- Nitric oxide; F- Protein carbonyls
Fig 1.4

Effect of lead (Pb) acetate on the activity levels of catalase and superoxide dismutase activity in head and body regions among young and adult male Drosophila melanogaster

Values are mean ± SE. (n = 50 flies per replicate (three replicates each).

Data analyzed by one way analysis of variance (ANOVA) followed by Tukey’s test.
* significant against control at p < 0.05.
Young and adult male flies were exposed to Pb acetate (1-10mM) in the media for 5 days.

A- Catalase (Head region); B- Catalase (Body region);
C- Superoxide dismutase (Body region)
Fig 1.5

Effect of lead (Pb) acetate on the activity levels of thioredoxin reductase and glutathione-s-transferase in head and body regions among young and adult male *Drosophila melanogaster*

Values are mean ± SE. (n = 50 flies per replicate (three replicates each).

Data analyzed by one way analysis of variance (ANOVA) followed by Tukey’s test.
* significant against control at p < 0.05.
Young and adult male flies were exposed to Pb acetate (1-10mM) in the media for 5 days.

A, B- Thioredoxin reductase; C, D- Glutathione-s-transferase
Fig 1.6

Mitochondrial complex I-III activity in head and body regions among young and adult male *Drosophila melanogaster* exposed to lead (Pb) acetate (5 d)

Values are mean ± SE. (n = 50 flies per replicate (three replicates each)).

Data analyzed by one way analysis of variance (ANOVA) followed by Tukey’s test. * significant against control at p < 0.05.

Young and adult male flies were exposed to Pb acetate (1-10mM) in the media for 5 days.

A, B-Complex I-III; C, D- MTT reduction
Fig 1.7

Cholinergic function and dopamine levels in head/ body regions among young/ adult male *Drosophila melanogaster* exposed to lead (Pb) acetate.

Values are mean ± SE. (n = 50 flies per replicate (three replicates each).

Data analyzed by one way analysis of variance (ANOVA) followed by Tukey’s test. * significant against control at p < 0.05.
Young and adult male flies were exposed to Pb acetate (1-10mM) in the media for 5 days.

A, B-Acetylcholinesterase; C, D-Butyrylcholinesterase; E, F-Dopamine
Fig 1.8

Lethality response (Young vs adult, male), gender differences (young male and female) and flying speed (young) in *Drosophila melanogaster* exposed to lead (Pb) acetate in sucrose media for 7 days

Values are mean ± SE. (n=6 vials of 25 flies/vial); Pooled data from 3 independent experiments.

Data analyzed by one way analysis of variance (ANOVA) followed by Tukey’s test.
* significant against control at p < 0.05 within the groups; ‘a’ significantly different at p < 0.05, compared to 10mM young/male flies. ‘b’ significantly different at p < 0.05, compared to 20mM young/male flies.

**A**- Percent mortality (Young/adult male flies)

**B**- Percent mortality (Young male and female flies)

**C**- Flying speed/Hyperactivity (Young male flies)
Table 1.1

Status of oxidative stress markers in young male *Drosophila melanogaster* exposed to lead (Pb) acetate in sucrose media.

<table>
<thead>
<tr>
<th>Markers</th>
<th>Pb acetate (mM)</th>
<th>0</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROSa</td>
<td>Head</td>
<td>2.45 ± 0.71</td>
<td>3.21 ± 0.44&lt;sup&gt;*&lt;/sup&gt;</td>
<td>5.92 ± 0.97&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Body</td>
<td>2.61 ± 0.18</td>
<td>3.11 ± 0.03&lt;sup&gt;*&lt;/sup&gt;</td>
<td>6.73 ± 0.47&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>HPb</td>
<td>Head</td>
<td>12.98 ± 0.02</td>
<td>11.68 ± 2.13</td>
<td>10.58 ± 0.11&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Body</td>
<td>21.94 ± 0.18</td>
<td>9.42 ± 0.28&lt;sup&gt;*&lt;/sup&gt;</td>
<td>7.80 ± 0.86&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>MDAc</td>
<td>Head</td>
<td>2.99 ± 0.02</td>
<td>1.13 ± 0.11&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1.12 ± 0.05&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Body</td>
<td>14.61 ± 0.23</td>
<td>11.45 ± 0.22&lt;sup&gt;*&lt;/sup&gt;</td>
<td>10.81 ± 0.87&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± SE.

Data analyzed by one way analysis of variance (ANOVA) followed by Tukey’s test.
* significant against control at p < 0.05.

Young male flies were exposed to Pb acetate (1-10mM) in the sucrose media for 5 days (data on 1mM is not shown since there were no significant differences compared to control).

<sup>a</sup> Reactive oxygen species, pmol DCF/mg protein
<sup>b</sup> Hydperoxides, nmol hydroperoxides/mg protein
<sup>c</sup> Manondialdehyde, nmol MDA/mg protein.
<sup>d</sup> Protein carbonyls, nmol carbonyls/mg protein
Table 1.2

Redox status (GSH and thiol levels) and nitric oxide levels in young male *Drosophila melanogaster* exposed to lead (Pb) acetate in sucrose media

<table>
<thead>
<tr>
<th>Markers</th>
<th>Pb acetate (mM)</th>
<th>0</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>GSH&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Head</td>
<td>1.88 ± 0.03</td>
<td>2.30 ± 0.13&lt;sup&gt;*&lt;/sup&gt;</td>
<td>2.20 ± 0.04&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Body</td>
<td>11.0 ± 0.3</td>
<td>11.6 ± 0.5</td>
<td>11.4 ± 0.3</td>
</tr>
<tr>
<td>TSH&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Head</td>
<td>715.9 ± 34.6</td>
<td>704.7 ± 0.2</td>
<td>630.1 ± 0.5&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Body</td>
<td>672.2 ± 56.5</td>
<td>447.3 ± 15.4&lt;sup&gt;*&lt;/sup&gt;</td>
<td>339.2 ± 26.7&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>NO&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Head</td>
<td>171.5 ± 2.57</td>
<td>151.8 ± 1.44&lt;sup&gt;*&lt;/sup&gt;</td>
<td>128.1 ± 2.13&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Body</td>
<td>94.4 ± 1.49</td>
<td>61.7 ± 0.17&lt;sup&gt;*&lt;/sup&gt;</td>
<td>47.9 ± 1.18&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± SE.

Young male flies were exposed to Pb acetate (1-10mM) in the media for 5 days (data on 1mM is not shown since there were no significant differences compared to control).

Data analyzed by one way analysis of variance (ANOVA) followed by Tukey’s test for comparison of means.

* significant against control at p < 0.05.

<sup>a</sup> Glutathione, μg GSH/mg protein.

<sup>b</sup> Total thiols, nmol DTNB/mg protein.

<sup>c</sup> Nitric oxide, nmol nitrite/mg protein.
### Table 1.3

**Effect of lead (Pb) acetate on activity levels of enzymic antioxidant defenses and glutathione-S-transferase activity in young male *Drosophila melanogaster***

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Regions</th>
<th>Pb acetate (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Catalase(^a)</td>
<td>Head</td>
<td>37.8 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Body</td>
<td>132.7 ± 1.4</td>
</tr>
<tr>
<td>SOD(^b)</td>
<td>Body</td>
<td>181.3 ± 7.6</td>
</tr>
<tr>
<td>TRR(^c)</td>
<td>Head</td>
<td>12.2 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Body</td>
<td>19.3 ± 1.0</td>
</tr>
<tr>
<td>GST(^d)</td>
<td>Head</td>
<td>162.9 ± 12.6</td>
</tr>
<tr>
<td></td>
<td>Body</td>
<td>239.3 ± 10.0</td>
</tr>
</tbody>
</table>

Values are mean ± SE.

\(^a\) nmol hydrogen peroxide decomposed/min/mg protein; \(^b\) Units/mg protein; \(^c\) µmol DTNB oxidized/min/mg protein; \(^d\) nmol GS-DNB/min/mg protein

### Table 1.4

**Activity of complex I-III and MTT reduction in young male *Drosophila melanogaster* exposed to lead (Pb) acetate**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Regions</th>
<th>Pb concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Complex I-III(^a)</td>
<td>Head</td>
<td>70.48 ± 1.19</td>
</tr>
<tr>
<td></td>
<td>Body</td>
<td>23.32 ± 0.22</td>
</tr>
<tr>
<td>MTT(^b)</td>
<td>Head</td>
<td>5.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Body</td>
<td>41.6 ± 1.3</td>
</tr>
</tbody>
</table>

Young male flies were exposed to Pb acetate (1-10mM) in the media for 5 days (data on 1mM is not shown since there were no significant differences compared to control).

Data analyzed by one way analysis of variance (ANOVA) followed by Tukey's test for comparison of means; \(^*\) significant against control at p < 0.05.

\(^a\) nmol cyt C reduced/min/mg protein; \(^b\) Absorbance/mg protein
Fig 1.9

Effect of Pb acetate on cholinergic function and dopamine levels in head region among young male *Drosophila melanogaster*

Values are mean ± SE. (n=6 vials with 25 flies/ vial).

Data pooled from 3 independent experiments. Data analyzed by one way analysis of variance (ANOVA) followed by Tukey’s test for comparison of means.

* significant against control (Pb-0mM) at p < 0.05.

A-Acetylcholinesterase; B-Butyrylcholinesterase; C-Dopamine
Fig 1.10

Protein enrichment as a response modifier of Pb (10mM)-induced lethality among young male *Drosophila melanogaster* in a 7 day treatment protocol

Values are mean ± SE. (n=6 vials with 25 flies/ vial).

Data pooled from 3 independent experiments. Data analyzed by one way analysis of variance (ANOVA) followed by Tukey’s test for comparison of means.

* significant against Pb 10mM at p < 0.05.

A-Casein enriched diet; B-Whey protein isolate; C-Soy protein isolate
Table 1.5

Status of oxidative markers in head and body regions of young male *Drosophila melanogaster* maintained on casein-enriched medium

<table>
<thead>
<tr>
<th>Markers</th>
<th>Region</th>
<th>Casein concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>ROS(^a)</td>
<td>Head</td>
<td>3.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Body</td>
<td>2.03 ± 0.11</td>
</tr>
<tr>
<td>HP(^b)</td>
<td>Head</td>
<td>16.5 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>Body</td>
<td>24.6 ± 2.13</td>
</tr>
<tr>
<td>MDA(^c)</td>
<td>Body</td>
<td>2.99 ± 0.02</td>
</tr>
</tbody>
</table>

Values are mean ± SE. (n=6 vials with 25 flies/ vial).

Data pooled from 3 independent experiments.
Data analyzed by one way analysis of variance (ANOVA) followed by Tukey’s test.
* significant against control at p < 0.05.
Flies were maintained on casein enriched diet for 5 days.

\(^a\) Reactive oxygen species, pmol DCF/mg protein
\(^b\) Hydroperoxides, nmol hydroperoxides/mg protein
\(^c\) Malondialdehyde, nmol MDA/mg protein.
### Table 1.6

**Status of Glutathione and protein carbonyl levels in head and body regions of young male Drosophila melanogaster maintained on casein-enriched medium**

<table>
<thead>
<tr>
<th>Markers</th>
<th>Regions</th>
<th>Casein concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>GSH(^a)</td>
<td>Head</td>
<td>2.26 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Body</td>
<td>10.0 ± 0.2</td>
</tr>
<tr>
<td>PC(^b)</td>
<td>Body</td>
<td>11.19 ± 0.6</td>
</tr>
</tbody>
</table>

Values are mean ± SE. (n=6 vials with 25 flies/ vial).

\(^a\) Reduced glutathione, µg GSH/mg protein

\(^b\) Protein carbonyls, nmol carbonyls/mg protein

### Table 1.7

**Status of antioxidant defenses in head and body regions of young male Drosophila melanogaster maintained on casein-enriched medium**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Region</th>
<th>Casein concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Catalase(^a)</td>
<td>Head</td>
<td>34.1 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Body</td>
<td>127.9 ± 0.0</td>
</tr>
<tr>
<td>SOD(^b)</td>
<td>Body</td>
<td>156.9 ± 1.3</td>
</tr>
<tr>
<td>TRR(^c)</td>
<td>Head</td>
<td>7.6 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Body</td>
<td>12.6 ± 0.6</td>
</tr>
</tbody>
</table>

Data pooled from 3 independent experiments. Data analyzed by one way analysis of variance (ANOVA) followed by Tukey’s test for comparison of means.

* significant against control at p < 0.05.

Flies were maintained on casein enriched diet for 5 days.

\(^a\) nmol hydrogen peroxide decomposed/min/mg protein; \(^b\) Units/mg protein.

\(^c\) µmol DTNB oxidized/ min/ mg protein.
Fig 1.11

Modulatory effect Casein enrichment (1 & 2%) on Pb (10mM) acetate induced lethality and incidence of hyperactivity phenotype (speed) among young male *Drosophila melanogaster*

Values are mean ± SE (n=6 vials with 25 flies/vial).

Data pooled from 3 independent experiments. Data analyzed by one way analysis of variance (ANOVA) followed by Tukey’s test for comparison of means.

* significant against control (Pb-0mM); # compared to Pb-5mM at p < 0.05.

A-Percent mortality; B-Hyperactivity (Speed)
Table 1.8

Modulatory effect of casein-enriched diet on Pb (5 mM) acetate-induced oxidative perturbations in head and body regions of young male *Drosophila melanogaster*

<table>
<thead>
<tr>
<th>Markers</th>
<th>Regions</th>
<th>CTR</th>
<th>Pb</th>
<th>Pb+CSN1%</th>
<th>Pb+CSN2%</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROS</td>
<td>Head</td>
<td>2.65 ± 0.37</td>
<td>3.56 ± 0.04*</td>
<td>2.54 ± 0.10</td>
<td>4.65 ± 0.17#</td>
</tr>
<tr>
<td></td>
<td>Body</td>
<td>3.83 ± 0.01</td>
<td>3.66 ± 0.03</td>
<td>3.86 ± 0.02</td>
<td>5.89 ± 0.17#</td>
</tr>
<tr>
<td>HP</td>
<td>Head</td>
<td>10.32 ± 0.14</td>
<td>7.97 ± 0.02*</td>
<td>12.11 ± 0.32#</td>
<td>15.48 ± 0.95#</td>
</tr>
<tr>
<td></td>
<td>Body</td>
<td>17.44 ± 0.57</td>
<td>13.37 ± 0.59*</td>
<td>14.07 ± 0.51</td>
<td>20.13 ± 0.83#</td>
</tr>
<tr>
<td>MDA</td>
<td>Body</td>
<td>2.99 ± 0.02</td>
<td>1.13 ± 0.11*</td>
<td>1.25 ± 0.02</td>
<td>2.34 ± 0.03#</td>
</tr>
<tr>
<td>GSH</td>
<td>Head</td>
<td>1.96 ± 0.01</td>
<td>2.26 ± 0.05*</td>
<td>2.34 ± 0.01</td>
<td>2.03 ± 0.02#</td>
</tr>
<tr>
<td></td>
<td>Body</td>
<td>11.25 ± 0.27</td>
<td>12.40 ± 0.44</td>
<td>11.95 ± 0.15</td>
<td>12.07 ± 0.05</td>
</tr>
<tr>
<td>NO</td>
<td>Head</td>
<td>171.5 ± 2.57</td>
<td>151.8 ± 1.44*</td>
<td>181.3 ± 1.01#</td>
<td>187.9 ± 0.68#</td>
</tr>
<tr>
<td></td>
<td>Body</td>
<td>94.4 ± 1.49</td>
<td>61.7 ± 0.17*</td>
<td>64.4 ± 0.17</td>
<td>61.0 ± 0.72</td>
</tr>
</tbody>
</table>

Values are mean ± SE (n=6 vials with 25 flies/ vial).

Flies were exposed to Pb acetate (5mM) for 5 days. Data pooled from 3 independent experiments. Data analysed by one way analysis of variance (ANOVA) followed by Tukey’s test for Comparison of means.

* significant against control at p < 0.05
# significant against Pb 5mM at p < 0.05

*a* Reactive oxygen species, pmol DCF/mg protein

*b* Hydroperoxides, nmol hydroperoxides/mg protein

*c* Malondialdehyde, nmol MDA/mg protein

*d* Reduced glutathione, µg GSH/mg protein

*e* Nitric oxide, nmol nitrite/mg protein
Fig 1.12

Modulatory effect of Casein enrichment on Pb acetate (5mM)-induced perturbations in antioxidant enzymes in young Drosophila melanogaster

Values are mean ± SE (n=6 vials with 25 flies/vial).

Data analyzed by one way analysis of variance (ANOVA) followed by Tukey’s test.
* significant against control (Pb-0mM); # compared to Pb-5mM at p < 0.05.

A-Catalase activity (Head); B-Catalase activity (Body); C-Superoxide dismutase activity (Body)
Fig 1.13

Modulatory effect of Casein enrichment on Pb acetate (5mM)-induced perturbations in antioxidant enzymes in young *Drosophila melanogaster*

Values are mean ± SE (n=6 vials with 25 flies/ vial).

Data analyzed by one way analysis of variance (ANOVA) followed by Tukey's test for comparison of means.

* significant against control (Pb-0mM); # compare to Pb-5mM at p < 0.05.

A, B-Thioredoxin reductase (A-Head; B-Body)
C, D-Glutathione-S-transferase (C-Head; D-Body)
Fig 1.14

Modulatory effect Casein enrichment on Pb acetate (5mM)-induced effect on acetylcholinesterase activity levels in young male *Drosophila melanogaster*

Values are mean ± SE (n=6 vials with 25 flies/ vial).

Data analyzed by one way analysis of variance (ANOVA) followed by Tukey’s test for comparison of means.
* significant against control; # compare to Pb-5mM at p < 0.05.

A, B-Acetylcholinesterase (A-Head; B-Body)
C, D-Dopamine (C-Head; D-Body)
Fig 1.15

Modulatory effect Casein enrichment on Pb acetate (5mM)-induced mitochondrial dysfunctions in young male *Drosophila melanogaster*

Values are mean ± SE (n=6 vials with 25 flies/vial).

Data analyzed by one way analysis of variance (ANOVA) followed by Tukey’s test for comparison of means.

* significant against control; # compare to Pb-5mM at p < 0.05.

A, B-Complex I-III (A-Head; B-Body)
C, D-MTT reduction (C-Head; D-Body)
Chapter 1

5.0 DISCUSSION

Although *Drosophila melanogaster* is a widely used system for studying the interaction between nutrition and lifespan (Bruce et al., 2013), very few researchers have utilized this model to understand the toxic implications of Pb (Hirsch et al., 2012). Accordingly, one of the primary aims of this study was to recapitulate the toxic effects of Pb in young flies so as to establish as a simple model to test the hypothesis that protein enrichment can alleviate the toxic response associated with Pb exposure in flies. Hence, as a primary requisite, toxicity of Pb was established in the fly model focusing on the hyperactivity phenotype, oxidative stress response, mitochondrial dysfunctions and neurotoxicity.

While the precise mechanism of Pb-induced toxicity is not clear, several experimental evidence suggest that Pb can cause generation of reactive oxygen species and also inhibit the activity of antioxidant enzymes in tissues (Toscano and Guilarte, 2005; Patrick, 2006; White et al., 2007; Neal and Guilarte, 2010; Prasanthi et al., 2010). In view of the relationship between Pb exposure and oxidative stress, much attention has been focused on compounds having antioxidant properties in order to alleviate Pb-induced neurotoxicity. However, not many studies have examined Pb-induced neurotoxic responses under no or low protein conditions in experimental models. This assumes relevance since inadequate nutrition (under situations of protein deficiency and/or malnutrition) during the pre- and postnatal period is considered a significant risk factor as it markedly alters brain development resulting in biochemical, physiological and anatomical changes (Bonatto et al., 2006). More importantly, exposure to Pb is often greater among children of low SES (Bellinger, 2008) and susceptibility to the neurotoxic effects of xenobiotics may be compounded under such conditions. It is in this context that it was hypothesized that casein enrichment is likely to alleviate Pb induced oxidative stress and toxicity.

In the present study, initially it was aimed to establish the toxicity profile of Pb in young and adult flies and also assess the gender differences. Interestingly, young flies appeared to relatively more susceptible to Pb exposure which
corroborates with the previous findings in animal models, as well as the higher susceptibility of children (Yang et al., 2003). Pb-induced higher lethality in males compared to female flies, an observation consistent with earlier reports in experimental animals (Mansouri et al., 2012). Hence, it was planned to establish some of the adverse effects of Pb in young male flies in terms of locomotor phenotype, induction of oxidative stress, redox status, and neurotoxicity. Significant elevations in global ROS levels suggested that Pb at higher concentrations induced significant oxidative stress and was associated with a diminution in the total thiol levels and an elevation in GSH levels. These findings are in agreement with the earlier reports wherein contribution of oxidative stress to the pathogenesis of Pb neurotoxicity in experimental animals (Adonaylo and Oteiza, 1999; Bokara et al., 2008) and in occupationally exposed workers (Gurer-Orhan et al., 2004) has been demonstrated.

Glutathione, an abundant and ubiquitous low-molecular-weight thiol plays important roles in antioxidant defense, nutrient metabolism, and regulation of cellular events (including gene expression, DNA and protein synthesis, apoptosis, cell proliferation and signal transduction). Accordingly, the elevated GSH levels observed with Pb exposure suggested an adaptive response to the survival needs of flies. Further, Pb exposure also caused an increase in the activity of GST, a Phase II detoxification enzyme involved in the metabolism of a range of electrophilic xenobiotics compounds by conjugation with GSH which is essential in the maintenance of normal physiological processes (Daggett et al., 1998).

Interestingly, we also found a marked increase in the activity of TRR, a selenoprotein known to be involved in many cellular redox processes in both head and body regions of flies suggesting its vital role in Pb exposure. Previously, Pb exposure was shown to enhance the activity levels of catalase, glutathione- S-transferase and TRR activity in the kidneys of rats (Conterato et al., 2007). It is speculated that increases in renal TRR expression and activity are early responses most likely representing a protective cellular mechanism. Further, Pb exposure also resulted in decreased nitric oxide levels in flies, an observation consistent with findings of decreased nitric oxide synthase activity.
and nitric oxide production in other animals exposed to Pb (Nava-Ruiz et al., 2012; Neal et al., 2012).

Further, Pb exposure caused a hyperactivity phenotype among flies which was demonstrable as increased locomotor behavior. We speculate that the Pb-induced hyperactivity phenotype manifested at concentrations beyond 5 mM in young flies may be attributed to enhanced activity of acetylcholinesterase and increased dopamine levels evidenced in the head region of flies. Our observation in flies is consistent with the hyperactivity caused by chronic Pb exposure in rats which was shown to be associated with increased histone acetylation in the hippocampus (Mansouri et al., 2012; Luo et al., 2014). Further, Pb intoxication in animal models affects both cerebral cortex and basal ganglia areas in brain involved in motor control. Chronic Pb exposure induces a hyperactivity phenotype and impaired motor coordination among male rats (Mansouri et al., 2013). In the present study, Pb exposure resulted in elevated DA levels in the head region of the flies. Recent findings suggest that Pb exposure is considered a high environmental risk factor for the development of ADHD (Luo et al., 2014). Since, DA systems are important for central regulation of motor activity, we speculate that the observed behavioral phenotype reflects the effect of Pb on DA levels and oxidative stress. Further, Pb exposure resulted in increased mitochondrial complex I activity and diminished MTT reduction in flies suggesting altered mitochondrial function. This corroborates earlier findings which have shown that that Pb induces significant ultrastructural changes such as vacuolization of cell cytoplasm, degeneration of mitochondria and, electron-dense inclusion bodies (Maier and Chan, 2002; Deveci, 2006).

Protein malnutrition (PMN) is a severe problem especially in developing countries affecting millions of unborn and young children during the most vulnerable stages of their development. PMN is known to interfere with protein synthesis, structure and alter enzyme activity (Morgane et al., 2002; Feoli et al., 2006). Dietary protein is a major source of essential amino acids which also serve as intracellular antioxidants and several studies have shown that decreases in dietary protein content could potentially increase oxidative stress. Further evidence suggests that PMN as well as under-nutrition induces impaired
learning and retention when imposed during the early postnatal period and in adulthood (Alamy and Bengelloun, 2012). Severe PMN provokes long-lasting deleterious oxidative effects on macromolecules by increasing lipid peroxidation and significantly decreases tyrosine and tryptophan content. In the present model, casein enrichment (1–2%) per se resulted in significantly enhanced ROS levels, elevated activities of antioxidant enzymes in both the head and body regions, while protein carbonyl levels were diminished. Dietary protein restriction may lead to an increase in oxidative damage by diminishing antioxidant defenses (Bonatto et al., 2005). Interestingly, Pb exposure under casein enrichment conditions caused further increases in the ROS levels, enhanced the activity of catalase in head regions and normalized SOD activity in body regions of the flies. We speculate that these biochemical alterations may be considered as specific adaptive/protective responses under Pb exposure.

Interestingly, under conditions of Pb exposure, casein enrichment significantly enhanced the activity of the key enzyme TRR, whose function mimics that of glutathione reductase catalyzing the NADPH-dependent reduction of active disulfide to dithiolTrx- (-SH)2 in Drosophila (Kanzok et al., 2001). Recent evidence suggests that TRR deficiency serves as a major factor that potentiates oxidative stress contributing to dopaminergic pathology (Lopert et al., 2012). Hence, it is quite probable that the higher activity levels of TRR evident with casein enrichment play a significant predominant role in the attenuation of Pb-induced oxidative stress in the fly model. GST’s which catalyze the transfer of glutathione to reactive electrophiles, may be particularly important given that their increased expression mitigates oxidative stress (Trinh et al., 2008). It can be reasonably assumed that the ability of casein to induce GST activity may also represent a major mechanism to protect the organism against Pb intoxication and in rescuing dopaminergic neuronal loss as hypothesized previously (Whitworth et al., 2005). Interestingly, casein supplementation reduced the Pb-induced increase in DA levels in the head region. Dopamine and norepinephrine are the main neurotransmitters involved in the pathophysiology of ADHD (Bokor and Anderson, 2014). In fact, a recent study showed that childhood Pb exposure was positively associated with ADHD diagnosis (Froehlich et al., 2009). Based
on the biochemical evidence obtained in the fly model, we propose that casein enrichment has the potential to alleviate the magnitude of Pb-induced oxidative stress and associated neurotoxic responses. Although further investigations are essential for elucidating the precise mechanism/s by which casein attenuates the Pb-associated adverse effects in this model, it can be reasonably speculated to be largely related to altered redox state (enhanced GSH/thiol levels, activity levels of thioredoxin and GST enzymes). However, one cannot rule out the contribution of specific amino acids present in the casein to remove Pb in the fly system. Recent findings (Pichaud et al., 2013) that diet (protein; carbohydrate ratio) can differentially affect mitochondrial functions in the *Drosophila* model emphasizes the need for further comprehensive studies to understand the modulatory influence of major nutritional factors (e.g. quality/quantity of protein levels) on the adverse effects of common neurotoxicants which are known to be mediated via oxidative stress and mitochondrial dysfunction.

In conclusion, these findings are the first to show that casein enrichment in young flies can significantly ameliorate Pb-induced damage and reverse Pb-induced changes in antioxidant/ mitochondrial enzyme activities. These findings are relevant to nutritional status since protein deficiency (low protein or no protein) conditions can in some cases significantly modify the risk for certain chemical-induced neurological diseases. More importantly, these new observations in the *Drosophila* model emphasize the need for further studies to understand the protective pathways and molecular events which are responsible for the neuroprotective action of casein protein under conditions of Pb-intoxication. Since casein enrichment effectively diminished the Pb-induced global oxidative stress in flies and attenuated some of the associated neurotoxic responses, it can be reasonably presumed that protein-enriched diet may be effective in alleviating the toxicities associated with low chronic Pb exposure levels especially among children of low SES and more importantly in third world countries where protein deficiency is prevalent. These observations also lend further support to the idea that nutrient intervention may play a vital role as a response modifier of Pb toxicity.
6.0 SUMMARY

1. This study provides evidence for the first time that the effect of Pb in terms of age and gender differences in the Drosophila system and the hyperactivity phenotype are similar to those reported in higher experimental rodents.

2. Salient findings such as induction of oxidative stress as evidenced by increased ROS generation are consistent with previous reports of oxidative stress mechanism/s in the rodent models.


4. Following Pb exposure, head regions of flies exhibited elevated AChE activity and dopamine levels.

5. In the co-exposure paradigm, casein (1 and 2%) enrichment rendered marked protection against Pb-induced lethality and hyperactivity phenotype.

6. Casein enrichment markedly ameliorated the levels of oxidative markers, enhanced the antioxidant enzyme (catalase, SOD, TRR and GST) activities in head and body regions of Pb intoxicated flies.

7. However, flies maintained on casein enriched medium exhibited a elevated levels of ROS/HP levels and normal NO levels in both head and body regions.

8. Interestingly, casein-enrichment normalized the Pb-induced diminution in the activity levels of SOD in body region of flies.

9. Casein enrichment further increased the Pb-induced increase in TRR activity levels in both head and body regions suggesting its vital role in attenuation of toxic response.

10. The ability of casein to induce GST activity may also represent a major mechanism to protect flies against Pb intoxication and in rescuing dopaminergic neuronal loss.
11. Casein enriched diet restored the activity levels of mitochondrial complex I-III in head and body regions.

12. In the co-exposure paradigm, casein (1 and 2%) normalized the dopamine levels.

13. Since casein enrichment in the fly model effectively diminished the global oxidative stress and attenuated some of the Pb mediated neurotoxic responses, it can be reasonably presumed that protein-enriched diet may be effective in alleviating the toxicities associated with low chronic Pb exposure levels especially among children populations exposed to low protein diets.