UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

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Neurotoxicological and Molecular aspects of methylmercury and monosodium glutamate exposure in the lobster cockroach, *Nauphoeta cinerea*

Porto Alegre
2019

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Tese apresentada ao Programa de Pós- Graduação em Ciências Biológicas: Bioquímica do Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de doutora em Bioquímica.

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Porto Alegre

2019

Dedication

I dedicate this thesis to my family, and everyone who supported me through this journey.

Acknowledgements

Blessing Ariyo Afolabi is a beneficiary of CNPq-TWAS postgraduate doctoral fellowship. I appreciate their financial support and assistance. My profound gratitude goes to the staff of the Department of Biochemical toxicology and Molecular biology UFSM and Biochemistry department UFRGS.

I am indeed grateful to Prof. Diogo Onofre Gomes de Souza (UFRGS) and Prof Joao Batista Teixeira da Rocha (UFSM) for the ample opportunity to work under their supervision and to learn under their expertise. These great men impacted my career in wonderful ways. My academic story is incomplete without them in it. I appreciate my colleagues for their support. Ultimately, I give all the glory to God Almighty for his mercies. He alone is to be adored, praised and worshipped.

Presentation

This thesis is put in three parts according to the dictates of the Post-Graduate Program of the Department of Biochemistry, Universidade Federal do Rio Grande do Sul (UFRGS).

Part I: Contains the abstract (written in English and Portuguese), list of abbreviations, introduction and objectives.

Part II: Contains results presented as published articles or manuscripts including materials and methods. Each article or manuscript is written in chapters.

Part III: Contains discussion, conclusion, perspectives, references, list of tables and figures. The discussion and conclusion is the interpretation of the results and inference from each chapter, the perspectives entails questions or further-works that can be done, the references are citations from Part I and III (references of Part II are incorporated after each chapter), thus the list of tables and figures cut across Parts I to III.

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PART I

This part contains the abstract (English and Portuguese), list of abbreviations, introduction and the objectives of the study.

RESUMO

O metilmercúrio (MeHg) é um neurotóxico capaz de se acumular no cérebro em desenvolvimento. Esta substância tem sido associada ao estresse oxidativo, alterações comportamentais e inibição da captação de glutamato, causando a ativação excessiva dos seus receptors e neurotoxicidade, em modelos de roedores. O glutamato monossódico (GMS) é um aditivo alimentar que também tem sido associado ao estresse oxidativo, alterações comportamentais, danos renais e hepáticos em roedores. Recentemente foi demonstrado que o GMS causou uma resposta adaptativa ao estresse oxidativo e diminuiu a sobrevivência de *D. melanogaster*. No entanto, estudos das possíveis interações de MeHg e GMS são raros na literatura. A *N. cinerea*, um inseto que representa um modelo promissor para esses estudos, foi utilizado para avaliar se existem interações sinérgicas, aditivas, antagônicas ou de potenciação após a co-exposição ao MeHg e GMS, avaliando as modificações no comportamento e na homeostase redox. A concentração de GMS utilizada foi baseada em estudos preliminares, enquanto a dose subtóxica de MeHg foi baseada num estudo anterior do nosso grupo de pesquisa. A primeira parte deste estudo envolveu a avaliação da coexposição simultânea das ninfas ao GMS ou NaCl e MeHg [Basal; 2% NaCl; 2% GMS; 0,125 mg/g MeHg; 0,125 mg/g de MeHg + 2% NaCl; 0,125mg/g MeHg + 2% GMS] por 21 dias e os seus efeitos no comportamento e nos índices de estresse oxidativo. Grupos tratados com MeHg [0,125 mg/g MeHg; 0,125 mg/g MeHg + 2% NaCl; 0,125mg/g MeHg + 2% GMS] apresentaram um aumento nos comportamentos avaliados, tais como a imobilidade, distância percorrida nas periferia e 'bottom zone' e uma diminuição no ângulo de rotação. MeHg + NaCl causou um aumento na distância percorrida nas periferia e 'bottom zone' enquanto MeHg + GMS causou um aumento na imobilidade. Houve também uma diminuição na atividade da acetilcolinesterase (AChE) e dos níveis totais de tiol, bem como um aumento nos níveis de TBARS nestes grupos [0,125 mg/g MeHg; 0,125 mg/g MeHg + 2% NaCl; 0,125mg/g MeHg + 2% GMS]. O grupo tratados apenas com MeHg apresentou um aumento ainda maior nos níveis de TBARS quando comparado aos grupos coexpostos e ao controle. O comportamento alterado observado nas ninfas sugere uma alteração no sistema colinérgico, o que poderia ser responsável pela redução da atividade da AChE. A análise de ICP-AES mostrou que os grupos tratados com MeHg [0,125 mg/g MeHg; 0,125 mg/g MeHg + 2% NaCl; 0,125mg/g MeHg + 2% GMS] apresentaram um aumento no conteúdo de Hg na cabeça em relação aos demais grupos. Por fim, a expressão dos genes que codificam proteínas do sistema antioxidante e de detoxificação (glutationa-s-transferase, tioredoxina, catalase, superoxido dismutase, peroxiredoxina) em ninfas foram avaliadas. Grupos tratados com MeHg [MeHg + NaCl; MeHg + GMS] mostraram um aumento na expressão das *GstT* e *GstD*. MeHg + NaCl causou um aumento nos níveis de mRNA de *GstT, GstD, Sod, Trx1 e Cat*; MeHg + GMS causou um aumento nos níveis de mRNA de *GstS, GstT* e *GstD.* Também, MeHg + GMS causou um aumento na expressão de *Trx5*, e uma diminuição de *Trx2* e *Trx1*. Os efeitos de NaCl neste estudo requer uma investigação mais aprofundada. Tomados em conjunto a co-exposição de MeHg e GMS causou uma alteração na homeostase redox em *N. cinerea*, também o MeHg + GMS mostraram mais interação aditiva do que interação sinérgica ou antagônica.

Palavras-chave: *Nauphoeta cinerea*; Metilmercúrio; Glutamato monossódico; Estresse oxidativo; ICP-AES; Expressão genetica.

ABSTRACT

Methylmercury (MeHg) is a neurotoxicant capable of accumulating in the developing brain. It has been associated with oxidative stress, alteration of behaviors and inhibition of glutamate uptake hence causing over-activation of glutamate receptors leading to neurotoxicity in rodent models. Monosodium glutamate (MSG) is a food additive that has also been linked with oxidative stress, alteration of behaviors, renal and hepatic damage in rodents. Recently MSG was shown to cause an adaptive response to oxidative stress while shortening life span in *Drosophila melanogaster*. The study of the potential interaction of MeHg and MSG is close to unavailable in literature, hence, *Nauphoeta cinerea* was used to evaluate if synergistic, additive, antagonistic or potentiation interaction existed after co-exposure of MeHg and MSG by evaluating modifications in behavioral and redox homeostasis. The concentration of MSG used was based on preliminary studies while the subtoxic dose of MeHg was based on the previous study by our research group. The first part of this study involved the evaluation of simultaneous co-exposure of nymphs to MSG or NaCl and MeHg [Basal; 2%NaCl; 2%MSG; 0.125mg/g MeHg; 0.125mg/g MeHg + 2% NaCl; 0.125mg/g MeHg + 2%MSG] for 21 days and its effects on behaviour and oxidative stress indices. MeHg treated groups [0.125mg/g MeHg; 0.125mg/g MeHg + 2% NaCl; 0.125mg/g MeHg + 2%MSG] showed an increase in behaviors such as immobility, distance traveled in the periphery and bottom zone and a decrease in turn angle. MeHg + NaCl caused an increase in distance traveled in the periphery and bottom zone, while MeHg + MSG caused a decrease in immobility. There was a decrease in acetylcholinesterase (AChE) activity and total thiol levels, as well as an increase in TBARS in the MeHg treated groups [0.125mg/g MeHg; 0.125mg/g MeHg + 2% NaCl; 0.125mg/g MeHg + 2%MSG]. MeHg alone caused a greater increase in TBARS levels than the other groups and control. The altered behavior observed in the nymphs suggested an alteration in the cholinergic system and this could be responsible for the reduction in AChE activity. ICP-AES analysis showed MeHg treated groups [0.125mg/g MeHg; 0.125mg/g MeHg + 2% NaCl; 0.125mg/g MeHg + 2%MSG] presented an increase in Hg content in the heads of the nymphs in relation to other groups. Ultimately, analysis of antioxidant and detoxification gene expression (glutathione-S-transferase, thioredoxin, peroxiredoxin, catalase, superoxide dismutase) in the cockroach nymphs was evaluated. MeHg treated groups [MeHg + NaCl; MeHg + MSG] showed an upregulation of *GstT* and *GstD*. MeHg + NaCl caused an upregulation of *GstT*, *GstD, Sod, Trx1* and *Cat*; MeHg + MSG caused an upregulation of *GstS, GstT* and *GstD*. Also, MeHg + MSG caused upregulation of *Trx5* and downregulation of *Trx2* and *Trx1*. No alteration in the mRNA levels of Prx4 was observed. However, the effect of MeHg + NaCl in the present study requires further investigation. Taken together, co-exposure of MeHg and MSG caused an alteration of redox homeostasis in *N. cinerea*, also MeHg and MSG showed more of additive interaction than synergistic or antagonistic interaction.

Keywords: *Nauphoeta cinerea*; Methylmercury; Monosodium glutamate; Oxidative stress; ICP-AES; Gene expression; Evolutionary history.

LIST OF ABBREVIATIONS

MeHg: Methylmercury

MSG: Monosodium glutamate

ROS: Reactive oxygen species

ATP: Adenosine 5' triphosphate

OP: Oxidative phosphorylation

OS: Oxidative stress

PUFA: Polyunsaturated Fatty Acid

FR: Free radicals

ROO·: Peroxyl radicals

RO· : Alkoxyl radicals

HOO: Hydroperoxyl radicals

OD: Oxidative damage

GSH: Glutathione

GST: Glutathione –S-transferase

GPX: Glutathione peroxidase

SOD: Superoxide dismutase

CAT: Catalase

qRT-PCR: Quantitative Reverse Transcriptase Polymerase Chain Reaction

ICP-AES: Induced Coupled Plasma-Atomic Emission Spectroscopy

NMDA: N-methyl D-Aspartate

ACh: Acetylthiocholine

nAChRS: Nicotinic acetylcholine receptors

LPO: Lipid Peroxidation

DSP: Diarrhetic shellfish poisoning

INTRODUCTION

1 MERCURY AND ITS FORMS

Mercury is a known non-essential metal prevalently distributed in the environment, which poses risk to human health due to its toxicity. It is released into the environment via anthropogenic activities such as mining, agriculture (pesticides, herbicides) and medicine (vaccine, dental amalgam). There are three forms of mercury; elemental mercury (metallic H^0), inorganic (Hg^+, Hg^{++}) and organic mercury. The elemental mercury is liquid at room temperature, the inorganic mercury exists as solid mercury salts while the organic forms, combined with carbon are methylmercury or ethylmercury (Park and Zheng, 2012).

The most widely known tragedy caused as a result of mercury poisoning is the 'Minamata disease' which occurred at the Minamata bay in 1956 in Japan due to a waste-water discharge from a chemical company (Chisso co. Ltd.) that produced plasticizers, industrial chemicals and fertilizers and this led to a high incidence of death amongst the people that consumed contaminated fishes from the bay. The affected people exhibited symptoms which include ataxia of the limbs and feet, mental retardation, primitive reflex, strabismus and congenital cerebral palsy of new-born of infected mothers (Harada 1995).

It is known that some women from Asia, Africa (Mali, Nigeria, Senegal, South Africa and Togo, 25%, 77%, 27%, 35% and 59% respectively) and some parts of U.S. and Europe use cosmetic soaps and creams containing mercury (in the form of ammoniated mercury, mercury iodide, mercurous chloride, mercurous oxide) in their composition with the aim of lightening their skin (Ladizinski *et al,* 2011). Mercury is transported through the epidermis consequently enabling the absorption of inorganic mercury through the skin via sweat glands, sebaceous

glands and hair follicles (Chan, 2011), hence inhibiting melanin formation by competing with copper (Cu) in tyrosinase enzyme and brightening the skin (Engler, 2004).

Methylmercury (MeHg) is a known neurotoxicant that can be generated by the bio-methylation of inorganic mercury by the aquatic sulfate-reducing bacteria which eventually bio-accumulates along the food chain, causing its levels in fish to be high enough to make its consumption the main source of MeHg exposure in humans (Oliveira *et al.,* 2018; Nogara *et al.,* 2019). MeHg could be absorbed via the skin of humans or muscles (in the case of fish), distributed via the bloodstream to all the tissues of the organism leading to its accumulation in the kidney, liver, and finally its' excretion via faeces (Farina *et al*., 2010). MeHg is known to cross the Blood-Brain Barrier (BBB) due to its capacity to form complexes with GSH, hence mimicking methionine a substrate of the LAT transporter which transports amino-acids in the central nervous system (Farina *et al.,* 2011).

MeHg preferentially affects the developing brain and numerous studies have shown that MeHg exposure alters the behavior of experimental animals, such as learning (Paletz *et al.,* 2006), memory (Dare *et al.,* 2003) and motor activity. In addition, the toxicity of MeHg at the nervous system is mediated by the inactivation of sulfhydryl-and selenohydryl containing proteins (Oliveira *et al.,* 2018; Nogara *et al.,* 2019) involved in the maintenance of cellular redox state (Fig. 1). Furthermore, MeHg targets neural proteins in the plasma membrane hence disrupting the homeostasis of different neurotransmission systems, such as the glutamatergic, the GABAergic (Atchison, 2005), and the cathecholaminergic system (Farina *et al.,* 2017).

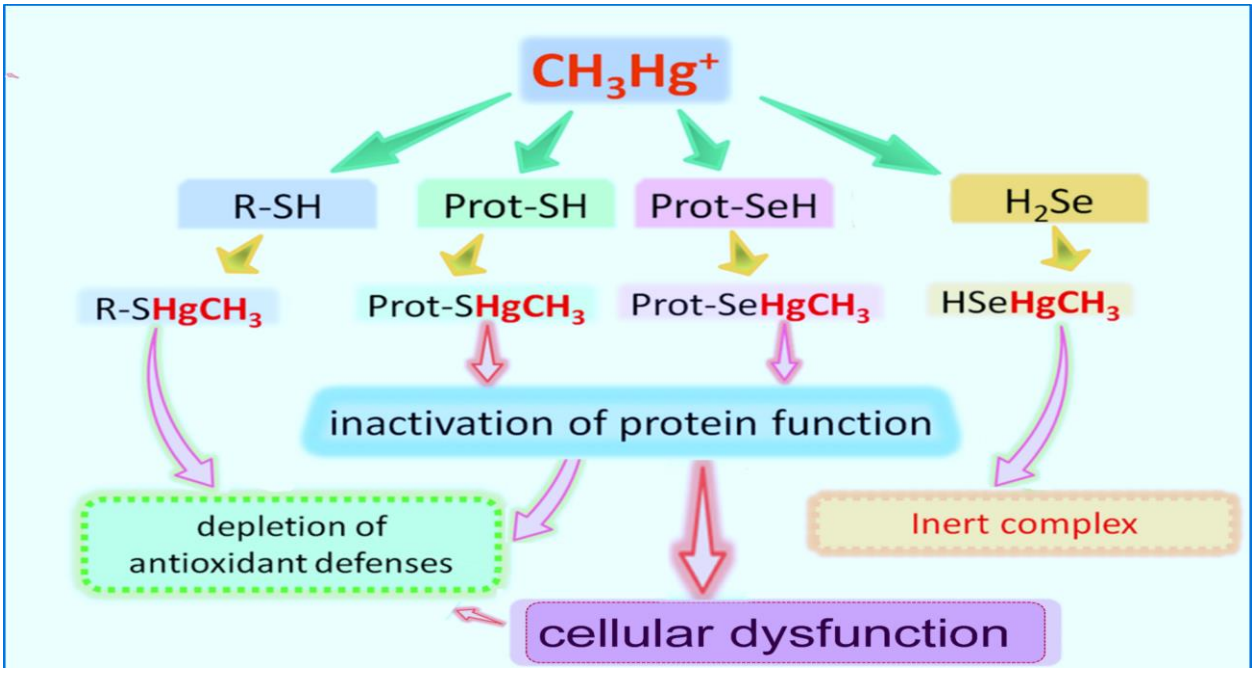


Figure 1: Mechanism of methylmercury (MeHg) induced toxicity. Molecular targets of MeHg (Aschner *et al.*, 2007, Modified by Rocha 'Brazillian congress of toxicology', 2019).

2 MONOSODIUM GLUTAMATE (MSG)

Monosodium glutamate [(MSG), (e-number E621)], is known to be one of the most widely used food enhancers (elicits umami taste) in industries (Ikeda, 2002), apart from the common table salt. MSG is a sodium salt of L-glutamic acid, an abundant non-essential amino acid in nature. Food enhancers are utilized in food industries and they play roles in food technology research (Zhang *et al.*, 2016). It has been shown that about 1200-3000 mg of MSG per day is consumed by the Asians (Brosnan *et al.*, 2014), although high doses of about 2000 to 8000 mg/kg body-weight have been reported to be toxic in rats but this is unexpected to be consumed in humans at any corresponding dose (Jubaidi *et al.* 2018). However, debates persist over the

health implications of MSG consumption, although, organizations and nutritionists endorse it and also reiterate its safety in humans (He *et al.,* 2011).

MSG is readily absorbed in the circulation, the liver is the major organ that metabolizes MSG making it more susceptible to the toxic effect of MSG via generation of ammonium ions and ROS (Tawfik *et al.,* 2012). The impairment of liver function could increase the blood levels of MSG and in-turn enhances its neurotoxic effects (Umukoro *et al.,* 2015). The probable toxic effects of MSG on the central nervous system (Husarova and Ostatnikov, 2013), female reproductive system (Mondal *et al.,* 2017), and hepatic system (Eweka et al., 2011) has been shown in animal studies. Recently, MSG has been shown to reduce lifespan and induce an adaptive response to oxidative stress in *Drosophila melanogaster* (Abolaji *et al*., 2017), increased mortality has also been seen in *Bactrocera dorsalis* after exposure to MSG and selected food additives (Chunyan *et al*., 2018), indicating that insects could be a good model to study MSG toxicity.

Based on Fig. 2, chronic consumption of MSG could release glutamate into cells (dissociation to Na^+ and glutamate), and glutamate may increase the activity of α–ketoglutarate dehydrogenase (α-KGDH) which generates ROS (Sharma *et al.,* 2014). Although glutamate contributes fuel to the Krebs cycle and modulates redox state of the cell at increased concentration, it can increase the mitochondrial proton gradient due to overproduction of oxidants (electron donors) by the Krebs cycle consequently increasing the production of mitochondrial superoxide. This proposed mechanism is supported by literature on brain tissues where α-KGDH is a potential site of ROS generation induced by glutamate (Zundorf *et al.,* 2009). On the other hand, glutamate could stimulate nerve cells to relay its signal but at high

concentration, glutamate can over-activate NMDA receptors leading to glutamate accumulation at the synaptic cleft. (Willard and Koochekpour 2013).

The amino acid transporter found in the central nervous system (CNS) (system Xc^-) mediates the exchange of extracellular L-cystine and intracellular L-glutamate across the plasma membrane, however, glutamate can cause inhibition of cystine which is required for the synthesis of glutathione (GSH) (Bridges *et al.*, 2012; Burdo *et al.*, 2006). Altogether, increased calcium influx (via over-activation of NMDA receptor), inhibition of cysteine uptake and GSH depletion can culminate and increase ROS production since GSH is essential for detoxification of xenobiotics.

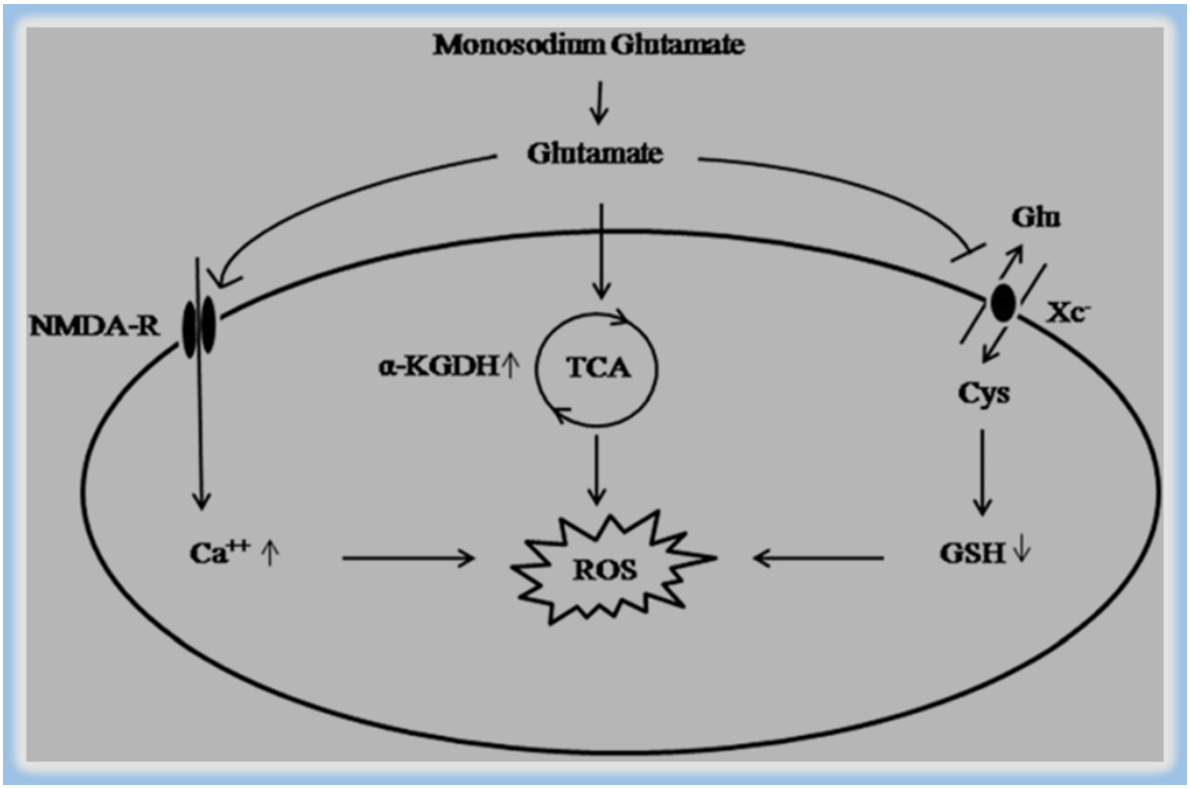


Figure 2: Proposed mechanism of production of ROS induced by MSG in the kidney of rodents (Sharma 2015). We propose that this mechanism of toxicity of MSG could occur in insects as well.

3 GLUTAMATERGIC SYSTEM

Glutamate is the principal excitatory neurotransmitter in the CNS in mammals, while in invertebrates, it acts as both excitatory and inhibitory neurotransmitter (Xu *et al*, 2015), but also located at the nerve-muscle junction (neuromuscular synapses) in most insects (James, 2016). More than twenty (20) glutamate receptors are identified in the central nervous system of mammals which are classified as ionotropic (voltage-sensitive) and metabotropic (ligand sensitive) receptors. The NMDA (N-methyl D- Aspartate) receptor is an ionotropic receptor permeable to calcium, sodium and potassium ions and is expressed in both neurons and astrocytes (Lee *et al,* 2010). Metabotropic glutamate receptors enhance the excitability of glutamatergic cells, regulate the degree of neurotransmission and contribute to synaptic plasticity (Lesage and Steckler, 2010).

The L-glutamate binding site has been demonstrated and characterized in the central nervous system of the *P. americana* (Satellite, 1992; Evans 1974). NR1 and NR2 subunits have been characterized and identified in the cockroach *Diploptera punctata* (Huang *et al.,* 2015) suggesting that these NMDAR subunits could also be present in our *N. cinerea*. Also, glutamate receptors have been shown to be present on the soma membrane of the dorsal unpaired median (DUM) neuron in the thoracic ganglia of the *P. americana,* although these receptors are pharmacologically different from analogous mammalian receptors (Washio 2002). In insects, glutamate plays roles in regulating physiological processes such as neuron sensibility modulation (Panek and Torkkeli, 2005), olfactory memory (McCarthy *et al*, 2011), rest/arousal neurons (El Hassani *et al*, 2012), locomotion and feeding (Wolstenholme, 2012). Glutamate related genes involved in signal transduction, biosynthesis and uptake have been characterized in *Chilo suppressalis* (Xu *et al*, 2015). Studies in *D. melanogaster* have shown that glutamate, (though

considered an insignificant neurotransmitter in the insect brain) needs to be maintained below toxic levels. There are about six transport systems in insects, a specific one for glutamic acid (Giordana *et al.,* 1989). The known high-affinity glutamate transporter in Drosophila, dEAAT1 has been shown to increase oxidative stress and reduce the life span of flies when inactivated suggesting that decreased regulation of extracellular glutamate levels or its accumulation could lead to various neurological defects in insects (Rival *et al.,* 2004).

The characterization of a cDNA encoding a Na^+ dependent glutamate transporter of the cockroach brain, *Diploptera punctata* (DipEAAT1), in a baculovirus- cell expression system, demonstrated its' stereo-selectivity (Donly, 2000). Meanwhile, this stereo-selectivity has not been confirmed in other insects (Donly *et al.,* 1997; Seal *et al.,* 1998) or mammals (Gazzola *et al.,* 1981). Although, studies have shown that most mammalian glutamate transporters are cloned from the brain except EAAT5 (Gegelashvili and Schousboe, 1998). Furthermore, an amino-acid sequence alignment revealed a sequence identity between DipEAAT1 and human glutamate transporters, hEAAT1 and hEAAT3 (Donly, 2000). Based on these findings, there is a possibility that *N. cinerea* possesses glutamate transporters that have almost identical functions to other insect glutamate transporters.

4 CHOLINERGIC SYSTEM

The brain cholinergic system plays roles in spatially and temporally organizations in mammals (Ballinger *et al,* 2016), participates in cognitive functions (Kim *et al,* 2019) and regulates sleep/wake cycles (Perry *et al,* 1999). Acetylcholine (ACh) is an important neurotransmitter in the CNS in mammals which acts as a neuromodulator and participates in cognitive processes, attention and memory (Picciotto *et al,* 2012).

In insects, the cholinergic system consists of ACh, choline acetyltransferase and acetylcholinesterase. The nicotinic acetylcholine receptors (nAChRs) can be found on the muscle fibres at the neuromuscular synapses, the neurons in the peripheral ganglia and in the brain and they can either be presynaptic or postsynaptic (Clarke *et al.*, 1986). In insects, the nAChRs mediate rapid synaptic neurotransmission, and they can be located at synaptic or extrasynaptic locations some of which gate cationic channels (David and Sattelle 1990; Beadle *et al.,* 1989). In Mammals, the muscarinic receptors regulate neurotransmitter signaling and participate in physiological functions such as smooth muscle contractility, learning and memory (Frank *et al*, 2003).

ACh is the major excitatory neurotransmitter in the nervous system of insects as observed in flies, locusts, honeybees and grasshoppers (Dupuis *et al*, 2012). Furthermore, cholinergic receptors are found exclusively at the CNS in insects and not at the neuromuscular junctions as present in vertebrates (James, 2016). Acetylcholine acts on various target tissues via two receptor types, the nicotinic and muscarinic receptors. In mammals, the muscarinic and the nicotinic receptors participate in synaptic activity in the brain (Frank *et al*, 2003).

5 MONOAMINERGIC SYSTEM

Monoamines such as serotonin, dopamine, norepinephrine, octopamine play roles in regulating behavior in animals (Swallow *et al*, 2016). Nevertheless, the evolutionary conservation of the monoaminergic system traverses both vertebrates and invertebrates, showing analogous effect in processes such as locomotor activity, stress responses, learning and memory (Bubak *et al* 2014; Kamhi *et al.*, 2013). In insects, dopamine is the most abundant monoamine neurotransmitter (also a melanin precursor) (Xu *et al*, 2015) released from the synaptic vesicles at the presynaptic terminals (Greer *et al*, 2005) which regulates behaviors such as locomotor

activity (Draper *et al*, 2007), learning, memory (Agarwal *et al*, 2011), and sucrose acceptance (Marrella *et al*, 2012). A previous study showed the monoaminergic system is involved in the reduction of immobility time in animals administered with magnesium and exposed to forced swimming test (Cardoso *et al*, 2009).

6 **REACTIVE OXYGEN SPECIES (ROS) AND REACTIVE NITROGEN SPECIES (RNS)**

ROS and RNS refer to reactive radicals and non-radical derivatives of oxygen and nitrogen respectively (Birben *et al*, 2012). Reactive oxygen species (ROS) are produced by living organisms due to normal cellular processes that are essential to maintain life (Genestra, 2007). They play roles in physiological processes at low to moderate concentrations, but when high they produce deleterious effects to cell components such as proteins, lipids and nucleic acid (Valko *et al*, 2006; Halliwell and Gutteridge, 1999). The three major ROS possessing physiological significance are superoxide anion (O_2^-), hydroxyl radical (**.**OH), and hydrogen peroxide (H_2O_2) as illustrated in the redox signaling (Fig. 3). The production of superoxide (O_2^-) has been confirmed in the mitochondria of the fat-body and muscle of cockroach, *Gromphadorhina coquereliana* (Malgorzata *et al*, 2013).

7 OXIDATIVE STRESS (OS)

Oxidative stress (OS) can be defined as an alteration in the pro-oxidant and antioxidant balance in favor of the pro-oxidant, hence resulting in oxidative damage (OD) (Halliwell, 1999). OD occurs via oxidative attack by reactive oxygen species (ROS). Oxygen is a reactive oxygen specie (ROS) important to biological systems and has both beneficial and deleterious effects. Oxygen participates in oxidative phosphorylation (OP) essential for the generation of energy in

the form of adenosine triphosphate (ATP). OP is crucial for the evolution of complex multicellular organisms, but in the same vein makes biological molecules such as protein, lipid, nucleic acid and carbohydrate prone to attack by ROS (Burton and Jauniaux, 2011).

Oxidative stress has been implicated in the pathophysiology of numerous disorders. The notion of the pro-oxidant-antioxidant balance highlights that the imbalance may be due to changes on either side of the equilibrium which could be an aberrantly high generation of ROS or, a deficit in the antioxidant defense system or both situations occurring simultaneously (Salisbury and Bronas, 2015). It has been shown that insects are not immune to the detrimental effects caused by oxidative stress, and ultimately the aerobic lifestyle of insects makes them more prone to oxidative stress (Gary and Clinton, 1995).

8 FREE RADICALS IN PHYSIOLOGICAL SYSTEM

Free radicals (FR) are molecules that contain one or more unpaired electrons in their electron shell and are formed when oxygen reacts with specific molecules, consequently making them more reactive (Chandrasekaran *et al,* 2017). These radicals can be formed by accepting or losing a single electron hence acting as oxidants or reductants (Lobo *et al,* 2010). Some FRs are produced via reactions with ROS. Hydroxyl radical (OH^-) can be produced by a series of reaction, such as the Fenton reaction that occurs in the presence of transition metals like Fe^{2+} or Cu^{2+}. Here, iron III is reduced to iron II which further reacts with hydrogen peroxide (H_2O_2) and breaks down to (OH^-) in a Haber-Weiss and Fenton reactions (Fenton, 1984).

$$Fe^{3+} + {}^{\bullet}O_2 \longrightarrow Fe^{2+} + O_2 \quad \textbf{Haber -Weiss}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + {}^{\bullet}OH \quad \textbf{Fenton reaction}$$

Furthermore, O_2^- can react with H_2O_2 to produce OH^- (Haber and Weiss, 1934; Liochev and Fridovich, 2002). The $OH^\bullet$ is the most reactive known radical that can damage biological molecules (Salisbury and Bronas, 2015) and initiate lipid peroxidation reaction by gaining electron from polyunsaturated fatty acid (PUFA). Other oxygen-derived free radicals are the peroxyl radicals ($ROO^\cdot$), alkoxy radicals ($RO^\cdot$), and hydroperoxyl radical (HOO) (Birben *et al*, 2012).

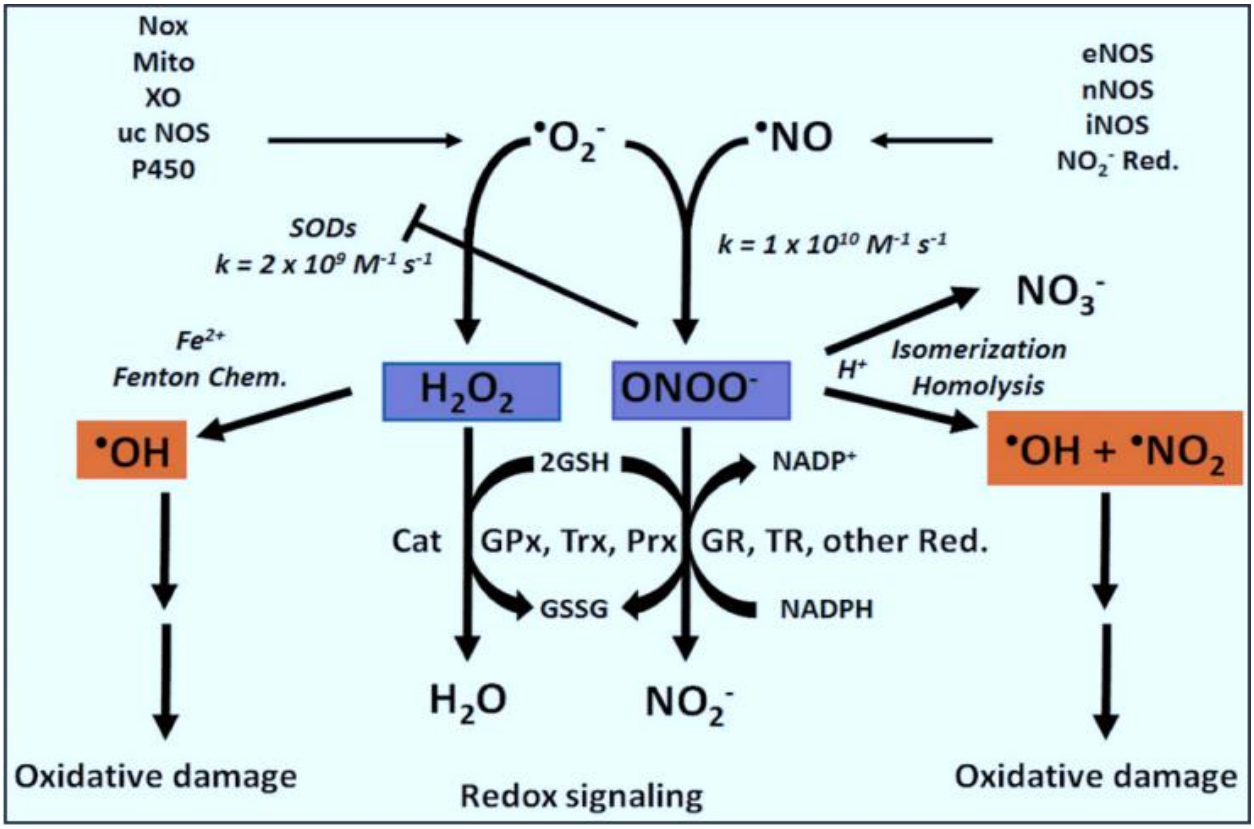


Figure 3: The chemical basis of redox signaling. Superoxide formation from NADPH oxidases (Nox), mitochondrial respiratory chain (mito), xanthine oxidase (XO,) NO synthase, P450, enhances redox signaling upon breakdown by self-dismutation or catalyzed by SODs to H_2O_2. H_2O_2 modulates thiol/disulfide equilibrium and modifies enzymatic activities. Detoxification of H_2O_2 via peroxiredoxins (Prx) and thioredoxins (Trx) or the selenol in glutathione peroxidase (Gpx) by reaction with thiol groups. Catalase catalyses H_2O_2 decomposition (Ullrich and Kissner, 2006; Daiber *et al.*, 2014).

9 ANTIOXIDANTS AND ANTIOXIDANT GENES

Antioxidants are classified as enzymatic and non-enzymatic antioxidants. They have the prospects to counteract the effects of oxidants. These antioxidant enzymes are coded for by their respective genes, thereby availing their physiological roles.

9.1 Enzymatic antioxidants and their Genes

Insects have detoxification enzymes crucial for detoxifying various toxic compounds which can be divided into three phases (I, II, and III). Phase I involves oxidation, hydrolysis, reduction; phase II involves conjugation while phase III comprises excretion (Wang *et al*, 2018). Insects have developed some endogenous antioxidant defense systems such as superoxide dismutase (SOD), catalase (CAT) and peroxidases to scavenge ROS, mitigate oxidative damage caused by heavy metals, mediate normal cellular redox status and adapt to oxidative stress (Wu *et al*, 2019).

SODs are the first line of defense against OD which plays roles in the dismutation of O_2^- to O_2 and H_2O_2 (Li *et al*, 2019). Three forms of SOD exist in insects, the CuZn-SOD found in the cytoplasm; Mn-SOD (mitochondria); and the extracellular form, EC-SOD. These are present in the hemolymph, and fluid secreted during ecdysis hence scavenges superoxide radicals (Zhang *et al*, 2014). Recent studies have revealed the involvement of CuZn-SOD during diapause in the oak silkworm *A. pernyi* (Bi *et al*., 2014).

In mammals, H_2O_2 produced by SODs is reduced to water by the activity of catalases (CATs) and the Glutathione peroxidases (GPXs) (Kirkman *et al,* 1999). In insects, CAT scavenges H_2O_2, hence impacting physiological functions (Felton and Summers, 1995; Zhang et al, 2016). Furthermore, the sequence and expression of CAT at the transcriptional and translational levels have been studied broadly in insects like *B.mori* (Yamamoto et al, 2005), *C.*

suppressalis (Xu et al, 2017), *H. armigera* (Wang et al, 2012) and *H. vitessoides* (Cheng et al, 2018). Additionally, insects utilize peroxiredoxins (Prxs) to scavenge H_2O_2, thereby recompensing for their lack of selenium containing GPX and glutathione reductases (GR), although they bring about signal transduction as well (Radyuk *et al*, 2003).

Peroxiredoxins (PRXs, also known as thioredoxin peroxidase) are ubiquitous in animals, plants and microbes (Perkins *et al*, 2015). They use electrons supplied by thioredoxins (Trxs) to catalyze the reduction of peroxides such as H_2O_2, peroxynitrite and other organic hydroperoxides (Lu and Holmgren, 2014). PRXs are crucial for survival in insects and also participates in protection from oxidative damage as observed in *Apis cerana cerana* (Yan *et al*, 2014).

Thiol containing enzymes such as thioredoxins (TRX 1, TRX 2), thioredoxin reductases, and glutaredoxins are also involved in the disposal of H_2O_2 in mammals (Gromer et al., 2004). Trxs are small evolutionary preserved predominant proteins required for antioxidant defense and crucial for maintaining cellular homeostasis (Arnér and Holmgren, 2000). Mammals have two isoforms of TRX. the cytosolic (TRX 1) and mitochondrial (TRX 2) (Lee *et al*., 2013), TRX 2 has been shown to be significantly expressed in the brain (Rybnikova *et al*, 2000). In insects, mitochondrial TRX has been predicted in *A. mellifera* (Corona and Robinson, 2006), hence they participate in antioxidant response in insects, namely *Apis cerana cerana* (Yao *et al*, 2013).

Glutathione-S-transferase (GST) is a phase II enzyme that participates in the detoxification of endogenous and exogenous electrophile compounds (Hayes *et al*, 2005) by catalyzing the conjugation of electrophilic compounds with thiol groups, enhancing the solubility of the electrophilic compounds and enabling its excretion (Habig et al, 1974), they also facilitate the

conjugation of MeHg (Espitia-Pérez *et al*, 2018). The most frequently described families of GSTs are the cytosolic, mitochondrial and membrane-associated GSTs (Robinson *et al*, 2004).

In insects, the microsomal GSTs are bound in membranes and located in the microsomes while the cytosolic-GSTs are evolutionarily and structurally different from the microsomal GSTs. Cytosolic-GSTs are more common in insects and are divided into six major classes such as Delta, Epsilon, Sigma, Theta, Omega, and Zeta (Hayes *et al*, 2005). GST *Theta* participates in detoxification of lipid peroxidation products in *B. mori* (Yamamoto *et al*, 2005) while Delta and Sigma GSTs are involved in an adaptive response to oxidative stress in *B. ignitus* (Kim *et al*, 2011).

9.2 Non-enzymatic antioxidants

These compounds confer protection against OD in organisms, they interact with ROS and terminate FR chain reactions. They are categorized as lipid-soluble and water-soluble antioxidants. Examples include ascorbic acid (vitamin c), α tocopherol (vitamin e), bilirubin, albumin, uric acid and glutathione [(L-γ glutamyl, L-cysteinyl, L-glycine) which contains a sulfhydryl (thiol) group found in-vivo] (Wu *et al*, 2013). Stilbene derivatives (resveratrol, phenolic acids, flavonoids), carotenoids are phenolic antioxidants while other antioxidants include lecithin, selenium and zinc (Pisoschi and 2015).

In insects, antioxidants such as ascorbic acid, carotenoids, glutathione and vitamin E, transferrin, ferritin, 3-hydroxykynurenine, uric acid have been reported, characterized, and shown to scavenge many oxidizing agents (Felton and summers, 1995). Vitamin C (water-soluble) provides intracellular and extracellular antioxidant potential and scavenges hydroxyl and superoxide radical anion in mammals (Pisoschi and 2015). It participates in protection against

UV stress in the cabbage looper, *Trichoplusia ni* and parsnip webworm *Depressaria pastinacella* (Susanne *et al*, 1999).

Vitamin E (α tocopherol, lipid-soluble) is abundantly found in the hydrophobic interior of the cell membrane and defends oxidant-induced membrane injury. In insects, tocopherols have been shown to scavenge radicals (Felton and Summer, 1995), extend lifespan in flies and promote sperm viability in bees (Canavoso *et a*l, 2001).

Glutathione (GSH) is abundant in cells, detoxifies hydrogen peroxides and lipid peroxides via the action of GSH-Px and also donates its electron to H_2O_2, hence reducing it to H_2O and O_2 (Masella *et al,* 2005). GSH is also a co-factor for various detoxifying enzymes like GSH-Px, glutathione transferases. GSH interacts with pro-apoptotic, anti-apoptotic signaling pathways, activates and regulates several transcription factors AP-1, NF-kB, Sp-1 (Masella *et al,* 2005). In insects, GSH participates in antioxidant defense (Felton and Summer, 1995) and plays roles in response to metals and other pollutants in caddisflies and mayflies (Lingtian *et al*, 2009).

Carotenoids are plant-based pigments that react with peroxyl, hydroxyl, and superoxide radicals (El-Agamey *et al*, 2004). B-Carotene inhibits the activation of oxidant-induced NF-kB and interleukin (IL)-6, as well as the production of tumor necrosis factor-α (TNF-α). Animals are unable to generate carotenoids on their own (Walter and Strack, 2011) while insects derive carotenoids from their diet or microbial symbionts (Kayser, 1982). In insects, carotenoids confer protection against light damage (Felton and Summers 1995; Dhinaut et al, 2017), participate in diapause (Veerman, 2001), egg coloration and formation of galls (Fox, 1976; Shamim *et al*, 2014).

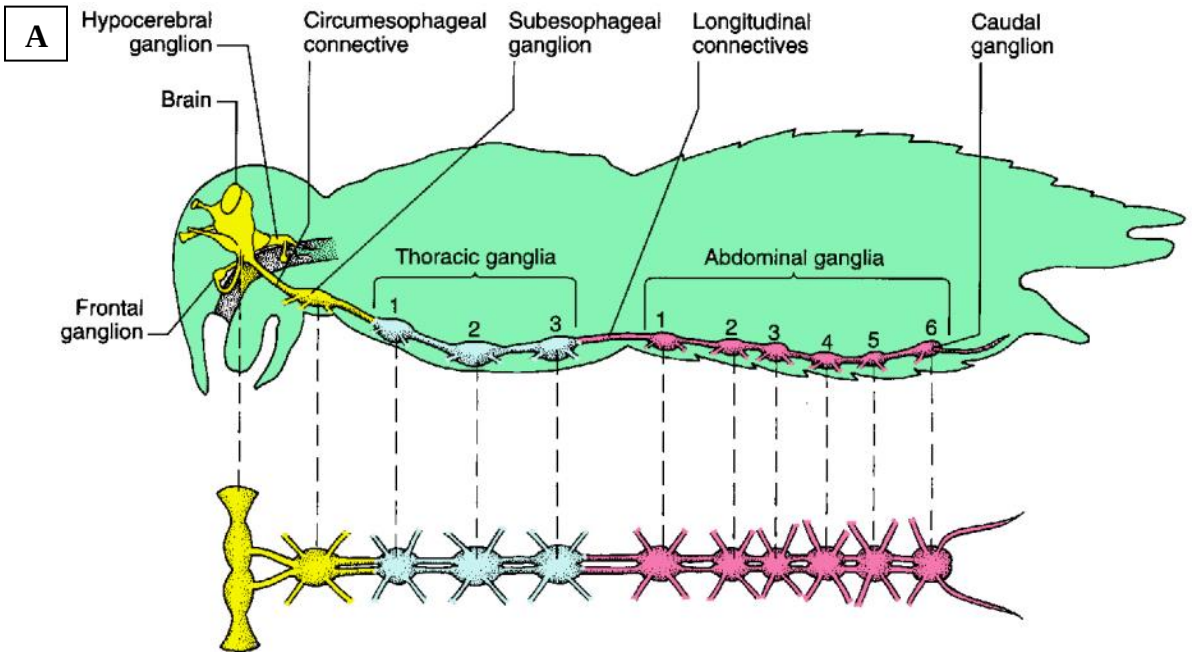
10 ALTERNATIVE MODELS OF RESEARCH

Ethical issues have posed many restrictions over the experimental use of higher model vertebrates like guinea pigs, dogs, monkeys, rabbits, rats and mice, hence alternatives to use of animals have been proffered to repress some of their limitations. A scheme involving 3Rs implying reduction, refinement and replacement of laboratory use of animals is being applied in recent research (Ranganatha and Kuppast, 2012). The most common alternative models (invertebrates) utilized in research include *Drosophila melanogaster, Danio rerio, Caenorhabditis elegans, Zootermopsis nevadensis*, *Manduca sexta, Bombyx mori, Apis mellifera* and micro-organisms such as *Saccharomyces cerevisae* and *Escherichia coli.*

Invertebrates possess numerous advantages over vertebrates such as a short life cycle, a small size enabling ease of manipulation, simple anatomy that facilitates the use of large sample size, ease of reproducibility of experiments and fewer or no ethical problems. Their cost of housing, rearing and maintenance is cheaper than rodents. They also possess genetic similarities to higher invertebrates such as mammals (Wilson-sanders, 2011). Cockroaches are potential insect models, the basic physiological and biophysical features of their nervous system is similar to mammals, and they are bigger in size than the fruit flies and mosquitoes (Stankiewicz *et al*., 2012; Harris and More, 2005). The *Nauphoeta cinerea* (lobster cockroach) is a propitious insect model that has contributed to toxicological, behavioral, and pharmacological studies (Afolabi *et al.,* 2018; Adedara *et al,* 2016; Rodrigues *et al,* 2013).

11 THE INSECT NEUROANATOMY

Major details regarding the neuroanatomy of insect were known only in the early 20th centuries (Strausfeld 1976). The central nervous system of insects is made up of the brain, ventral nerve cord, and ventral ganglia (Fig. 4 A&B). The brain consists of some fused ganglia that make up the protocerebrum, deutocerebrum, and tritocerebrum (James 2016). The protocerebrum receives sensory input from the compound eyes; the deutocerebrum receives sensory input from the antennae and sends motor output to the antennae while the tritocerebrum sends motor neurons to muscles in the labrum and pharynx and innervates the stomatogastric (foregut) nervous system controlling foregut muscles. Sensory or afferent neurons deliver signals to the CNS; motor or efferent neurons deliver an output to muscles, glands, and organs; interneurons mediate between sensory and motor neurons and are located within the CNS (James 2016).



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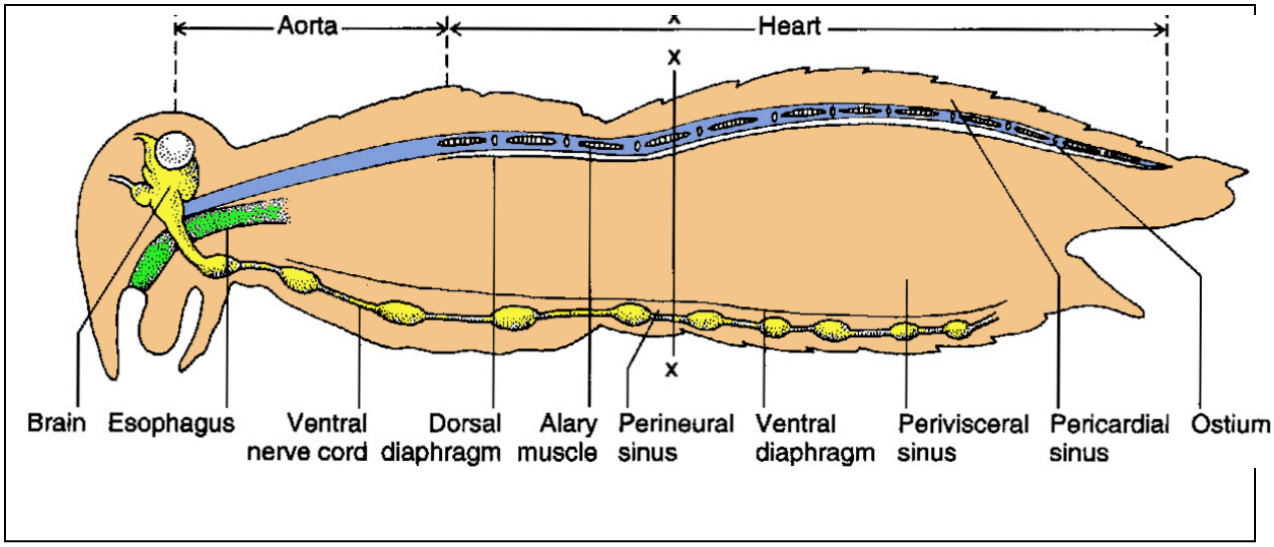

Figure 4: **The internal anatomy of insects.** **A** is the central nervous system showing the brain and ventral ganglia and **B** the circulatory system showing the ventral nerve cord (Underwood 2009).

11.1 The Insect Brain

The insect brain is made up of supraesophageal ganglion (protocerebrum, deutocerebrum and tritocerebrum) (Fig. 5 A&B). The protocerebrum is the main integrative center that process incoming information from many sensory sources. The optic lobe (Fig. 5A) contains several neuropil regions, processes information from the compound eyes and forms part of the protocerebrum (James, 2016). The nervus corporis cardiac I and II, link the protocerebral neurosensory cells (NSC) with the corpora cardiac (CC) and corpora allata (CA) (Fig. 5B). The protocerebrum contains the mushroom bodies (corpora pedunculata) that are big, bilateral integrative centers known to be involved in olfactory learning (Fig. 6). The size of mushroom bodies varies in different insects (James, 2016).

The **protocerebrum** is also made up of the central body complex (Pfeiffer and Homberg, 2014) containing glutamate, acetylcholine as the major transmitters. The other neurotransmitters and neuromodulators contained in the central body complex include γ–aminobutyric acid

(GABA), histamine and dopamine (Nässel, 1999). Some neuropeptides have been detected in the brain (Nässel, 1993) and central complex neurons (Nässel, 2002; Nässel and Homberg, 2006) of insects. SIFamide, a neuropeptide has been identified in *Manduca sexta* and *Drosophila* (Heuer *et al.,* 2012; Kahsai and Winther 2011). However, nitric oxide (NO) has been found at synaptic endings of neurons present in locust, *Shistocerca gregaria* and grasshopper (Muller 1997; Kurylas *et al.,* 2005; Wenzel *et al.,* 2005; Siegl *et al.,* 2005). These neuropeptides and or neurotransmitters have various functions, although their specific physiologic reactions are unknown. However, acetylcholine, GABA, and NO participate in sound production in grasshoppers (Kunst *et al.,* 2010) while tachykinin and neuropeptide F have been demonstrated to modulate locomotor behavior in Drosophila (Kahsai *et al.,* 2011).

The **deutocerebrum** derives sensory input from mechano- and chemosensory receptor neurons on the antennae (Homberg *et al*., 1989; Stocker 1994; Hildebrand, 1995) and sends motor signals to muscles of the antennae. The neuropil regions in the deutocerebrum process the information from the chemosensory and mechanosensory neurons. Chemosensory input is linked to the antennal lobe (AL) neuropil (Fig. 7), whereas the antennal mechanosensory and motor center (AMMC) receives the mechanoreceptor input and releases the motor information. The AL and AMMC are represented on the left and right sides of the brain, meanwhile, the AMMC is located at the posterior and appears ventral to the AL (Marion-Poll and Tobin 1992; Hildebrand, 1995). The chemosensory inputs are partitioned into different synaptic sites within the AL based on the stimuli the receptor responds to such as pheromone, food, host odors or carbondioxide (James, 2016).

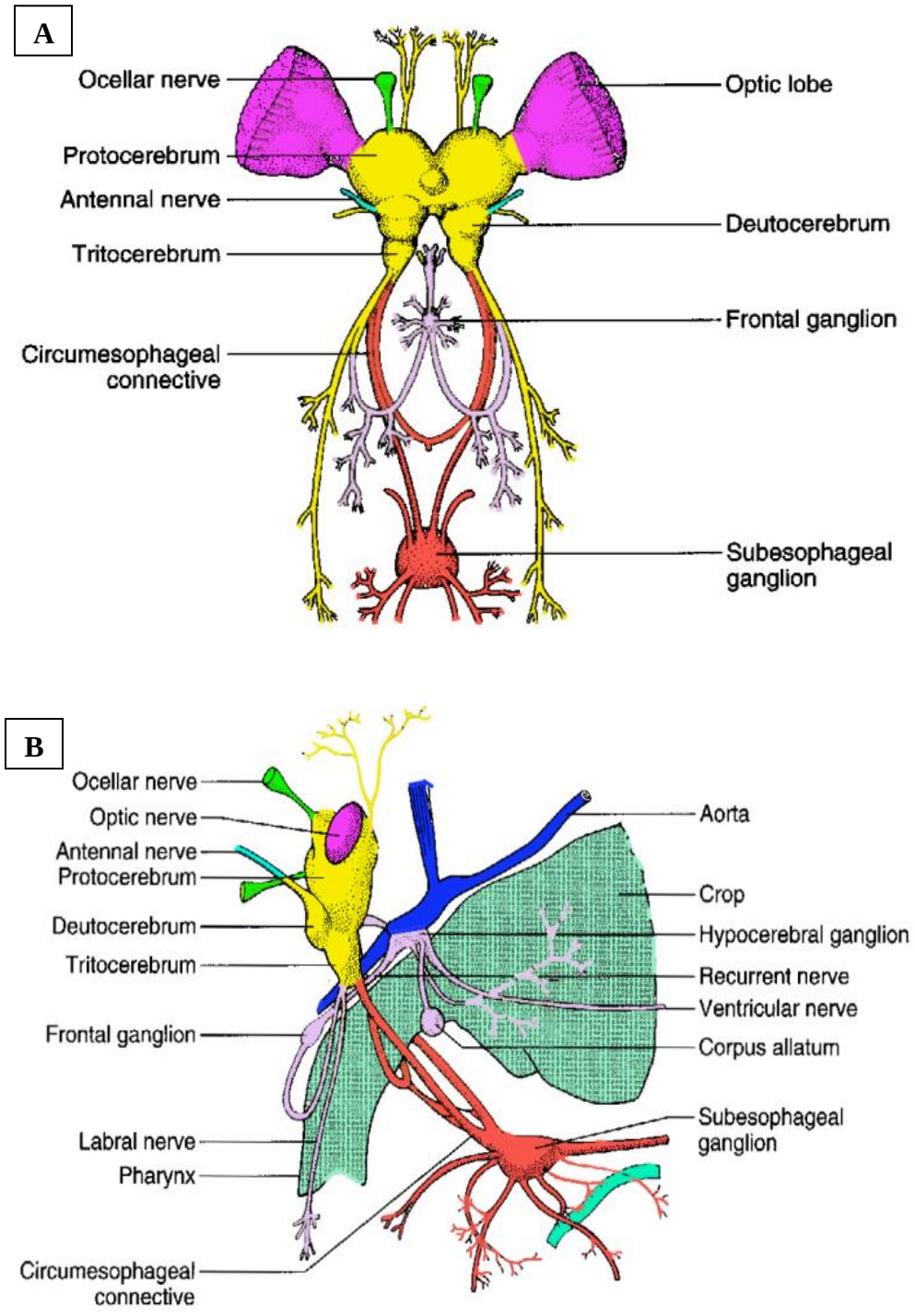

Figure 5: **The internal anatomy of insects.** **A** is the visceral nervous system and **B** the peripheral nervous system (Underwood 2009).

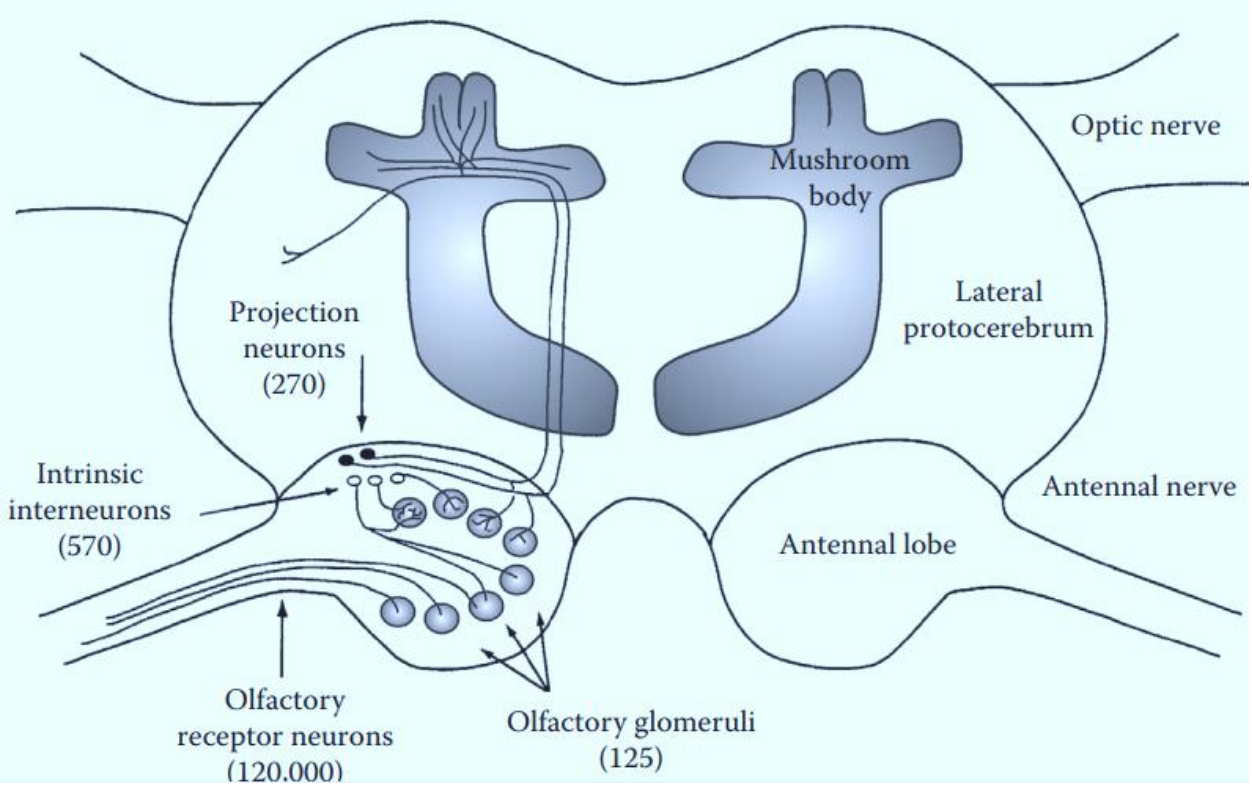

Figure 6: **The frontal section of the brain of a female cockroach demonstrating the antennal lobes and mushroom bodies**. The sensory neurons from the antennae pass into the deutocerebrum where the synapse in glomeruli with interneurons projecting to the protocerebrum. The numbers in bracket indicate the approximate numbers of neurons in the olfactory system in a cockroach (Culled from James L. Nation 2016, used with permission from Lemon and Getz, 1999).

The **tritocerebrum** directs motoneurons to muscles in the labrum and pharynx and innervates the stomatogastric nervous system, a system of several small ganglia such as the frontal ganglion, hypocerebral ganglion, and ingluvial ganglia that control the foregut muscles (Fig. 7). The commissural neurons from each side of the tritocerebrum move around the esophagus and transmit a cross-talk between the two halves, and lateral connectives connect each half of the tritocerebrum with the subesophageal ganglion (James, 2016) (Fig. 7&8). The frontal ganglion situated anterior to the brain is connected to the tritocerebrum by lateral connectives.

Nerves from the frontal ganglion innervate the pharynx. The recurrent nerve runs through the esophagus, moves underneath the brain, and connects behind the brain with the hypocerebral ganglion reclining on the surface of the esophagus (Fig. 7&8) (James, 2016). The tritocerebrum has lateral nerve cord connections to the subesophageal ganglion. The brain rests on the gut which proceeds between the lateral nerve cord connectives to the subesophageal ganglion (SG). The SG is formed from the fusion of three pairs of ganglia. It is made up of sensory and motor connections to sensory structures and muscles of the mouthparts, salivary glands, neck receptors in some insects and neck muscles. The SG influences motor patterns that emanate from other ganglia and these motor patterns pertain to walking, flying, and breathing in insects (James, 2016).

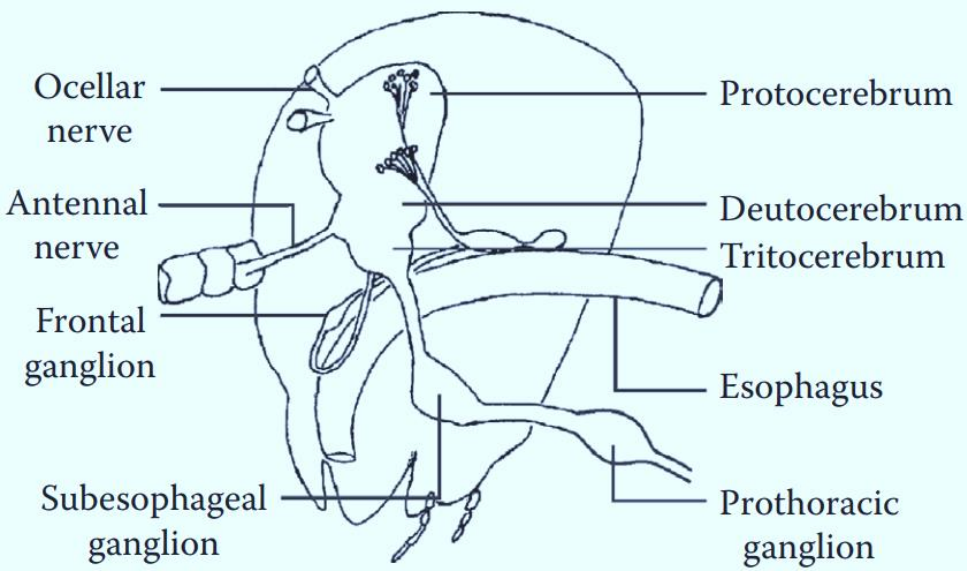

Figure 7: **A side view of the main parts of the brain with associated head connections and ventral connectives to the subesophageal ganglion** (Culled from James, 2016. Diagram modified from Snodgrass, 1935 and Jenkin, 1962).

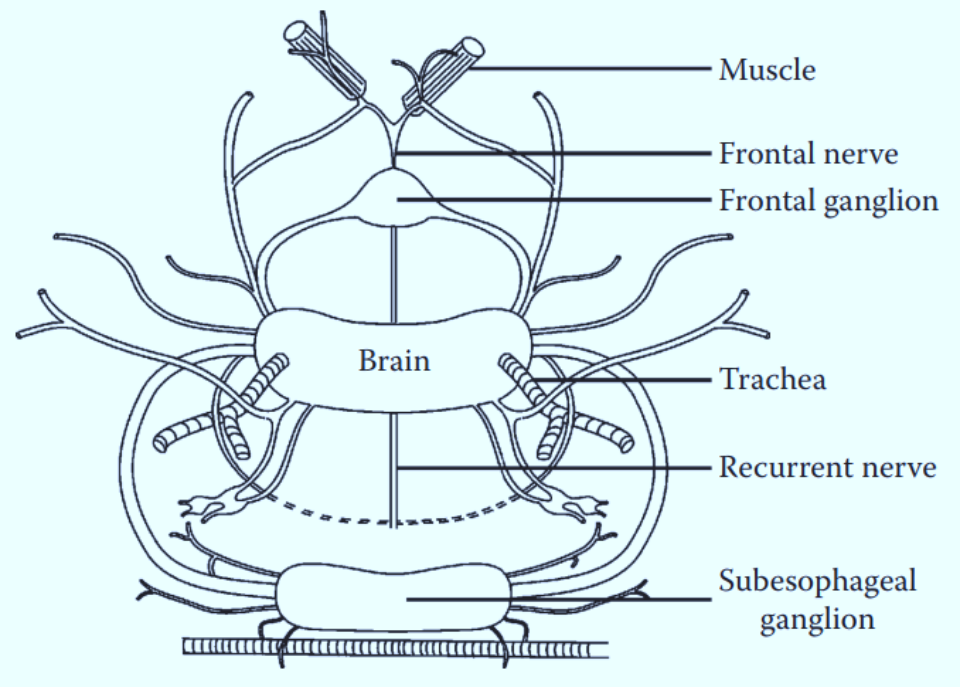

Figure 8: **Vertical view of the brain and linked head ganglia and nerves of a larval tomato hornworm *Manduca spp*. Lepidoptera Sphingidae** (Culled from James, 2016). The tracheal trunks penetrate the brain tissue and provide for gas exchange in the brain.

11.2 The Insect Ventral Ganglia

Most insects possess three thoracic ganglia- the pro, meso, and metathoracic ganglia, situated in corresponding thoracic segments (based on early evolutionary discovery), though changes have been observed with this arrangement in insects (Fig. 9). The Hemiptera and Diptera have fused meso-and metathoracic ganglia usually with a fusion of some of the abdominal ganglia forming a large thoracic ganglion (Fig. 10). Each thoracic ganglion directs motor axons to the leg muscles of its segment and receives sensory axons from sensory receptors in the tarsi and leg joints. The meso- and metathoracic ganglia supply motor nerves to the wing muscles of the meso- and metathoracic segments.

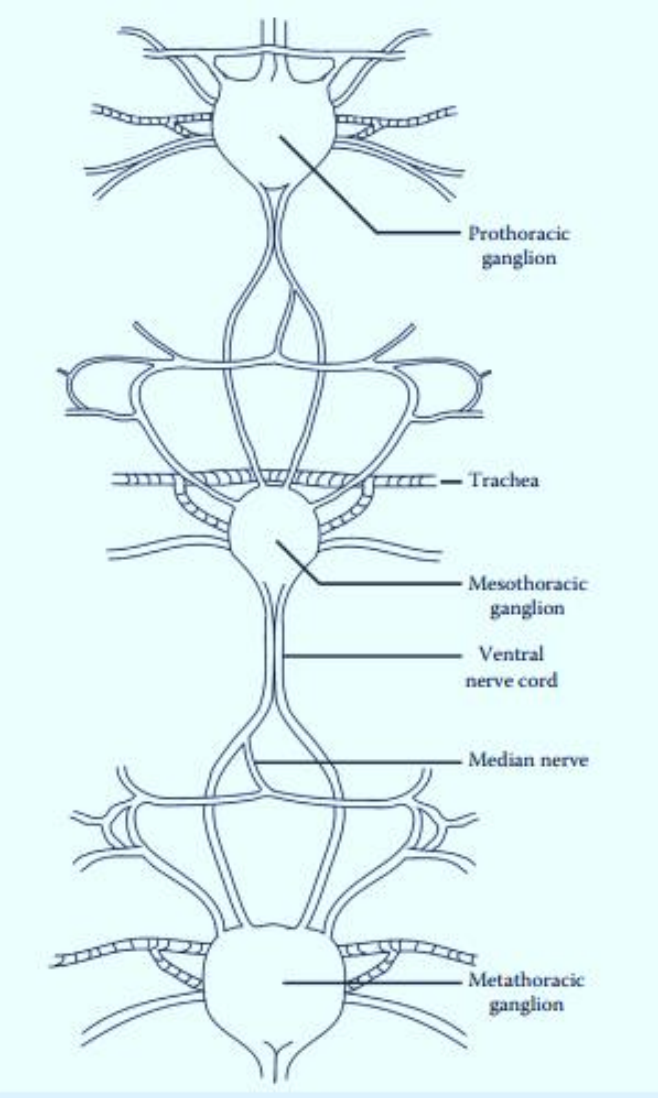

Figure 9: Posterior view of the thoracic ganglia of a larval tomato hornworm. The median nerve arises from the ganglion, travels with the connective tissue, detaches from the ventral connective and divides into two branches passing to each side of the posterior segment. The large tracheal trunks supply several smaller branches to the ganglia (Culled from James, 2016).

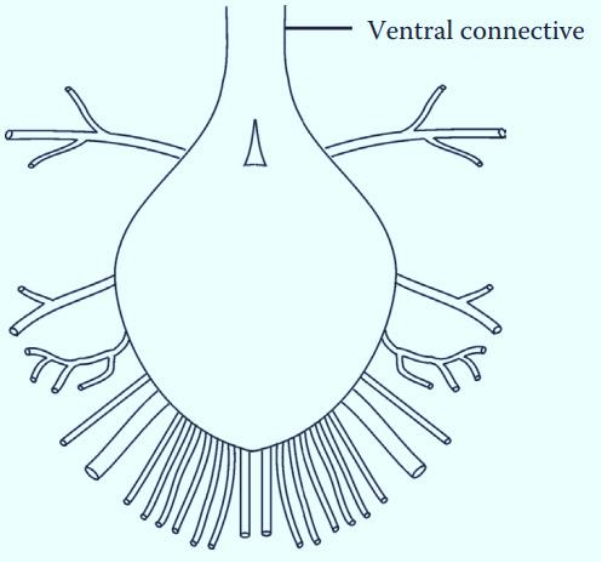

Figure 10: **The big second thoracic ganglion of the hemipteran, *Oncopeltus fasciatus*, the large milkweed bug.** The ganglion is made up of fused neuromeres from the abdomen that is nerves that originates from the ganglion and reaches the abdomen (Culled from James, 2016).

11.3 The Insect Abdominal Ganglia

The quantity of abdominal ganglia (AG) varies in different insect orders. One of the ganglions in each segment was the evolutionary primitive condition, but in all live insects, some merging of abdominal ganglia has taken place. Some Apterygota have eight AGs; Odonta larvae have seven; Orthoptera have about six; some insects have fewer than five AGs (Fig. 11); while some highly developed dipterans and hemipterans have their abdominal ganglia fused with thoracic ganglia forming a big metathoracic ganglion (James, 2016). In the *Periplaneta americana*, the big sixth abdominal ganglion is the terminal abdominal ganglion (TAG) and is a fusion of neuromeres (ganglionic masses associated with a particular segment) from the posterior segments (Fig. 12). However, the thoracic and abdominal ganglia are divided in to three anatomical and functional parts- a dorsal motor neuropil, middle integrative neuropil and ventral

sensory neuropil. These parts are involved in the communication and maintenance of the motor and sensory nerves (James, 2016).

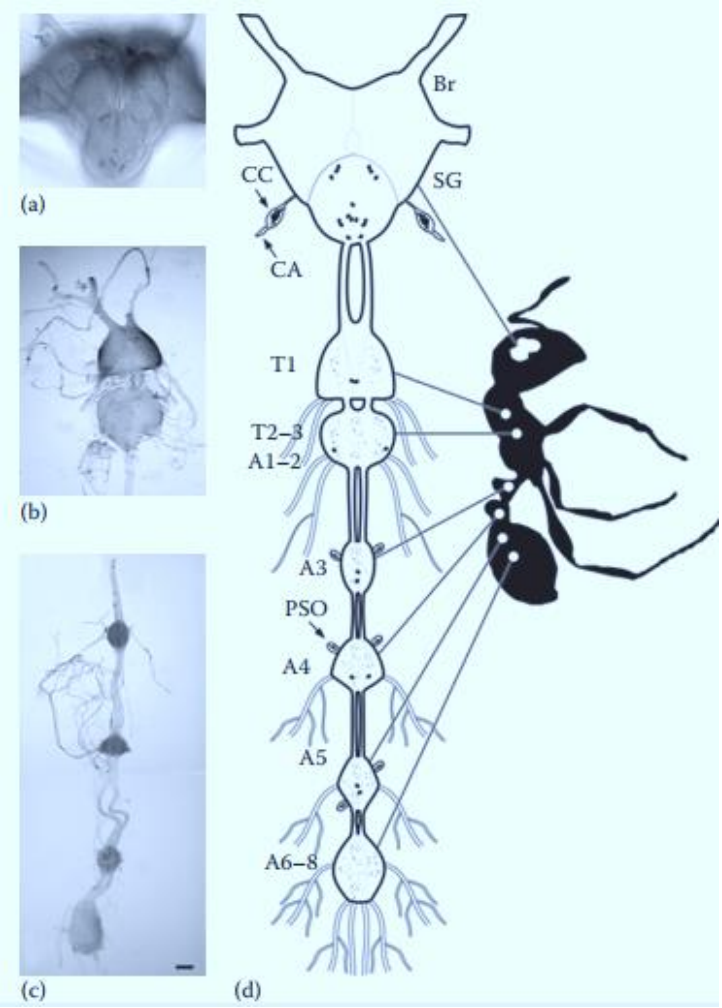

Figure 11: **The central nervous system (CNS) of an adult fire ant showing the fusion of ganglia in the thoracic and abdominal ganglia**. (a) the brain (b) the two big thoracic ganglia (c) the four abdominal ganglia (d) a diagram of the CNS of ant adjacent to the photographs. Br, brain; SG, subesophageal ganglion; CC, corpora cardiac; CA corpora allata; PSO perisympathetic organ; T1-T3, first to third thoracic ganglia; A1-A8, first to eight abdominal ganglia (Culled from James, 2016; used with permission from Choi et al, 2009).

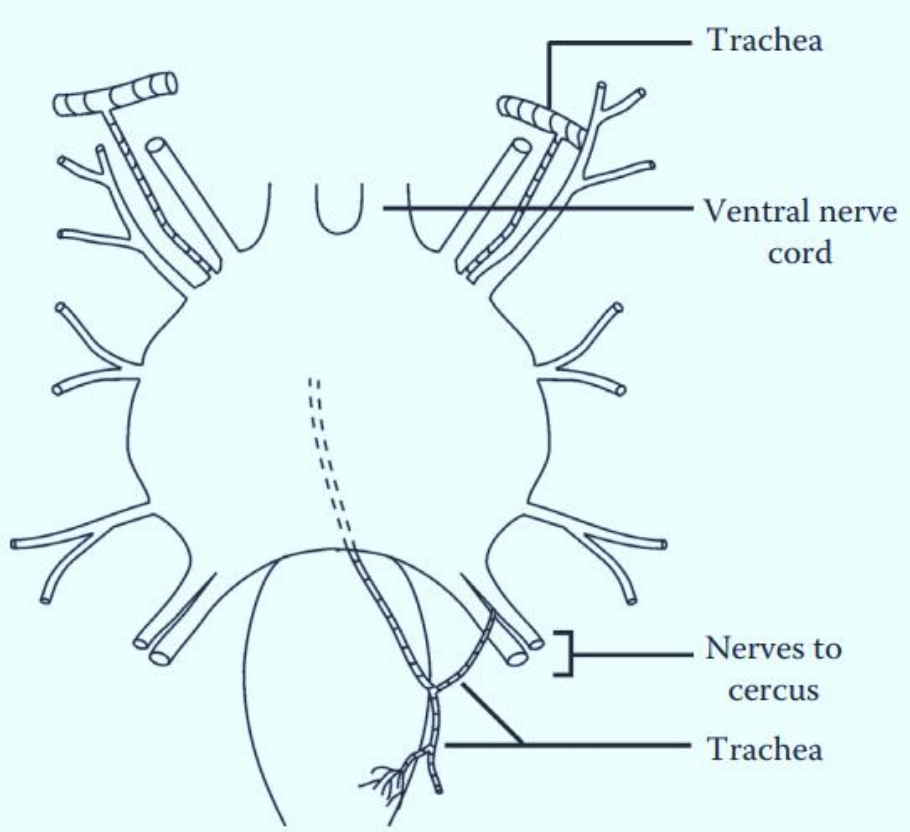

Figure 12: **The sixth and terminal abdominal ganglion (TAG) of *Periplaneta americana*.** The TAG contains fused neuromeres from several posterior abdominal segments. Nerves from the TAG moves into the cerci, and sensory neurons from the cerci synapse in the TAG with huge axons that project forward to the thoracic ganglia and brain (Culled from James, 2016).

12 Nauphoeta *cinerea* (An Alternative Organism of Research)

Nauphoeta cinerea (Olivier, 1789) or 'lobster cockroach' (Blattodea: Blaberidae) is a tropical animal that originated in northeastern Africa, belonging to the superfamily Blaberoidae and genus *Nauphoeta*. It is also identified as the speckled cockroach. *N. cinerea* is identified by its brown color (nymphs are darker) (Fig. 13), inability to fly at the adult stage despite having wings (nymphs possess no wings) and high reproductive capacity. They undergo moulting at both nymph and adult stages (Fig. 14), the nymphs moult about 8 times in 21 days. The male and female nymph and adult *Nauphoeta* are differentiated by their shape at the lower abdomen; the males are oval-shaped while the females are round-shaped (Fig 15 & 16). Also, the females of the same age appear bigger in size than the males. *N. cinerea* undergoes incomplete metamorphosis, the egg takes about 40 days to form the nymph, and the nymphs take about 90 days to become an adult. This cycle is similar to that of the rodents in terms of duration.

N. cinerea is omnivorous, can survive without food and water for a few weeks and can also walk and live for a few hours when rendered headless as observed during experiments. This could be due to a decentralized sensory and motor system as described by Strausfield, demonstrating the location of the insect brain in several body segments ranging from the posterior to the anterior end of the insect body (Strausfield, 1976). The *Nauphoeta* is able to walk on both vertical and horizontal surfaces, thus studies have shown that *P. americana* walks on these type of surfaces as well using the tarsi (Roth and Willis, 1952), while they utilize the arolium to walk on smooth surfaces [Fig. 17 (Roth and Willis, 1952; Beutel and Gorb, 2001)]. The structure of the legs of *P. americana* is possibly the same with *N. cinerea.*

N. cinerea is known to reproduce asexually via facultative parthenogenesis, a natural form of reproduction that involves the growth and development of embryos without fertilization, this occurs when the female *Nauphoetas* are separated from their male counterparts (Laura *et al.*, 1999; Corley *et al*, 2001); they also reproduce sexually. The *Nauphoeta* females housed in groups promote and stabilize the production of asexual libraries better than the *Periplaneta americana* females, and this is triggered by specific chemosensory signals of females which is detected mainly by the antennas (Katoh *et al.*, 2017). Although most species of cockroaches are oviparous in nature, *N. cinerea* is ovoviviparous in nature (Bell *et al.*, 2007) just like the Madagascar hissing cockroach.

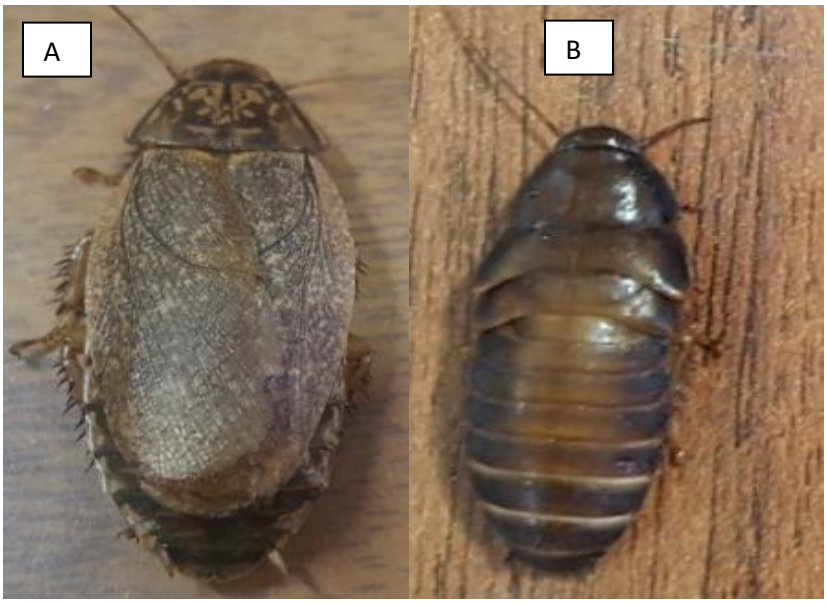

Figure 13: **Dorsal view of *N. cinerea***. A - Adult; B- Nymph (Image by Author).

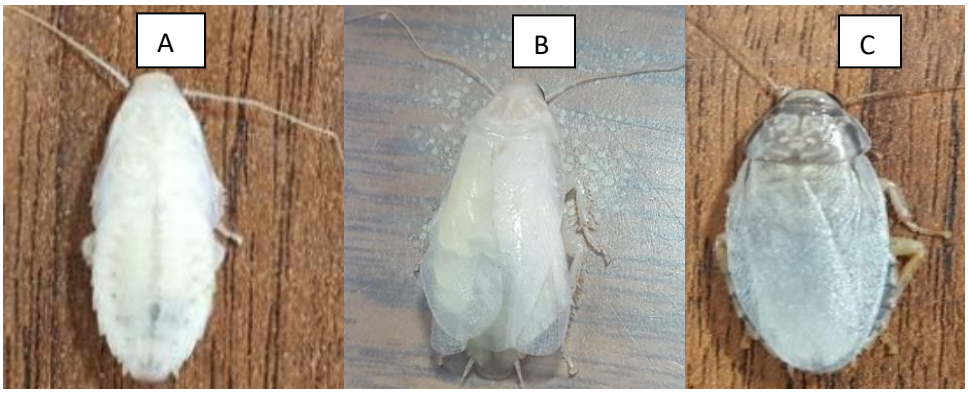

Figure 14: **Dorsal view of *N. cinerea* showing moulting at various stages of the life cycle**.
A –Nymph; B- Adult; C- almost completely formed adult *N. cinerea* (Image by Author).

Figure 15: **Ventral view of a male and female nymph of *N. cinerea***. (Image by Author).

Figure 16: **Ventral view of a male and female Adult *N. cinerea*.** (Image by Author).

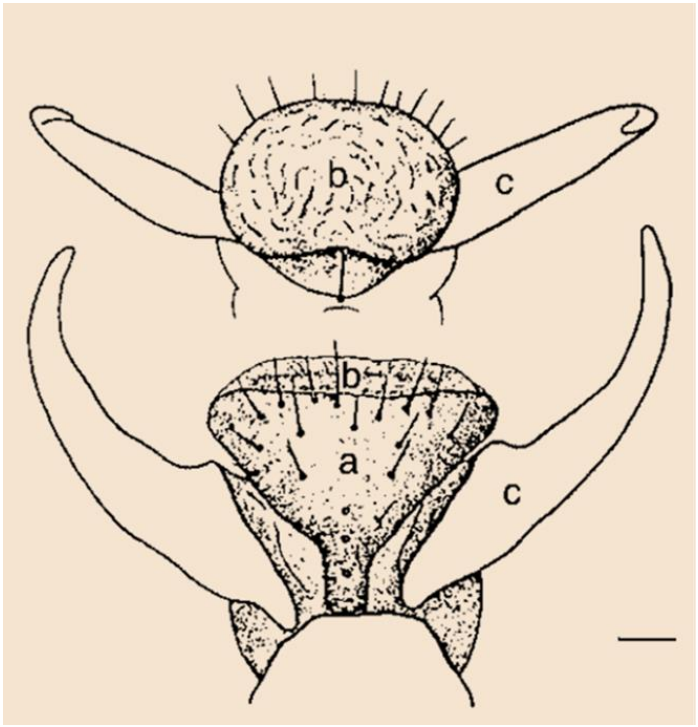

Figure 17: **Structures of the legs of *Periplaneta americana***. a-arolium; b-aroliar pad; c-tarsal claw. (Roth and Willis 1952b). This leg structure could be similar to the legs of *N. cinerea*.

JUSTIFICATION

Insects are having wide recognition in research areas such as toxicology, molecular biology, ethology, biotechnology, immunology, endocrinology and so on. *D. melanogaster* is known to be the most utilized insect model, although recently there has been the emerging use of other insects namely *B.mori, G. mellonella, A. mellifera, M. sexta, H. armigera, C. suppressalis, Z. nevadensis, C. secundus, M. domestica, A. aegypti* and so forth. *N. cinerea* is a prospective insect model which has been employed in toxicological, pharmacological and behavioral studies (Adedara *et al,* 2016; Rodrigues *et al,* 2013), furthermore, its' head and fat-body transcriptome has been recently published by our group, providing genetic information necessary for molecular studies (Segatto et al, 2018). Consequently, *N. cinerea* presents advantages as an alternative research model such as prolific reproductive cycle, similar life span to rodents and many more, however, there are no reports in the literature on the sensibility of the nymphs of *N. cinerea* to certain toxic chemical mixtures.

To offer new contributions for toxicology, it is necessary to evaluate the additive, synergistic, potentiation or antagonistic effects of co-exposure of potentially toxic compounds, which illustrates real life and public health issues; this present study evaluated the effects of MSG and MeHg. Glutamate is an excitatory neurotransmitter in the CNS, it performs crucial roles in development, memory and behavior. MeHg is a neurotoxicant that crosses the BBB and accumulates in the brain. MeHg disrupts the glutamatergic neurotransmission system, increases extracellular glutamate levels via inhibition of its uptake and release into the synaptic cleft, consequently causing over activation of glutamate receptors and increase in calcium release which can lead to its accumulation in the mitochondria and eventually result in an increase in the generation of reactive oxygen species, hence culminating to excitotoxic events.

OBJECTIVE

GENERAL

The present study aims to evaluate modifications in behaviour and redox homeostasis in the nymphs of lobster cockroaches co-exposed to methylmercury and monosodium glutamate, estimating the possible additive or synergistic interaction of these compounds in a *Nauphoeta cinerea* model.

SPECIFIC

CHAPTER 1

-To analyze the vertical exploration and locomotor activity of the nymphs of *N. cinerea* co-exposed to a diet containing methylmercury and monosodium glutamate.

- To investigate the possible additive or synergistic interaction between MeHg and MSG by the evaluation of biochemical parameters related to oxidative stress and acetylcholinesterase activity in the heads of the nymphs of *N. cinerea*.

CHAPTER 2

- To estimate the levels of Hg in the heads of cockroaches exposed to MeHg and MSG

-To evaluate the expression of antioxidant genes (glutathione-S-transferase, superoxide dismutase, catalase, thioredoxins, peroxiredoxins) and genes associated with detoxification, namely GST *sigma,* GST *theta,* GST *delta, Sod, Cat, Trx1, Trx2, Trx5 and Prx4* in the heads of the nymphs of *N. cinerea* exposed to MeHg and MSG.

MATERIALS AND METHODS

The materials and methods have been incorporated into each chapter of Part II of this thesis.

PART II

This part features the experiments and results according to chapters.

CHAPTER ONE

DIETARY CO-EXPOSURE TO METHYLMERCURY AND MONOSODIUM GLUTAMATE DISRUPTS CELLULAR AND BEHAVIORAL RESPONSES IN THE LOBSTER COCKROACH*, NAUPHOETA CINEREA* MODEL.

Blessing A. Afolabi, Isaac A. Adedara, Diogo O. Souza, João B. T. Rocha

Article published in the Journal of Environmental Toxicology and Pharmacology.

Dietary co-exposure to methylmercury and monosodium glutamate disrupts cellular and behavioral responses in the lobster cockroach, *Nauphoeta cinerea* model.

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Abstract

The present study aims to investigate the effect of monosodium glutamate (MSG) both separately and combined with a low dose of methylmercury (MeHg) on behavioral and biochemical parameters in *Nauphoeta cinerea* (lobster cockroach). Cockroaches were fed with the basal diet alone, basal diet + 2% NaCl, basal diet + 2% MSG; basal diet + 0.125mg/g MeHg, basal diet + 0.125mg/g MeHg + 2% NaCl; and basal diet + 0.125mg/g MeHg + 2% MSG for 21 days. Behavioral parameters such as distance traveled, immobility and turn angle were automatically measured using ANY-maze video tracking software (Stoelting, CO, USA). Biochemical end-points such as acetylcholinesterase (AChE), glutathione-S-transferase (GST), total thiol and TBARS were also evaluated. Results show that MeHg + NaCl, increased distance traveled while MeHg + MSG increased time immobile. AChE activity was significantly reduced in cockroaches across all the groups when compared to the control. There was no significant alteration in GST activity and total thiol levels. It could be that both NaCl and MSG potentiates the neurotoxic effect of MeHg in cockroaches.

KEYWORDS: *Nauphoeta cinerea;* methylmercury; monosodium glutamate; locomotor behavior; oxidative stress.

1. Introduction

Methylmercury (MeHg) has been established as a precarious environmental contaminant that causes neurological deficits in both experimental animals and humans (Syversen and Kaur, 2012). The electrophilic nature of MeHg makes it react with nucleophiles such as sulfhydryl-, and selenol-containing proteins and low molecular mass thiol-containing molecules, which will be responsible for most of its toxicological effects. The potent involvement of MeHg with the activation of Nrf2, the transcription factor involved in the regulation of antioxidant system also suggests there are several mechanisms involved MeHg-induced oxidative stress and cell toxicity (Antunes dos Santos et al., 2018; Unuki et al. 2018). Experimental points of evidence have shown that methylation of inorganic mercury by methanogenic bacteria in an aquatic environment allows MeHg bioaccumulation in the aquatic food chain, eventually reaching the human diet (Honda *et al*., 2006; Moreira and Farina, 2014). Accordingly, seafood represents a major source of MeHg, and about 90-95% is absorbed in the gastrointestinal tract (Bradley *et al*., 2017). Depending on the exposure, MeHg can cause long-term neurological disturbances and can also be teratogenic (Abbott et al., 2017). An increased severity of methylmercury poisoning in the population of a MeHg polluted area in Japan who fed on high intake of fish has been observed, demonstrating for the first time dose-response effects of methylmercury in Japan (Takaoka *et al*., 2018). A wide range of sensory disturbance and other complications such as malignant diseases, renal diseases, respiratory diseases, amongst others were also observed in these subjects suggesting other symptoms of MeHg poisoning (Takaoka *et al*., 2018).

A broad toxic effect of MeHg toward different systems should be expected considering the pervasive distribution of sulfhydryl-and selenohydryl containing proteins in the nervous system, as well as the significance of the proper redox state in such proteins, which allows their

correct functioning (Farina and Aschner, 2017). Apparently, metabolites of selenium have been shown to mitigate the toxicity of methylmercury (Oliveira *et al*.,2017). In view of this, MeHg has been described to disrupt the homeostasis of different neurotransmission systems, including the glutamatergic (Aschner *et al*., 2007), the GABAergic (Basu *et al*., 2010), the dopaminergic (Bridges *et al*., 2017), and the cathecholaminergic system (Farina et *al*.,2017).

The most important excitatory neurotransmitter in the mammalian CNS, serving vital roles in brain development, and memory formation is glutamate (Briguglio *et al*., 2018). MeHg increases extracellular glutamate levels, which result from both inhibitions of glutamate uptake and stimulation of its release into the synaptic cleft (Farina *et al*., 2003), which can culminate in excitotoxic events (Aschner *et al*., 2007). In fact, an increased influx of Na^+ and Ca^{2+} occurs due to over-activation of the NMDA subtype glutamate receptors, which can cause oxidative stress and neurotoxicity (Xin *et al*., 2005).

Monosodium glutamate (MSG), a derivative of one of the most abundant non-essential amino acid in nature, glutamic acid, is a food additive used on a daily basis. Excessive daily intake of MSG causes an increase in the levels of glutamic acid in the blood (Garattini, 2000). MSG has been shown to be cytotoxic in tissues such as liver and brain, possibly via induction of oxidative stress (Pavlovic *et al*., 2009; Paul *et al*., 2012). A reduction in learning capabilities and short memory has been observed in rats exposed to low dose MSG, indicating its neurotoxic effect on forebrain in the hippocampus (Moneim *et al*., 2018; Jin *et al*., 2018). Studies have shown that exposure to MSG causes inhibition of Na^+K^+ATPase and memory impairment of the hippocampus of rats (Ramalho *et al*., 2018). Sodium-dependent Glu transporters (GluTs) and Na^+K^+ATPase are closely connected in the brain tissue and apparently are a part of identical

macromolecular complexes that work as a functional unit to regulate glutamatergic neurotransmission (Rose *et al*., 2009).

Recently, MSG has been shown to reduce lifespan and induce an adaptive response to oxidative stress in *Drosophila melanogaster* (Abolaji *et al*., 2017), indicating that insects can be a good model to study MSG neurotoxicity. MSG has been shown exert a decline in short-term memory of *Drosophila melanogaster* exposed over a period of time (Xia *et al.,* 1997).

Studies have shown the challenges encountered with toxicity of mixtures of chemicals, but mixture of chemicals that interact with the same toxicity pathway could be tested over a wide dose ranges quickly and at a small expense, thereby enabling data obtained to allow a brilliant and focused procedure in evaluating risk in human populations exposed to mixtures (Krewski *et al*., 2010).

The lobster cockroach, *Nauphoeta cinerea* has been identified as a suitable alternative organism for carrying out basic toxicological studies that could provide new comprehension for translational neuroscience research before the common use of vertebrate in toxicological experiments (Adedara *et al*., 2015). Apart from the similitude in the basic physiological and biophysical features of the nervous system between insects and mammals, cockroaches are bigger and also yield more tissues when compared to fruit flies (Stankiewicz *et al*., 2012). Thus, the present study investigated the potential neurotoxic interaction between methylmercury and monosodium glutamate in the heads of the nymphs of the lobster cockroach, *Nauphoeta cinerea* by assessing behavioral and biochemical endpoints of neurotoxicity, including antioxidant, cholinergic and oxidative stress indices in the heads of the cockroach for a period of twenty-one (21) days. The dose of MeHg chosen in the present study was based on the work done by Adedara *et al*., (2015).

2. Materials and methods

2.1.1 Chemicals

Monosodium glutamate (MSG 99 % purity Ajinomoto®) was obtained from a supermarket in Santa Maria, sodium chloride, glutathione, 1- chloro-2,4 dinitrobenzene (CDNB), 5′,5′-dithiobis-2-nitrobenzoic acid, (DTNB), acetylthiocholine iodide, trichloroacetic acid (TCA), thiobarbituric acid (TBA) and hydrogen peroxide (H_2O_2) were obtained from Sigma Aldrich (St. Louis, MO, USA.

2.1.2 Cockroach experimental protocol

Nymphs of lobster cockroach (*Nauphoeta cinerea*) were obtained from the Departamento de Bioquímica e Biologia Molecular, Centro de Ciências Naturais e Exatas (CCNE), Universidade Federal de Santa Maria, Brasil. The insects were reared in plastic boxes (45.5 cm x 40.2 cm x 29.5 cm). Nymphs were selected and put in small transparent boxes (10.8 cm x 10.8 cm x 7.9 cm) for the period of the experiment. The nymphs were adapted in the new box for 10 days before they were exposed to the treatment. The temperature differed between 21 -27^{0}C and humidity between 57-75% during the period of exposure (14th February to 7th March 2017). The insects had free access to water and standard dog food (composition in supplemental table 1) procured from (Total Alimentos LTDA. D.Industria- Três corações, Minas Gerais, Indústria Brasileira) during rearing and adaptation. In the course of the experiment, the cockroaches were exposed to a formulated diet containing MSG, NaCl or MeHg dissolved in ethanol. The basal diet contained (per 20g of diet): 10g milled corn flour, 7g wheat flour, 2g granulated sugar (sucrose), 0.5g casein, 0.4g powder milk and 0.1g table salt (NaCl). The components were mixed with a blender. In the case of diets containing MSG and NaCl, they were added to the basal diet to the final concentration of 2%. MeHg was added dissolved in ethanol, and identical volume of

ethanol was added to the diets that did not contain MeHg. All the diets were left in the open air until all ethanol was evaporated, then stored at -20^{o}C.

The cockroaches were divided into six (6) groups consisting of 40 nymphs each of length varying between 1.02 and 1.26 cm at the start of the experiment. At the end of the experiment, the length varied between 1.59 and 1.76cm. Cockroaches were fed with the basal di*et al*one, the basal diet with 2% NaCl, the basal diet with 2% MSG; the basal diet with 0.125mg/g MeHg, the basal diet mixed with 0.125mg/g MeHg and 2% NaCl, and the basal diet mixed with 0.125mg/g MeHg and 2% MSG for 21 days. The sub-toxic dose of MeHg was selected from the study by Adedara *et al*., (2015).

2.2 Behavioral experiments in a novel environment.

The cockroaches were placed in a transparent round bowl (26cm x 12cm x13 cm) as shown in fig 1 and their behavior was recorded during 10-min using a closed circuit TV, with a camera supplied by Intel bras Vm1120IR, Pelotas, Rio Grande do Sul. The experiments were conducted between 8:00 am to 2:00 pm. The behavior parameters were automatically measured using suitable video-tracking software (ANY-maze, Stoelting, CO, USA). The following parameters were evaluated, the locomotor activity of the cockroaches represented by the total distance traveled; immobility and absolute turn angle (which represent the changes in the direction of the center point of the animal, and a marker of grooming). These two parameters were shown to be reduced in chlorpyrifos toxicity according to Adedara *et al.* (2016). ANY-maze software has been used successfully with different animal models (Fontana et al. 2016; Baune et al. 2008), including insects (Baggio et al. 2013; Adedara et al. 2015; 2016).

2.2.1 Vertical exploration

The vertical behavior of cockroaches in the new environment designates its natural tendency to explore the novel situation (Durier and Rivault, 2002; Goldman *et al*., 2006). Two types of movement were considered as shown in fig 1. The bottom zone of the apparatus (clear bottom) and the periphery zone which is the inclined walls of the apparatus (the green area). The exploratory profile of the cockroaches was represented by track plots and heat map plots, which shows a graphical representation of the movement of the cockroaches using lines and color intensity respectively.

2.3 Biochemical analysis

Cockroaches were put on ice for 15 minutes, the heads were removed, weighed and homogenized in ice-cold 0.1M phosphate buffer, pH 7.4 in ratio 1:10 (g head: ml buffer) and centrifuged at 2,500G for 10 Minutes at 4^0C to obtain the supernatant. The supernatant was further diluted to ratio 1:15 and subsequently used for the biochemical estimations.

The protein contents of the head of cockroaches were determined at 280nm using UV-visible 1650 PC Spectrophotometer produced by Shimadzu.

2.3.1 Determination of acetylcholinesterase activity

Acetylcholinesterase activity was determined using the method of Ellman *et al*. (1959). The system consisted of 110µL of distilled water, 50µL of initial concentration 0.1M potassium phosphate buffer (pH 7.4), and 30µL of a sample whose protein was standardized to a concentration of 0.8mg/ml, 20µL of 10mM DTNB, and 20µL of 8mM acetylthiocholine as the substrate. The degradation of acetylthiocholine iodide was measured for 24 minutes (30 seconds

interval) at 412 nm using a spectra Max plate reader (Molecular Devices, CA, USA) and the results were expressed as μmol thiocholine formed /min/mg protein.

2.3.2 Determination of glutathione-s-transferase activity.

Glutathione-S-transferase activity was determined according to the method of Habig and Jakoby (Habig and Jakoby, 1981), with slight modifications (Gweshelo *et al*., 2016) using 1-chloro-2,4dinitrobenzene (CDNB) as the substrate. Briefly, the assay mixture consists of 135μL of initial concentration 0.1M potassium phosphate buffer (pH 6.5), 50μL of tissue sample whose protein was standardized to a concentration of 0.2mg/ml, 100μL of glutathione 3mM, and 15μL of 20mM CDNB. The assay is based on the formation of a conjugated complex of CDNB with glutathione (GSH) at 340 nm and was monitored for 18 minutes (30 Seconds interval) using a spectra Max plate reader (Molecular Devices, CA, USA) and the results were expressed as μmol/min/mg protein.

2.3.3 Determination of total thiol concentration

Total thiol level was determined according to the method of Ellman, (1959). The assay mixture consisted of 160 μL of initial concentration 0.1M potassium phosphate buffer (pH7.4), 20 μL of tissue sample whose protein was standardized to a concentration of 10mg/ml, using the NanoDrop spectrophotometer and 20 μL of 10 mM DTNB. The mixture was incubated for 30 minutes at ambient temperature (25^0C). The absorbance was measured at 412nm. The results were expressed as units/mg protein.

2.3.4 Determination of thiobarbituric acid reactive substances

Lipid peroxidation products were estimated as thiobarbituric acid reactive substances (Puntel *et al*., 2007). Briefly, tissue samples were obtained by homogenizing the heads of

cockroaches in ice-cold 0.1M potassium phosphate buffer (pH 7.4) in a ratio of 1:5 (mg head: µL buffer). The stock reagent contained equal volumes of trichloroacetic acid (10%, w/v) and 2-thiobarbituric acid (0.75%, w/v) in 0.1M HCl. The assay medium consisting of 200 µL of tissue supernatant and 400 µL of stock reagent was incubated at 95^0C for 60 min. The samples were then cooled and centrifuged at 8,000 x g for 10 min and the absorbance of the supernatant was analyzed at 532 nm. The values of TBARS were normalized by the cockroach head weight and tissue levels were expressed as nmol MDA/g tissue.

2.4 Statistical analyses

The data were expressed as the mean±standard error of the mean. Statistical analyses were carried out by two-way analysis of variance (ANOVA) followed by Newman-Keuls *post-hoc* test. Significance was set at $p<0.05$. The study consists of a factorial design 2 (with and without 0.125mg MeHg/g of diet) by 3 (Basal/2%NaCl/2%MSG). The statistical analyses were performed using Graph Pad Prism 6 and Statistica software packages.

3 Results

3.1.1 Locomotor activity

Two-way ANOVA indicated a significant main effect of MeHg **[**F (1, 53) =10.803: P=0.00180], and diet [F (2, 53) =14.437: P< 0.00001]. Similarly, MeHg x diet interaction [F (2, 53) =8.8289: P=0.00049] was significant for distance travelled by the cockroaches. Also, the mean of the MeHg treated group was higher than the non-MeHg treated group (Fig.2). In respect of immobility, the ANOVA showed a significant main effect of MeHg [F (1, 54) =29.928: P<0.00001], and diet [F (2, 54) =20.851: P< 0.00001]. Likewise, MeHg x diet interaction was significant [F (2, 54) =49.693: P< 0.00001]. Also, the mean of the MeHg treated group was higher than the non-MeHg treated group (Fig.3). The absolute turn angle showed a significant

main effect of diet [F (2, 54) =17.024: P< 0.00001] and significant MeHg x diet interaction [F (2, 54 =3.7461: P=0.02996] (Fig. 4).

3.1.2 Vertical exploration

Concerning the distance traveled in the bottom zone, there was a main effect of MeHg [F (1, 55) = 7.4734: P=0.00841] and diet [F (2, 55) =18.382: P< 0.00000] (Fig. 5). Furthermore, there was no significant MeHg x diet interaction. The mean of the MeHg treated group was higher than the mean of the non-MeHg treated group. Significant main effect of MeHg [F (1, 54) =5.3792: P=0.02419] and diet [F (2, 54) =9.8571: P=0.00022] was revealed in the distance travelled in the periphery zone. Also, a significant MeHg x diet interaction [F (2, 54) = 6.3341: P=0.00338] was observed in this zone. The mean of the MeHg treated group was higher than the mean of the non-MeHg treated group (Fig. 6).

3.1.3 Exploratory profile

The track plots of a representative cockroach of each group are shown in Fig.7. The treated cockroaches showed different behavior in their exploration of both the bottom and periphery zone. The track plots correlate with the distance travelled in the bottom and periphery zone. The heat-map plots of a representative cockroach are shown in Fig. 8. The heat-map plots show an overall view of the time each cockroach stayed in a particular spot in the apparatus by the intensity of the color. The spot with more intense color shows the cockroach stayed longer at that particular spot. The heat map plots also correspond to the distance travelled in the bottom and periphery zone.

3.1.4 Acetylcholinesterase activity, oxidative stress indices and Mortality Profile.

The activity of acetylcholinesterase and oxidative stress indices in heads of cockroaches, used for this experiment for 21 days is shown in Fig 9-12. Two-way ANOVA showed a main effect of MeHg [F (1, 76) =21.211: P=0.00002] and diet [F (2, 76) =3.0163: P=0.05473] in the acetylcholinesterase activity. Furthermore, there was no significant MeHg x diet interaction (Fig. 9). There was no significant main effect of MeHg and diet in the GST activity. The MeHg x diet interaction was not significant as well as shown in Fig. 10.

In respect of total thiol levels, there was a main effect of MeHg [F (1, 36) =6.2319: P=0.01726]. Furthermore, the mean of the non-MeHg treated group was higher than the MeHg treated group. This signifies that the MeHg treated groups had the tendency to decrease the total thiol levels when compared to the Non-MeHg treated groups Fig 11.

A significant main effect of MeHg [F (1, 29) = 40.830: P< 0.00001] and diet [F (2, 29) =5.9047: P=0.00706] was revealed in the TBARS levels. There was also a significant MeHg x diet interaction [F (2, 29) =41.224: P< 0.00001] Fig. 12.

This present study also showed no significant difference in the mortality rates of the cockroaches across all the groups treated (Data not shown).

4 Discussion

There has been substantial support in the use of alternate models in neuroscience to evaluate the safety and toxic effects of chemical substances to the brain (Peterson *et al*., 2008). The present study evaluated for the first time, possible interactions between MeHg and MSG analysing behavioral tests to assess locomotor parameters, motor coordination, exploratory behavior, as well as, biochemical parameters.

The absolute turn angle behavior can be interpreted as in whirling movement of the cockroaches (Spink *et al*., 2007) and it is a locomotor parameter used to indicate motor coordination during bodily movements (Riemann and Lephart, 2002). Moreover, distance traveled and time immobile are motor activity measurements used to evaluate the effect of neurotoxicants (Cory-Slechta *et al*., 2001). Taking all the behavioral parameters evaluated into consideration, we propose that cockroaches exposed to MeHg and MSG exhibited impairments in the motor posture, a kind of stereotyped behavior, as observed in the increased absolute turn angle and time immobile as well as reduced total distance traveled. Previous studies have reported that MSG decreased both vertical and horizontal locomotion in rats most likely due to its interaction with dopamine, as a dopamine antagonist was found to abolish the induction of locomotion by glutamate antagonist (Dalia *et al*., 1996). Moreover, Shivasharan *et al*, (2013) and Umukoro *et al*., (2015) also reported that administration of MSG resulted in significant decrease in mobility of rats and mice respectively, an indication of its toxicity.

Acetylcholine is a neurotransmitter, involved in the control of motor function, locomotor, and exploration (Nagy and Aubert, 2013). Acetylcholinesterase hydrolyzes acetylcholine at the synapses, therefore playing an essential role in cholinergic neurotransmission. In addition to its role, it is deemed to be an important biomarker of neuropathological conditions such as mild cognitive impairment, dementia and Alzheimer's disease (Kan *et al*., 2010; Hariharan *et al*., 2014). Recently, we and other laboratories have demonstrated that AChE can be a biomarker of MeHg and other toxic electrophile chemicals (Cheng *et al*., 2005; Kang *et al*., 2014), although, the mechanism of inhibition of MeHg on AChE requires further investigation. In the present study, the decrease in the acetylcholinesterase activity as observed in the MeHg treated group of cockroaches, could alter a normal synaptic transmission and account for the alteration in the

locomotor and coordination activities seen in the MeHg + NaCl and MeHg + MSG groups of cockroaches. This observation aligns with a previous study which reported that MSG significantly reduced the acetylcholinesterase activity in the brain of mice (Abu-Taweel, 2016). However, NaCl only group also tended to decrease the acetylcholinesterase activity. It is known that one of the possible molecular targets for MeHg toxicity in a developing brain is the glutathione antioxidant system (Bisen-Hersh *et al*., 2014). The conjugation of GSH with MeHg requires GST, a key detoxification enzyme (Barcelos *et al*., 2012). Evaluation of total thiol levels is an indirect oxidative stress biomarker of damage to proteins and peptides of diverse length (Saniova *et al*., 2006). In the present study, there was no significant alteration in the total GST activity. It is possible that the duration of exposure to MeHg did not affect the GST activity, more so not all antioxidants are affected by MeHg in a similar manner. This correlates with the work of (Espitia- Pérez *et al*., 2018) that found no difference in GST activity in liver and heart of pregnant rats exposed to MeHg but found an increased GST activity in the rats' kidney. Vorojeikina *et al.*, (2017) equally reported increased MeHg susceptibility in GST knockout flies, treatment with the human GSTP1 ameliorated the toxicity in some cases, but failed to reduce the insult in other instances, as the dose used in this study in relation to weight is in the same range with the present study.

There was a significant main effect of MeHg in the total thiol levels. There was a decrease in the general means of all the three groups treated with MeHg when compared with the groups not treated with MeHg, the effect observed was smaller than previously reported (Amos *et al*., 2015; Farina *et al*., 2013).

It has been reported that the pathological process of MeHg toxicity involves disruption of redox cellular homeostasis and eventual formation of reactive oxygen species (ROS) (Sanfeliu *et al*.,

2001; Huang *et al*., 2008). In the present study, both the MeHg + NaCl and MeHg + MSG cockroaches showed an increase in TBARS levels establishing an increase in lipid peroxidation. Also, cockroaches treated with sodium alone also increased TBARS levels. This could be as a result of oxidative stress in the heads of the cockroaches treated.

The present study also revealed that sodium chloride alone also caused oxidative stress in the treated cockroaches. This is similar to the observation of sodium chloride induced oxidative stress in parts of the brain of mice as reported by Liu *et al*., (2014). Other studies have also reported neurotoxicity of NaCl, however, its neurotoxic mechanism is yet to be fully elucidated (Morland *et al*., 2016).

In conclusion, the findings from the present study indicate that the monosodium glutamate could potentiate the toxic effect of MeHg, as observed in altered mobility, absolute turn angle and some biochemical parameters in the cockroaches exposed. The positive correlation between our study and some earlier studies on the neurotoxicity of MSG using mammalian models further supports the use of *Nauphoeta cinerea* as a valid alternative model for basic toxicological studies.

Conflicts of interest

The authors have no conflict of interest to declare.

Acknowledgements

B.A. Afolabi is indebted to TWAS-CNPq for financial support.

This work was supported by FAPERGS-PRONEX-CNPQ, CNP-SWB.

References

1. Abbott, L.C., Moussa, E.M., Carl, T.L., Cortez, D., Clayton, H.L., Holland, C.S., *et al*., 2017. Early Exposure to Mercuric Chloride or Methylmercury Alters Zebrafish Embryo (*Danio rerio*) Development. Poult Fish Wildl Sci. 5, 178.
2. Abolaji, A.O., Olaiya. C.O., Oluwadahunsi, O.J., Farombi, E.O., 2017. Dietary consumption of monosodium L-glutamate induces adaptive response and reduction in the lifespan of *Drosophila Melanogaster*. Cell. Biochem. Funct. 35(3), 164-170.
3. Abu-Taweel, G.M. (2016). Effect of monosodium glutamate and aspartame on behavioral and biochemical parameters of male albino mice. African Journal of Biotechnology. 15(15), 601-612.
4. Adedara, I.A., Rosemberg, D.B., de Souza, D., Kamdem, J.P., Farombi, E.O., Aschner, M., Souza, D.O., Rocha, J.B.T., 2015. Biochemical and behavioral deficits in the lobster cockroach *Nauphoeta cinerea* model of methylmercury exposure. Toxicol. Res. 4, 442-451.
5. Adedara, I.A., Rosemberg, D.B., Denis B, Farombi, E.O., Aschner M., Souza, D.O., Rocha, J.B.T., 2016. Neurobehavioral and biochemical changes in *Nauphoeta cinerea* following dietary exposure to chlorpyrifos. Pesticide Biochemistry and Physiology. 130, 22-30.
6. Amos, O.A., Jean, K.P., Thiago, H.L., Ebenezer, O.F., Diogo, O.S., Elgion, L.S.L., João, B.T.R., 2015. Ovotoxicants 4-vinylcyclohexene 1,2-monoepoxide and 4-vinylcyclohexene diepoxide disrupt redox status and modify different electrophile sensitive target enzymes and genes in *Drosophila melanogaster*. Redox. Biol. 5, 328-339.

7. Antunes dos Santos, A., Ferrer B., Gonçalves, F.M., Tsatsakis, A.M., Renieri, E.A., Skalny, A.V., Farina, M., Rocha, J.B.T., and Aschner M., 2018. Oxidative stress in methylmercury-induced cell toxicity. Toxics, 6(3), 47.

8. Aschner, M., Syversen, T., Souza, D.O., Rocha, J.B., Farina, M., 2007. Involvement of glutamate and reactive oxygen species in methylmercury neurotoxicity. Braz. J. Med. Biol. Res. 40, 285–291.

9. Baggio, F., Bozzato, A., Benna C., Leonardi E., Romoli O., Cognolato, M., Silvio, C.E.T., Costa, R., Sandrelli, F. 2013. 2mit, an Intronic Gene of Drosophila melanogaster timeless2, Is Involved in Behavioral Plasticity. PLoS ONE 8(9): e76351.

10. Basu, N., Scheuhammer, A.M., Rouvinen-Watt, K., Evans, R.D., Trudeau, V.L., Chan, L.H., 2010. In vitro and whole animal evidence that methylmercury disrupts GABAergic systems in discrete brain regions in captive mink. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 151 (3), 379–85.

11. Barcelos, G.R., de Marco, K.C., Grotto, D., Valentini, J., Garcia, S.C., Braga, G.U., Barbosa, F., 2012. Evaluation of glutathione S-transferase GSTM1 and GSTT1 polymorphisms and methylmercury metabolism in an exposed Amazon population. J. Toxicol. Environ. Health. Part A, 75, 960–970.

12. Baune, B.T., Wiede, F., Braun, A., Golledge, J., Arolt, V., and Koerner H. 2008. Cognitive Dysfunction in Mice Deficient for TNF- and Its Receptors. American Journal of Medical Genetics Part B (Neuropsychiatric Genetics) 147B:1056–1064.

13. Bisen-Hersh, E.B., Farina, M., Barbosa, F.Jr., Rocha, J.B.T., Aschner, M., 2014. Behavioral effects of developmental methylmercury drinking water exposure in rodents. J. Trace. Elem. Med. Biol. 28, 117–124.

14. Bradley, M.A., Barst, B.D., Basu, N., 2017. A Review of Mercury Bioavailability in Humans and Fish. Int. J. Environ. Res. Public Health. 14, 169.

15. Bridges, K., Venables, B., Roberts, A. 2017. Effects of dietary methylmercury on the dopaminergic system of adult fathead minnows and their offspring. Environmental Toxicology and Chemistry 36(4), 1077-1084.

16. Briguglio, M., Dell'Osso, B., Panzica, G., Malgaroli, A., Banfi, G., Zanaboni, C *et al*., 2018. Dietary Neurotransmitters: A Narrative Review on Current Knowledge. Nutrients. 10, 591.

17. Cheng, J.P., Yang, Y.C., Hu, W.X., Yang, L., Wang, W.H., Jia, J.P., Lin, X.Y., 2005. Effect of methylmercury on some neurotransmitters and oxidative damage of rats. J. Environ. Sci 17, 469–473.

18. Cory-Slechta, D.A., Crofton, K.M., Foran, J.A., Ross, J.F., Sheets, L.P., Weiss, B., Mileson, B. 2001. Methods to Identify and Characterize Developmental Neurotoxicity for Human Health Risk Assessment. I: Behavioral Effects. Environmental Health Perspectives 109(1), 1-13.

19. Dalia, A., Uretsky, N.J., Wallace, L.J. 1996. Induction of locomotor activity by the glutamate antagonist DNQX injected into the ventral tegmental area. Brain Res. 728, 209-214.

20. Durier, V., Rivault, C., 2002. Influence of a novel object in the home range of the cockroach *Blattella Germanica*. Med. Vet. Entomol. 16, 121–125.

21. Ellman, G.L., 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82, 70–77.

22. Espitia-Pérez, P., Albino, S.M., Tais da Rosaa, H., Silveira, A.K., Espitia-Pérez, L., Brango, H., Moraes, D.P., Hermann, P.R.S., Mingori, M., Barreto, F., Kunzler, A.,

Gelain, D.P., Schnorr, C.E., Moreira, J.C.F., 2018. Effects of methylmercury and retinol palmitate co-administration in rats during pregnancy and breastfeeding: Metabolic and redox parameters in dams and their offspring. Ecotoxicology and Environmental Safety. 162, 603–615.

23. Farina, M., Aschner, M., Rocha, J.B.T., 2017. Chapter Two- The Catecholaminergic Neurotransmitter System in Methylmercury-Induced Neurotoxicity. Advances in Neurotoxicology. Volume 1, 2017, pages 47-81.

24. Farina, M., Aschner, M., 2017. Methylmercury-Induced Neurotoxicity: Focus on Pro-oxidative Events and Related Consequences. Adv. Neurobiol. 18, 267-286.

25. Farina, M., Avila, D.S., Rocha, J.B.T., Aschner, M., 2013. Metals, oxidative stress and neurodegeneration: A focus on iron, manganese and mercury. Neurochem. Int. 62, 575–594.

26. Farina, M., Dahm, K.C., Schwalm, F.D., Brusque, A.M., Frizzo, M.E.S., Zeni, G., Souza, D.O., Rocha, J.B.T. 2003. Methylmercury Increases Glutamate Release from Brain Synaptosomes and Glutamate Uptake by Cortical Slices from Suckling Rat Pups: Modulatory Effect of Ebselen. Toxicological Sciences 73, 135–140.

27. Fontana, B.D., Meinerz, D.L., Rosa, L.V.C., Mezzomo, N.J., Silveira, A., Giuliani, G.S., Quadros, V.A., Filho, G.L.B., Blaser, R.E., Rosemberg, D.B. 2016. Modulatory action of taurine on ethanol-induced aggressive behavior in zebrafish. Pharmacology, Biochemistry and Behavior 141: 18–27.

28. Garattini., S., 2000. Glutamic acid, twenty years later. J. Nutr. 30 (4), 901S–909S.

29. Goldman, D.I., Chen, T.S., Dudek, D.M., Full, R.J., 2006. Dynamics of rapid vertical climbing in cockroaches reveals a template. J. Exp. Biol. 209, 2990–3000.

30. Gweshelo, D., Muswe, R., Mukanganyama, S., 2016. In vivo and in vitro inhibition of rat liver glutathione transferases activity by extracts from *Combretumzeyheri* (Combretaceae) and *Parinaricuratellifolia* (Chrysobalanaceae). BMC Complement. Altern. Med. 16, 238.

31. Habig, W.H., Jakoby, W.B., 1981. Assays for differentiation of glutathione S-transferases. Methods. Enzymol. 77, 398–405.

32. Hariharan, A., Shetty, S., Shirole, T., Jagtap, A.G., 2014. Potential of protease inhibitor in 3-nitropropionic acid induced Huntington's disease-like symptoms: Mitochondrial dysfunction and neurodegeneration. Neurotoxicol. 45, 139–148.

33. Honda, S., Hylander, L., Sakamoto, M., 2006. Recent advances in evaluation of health effects on mercury with special reference to methylmercury a mini review. Environ. Health Prev Med 11, 171–176.

34. Huang, C.F., Hsu, C.J., Liu, S.H., Lin-Shiau, S.Y., 2008. Ototoxicity induced by cinnabar (a naturally occurring HgS) in mice through oxidative stress and down-regulated Na^+-K^+ ATPase activities. NeuroToxicol. 29(3), 386–396.

35. Kang, S.J., Jeong, S.H., Kim, E.J., Park, Y.I., Park, S.W., Shin, H.S., Son, S.W. Kang, H.G., 2014. Toxic effects of methylmercury, arsanilic acid and danofloxacin on the differentiation of mouse embryonic stem cells into neural cells. J. Vet. Sci 15, 61–71.

36. Jin, L., Lin, L., Li, G.Y., Liu, S., Luo, D.J., Feng, Q., Sun, D.S., Wang, W., Liu, J.J., Wang, Q., Ke, D., Yang, X.F., Liu, G.P., 2018. Monosodium glutamate exposure during the neonatal period leads to cognitive deficits in adult Sprague-Dawley rats Neuroscience Letters, 682, pp. 39-44.

37. Kim, W.T., Choi, H.J., Park, Y., Kim, A.Y., Kim, Y.H., Seo, J.B., Lee, S.H., Koh, Y.H., 2010. Alterations in both acetylcholinesterase activity and synaptic scaffolding protein localization in the nervous system of *Drosophila* presenilin mutants, J. Asia. Pac. Entomol. 13, 339–343.

38. Krewski, D., Acosta, Jr D., Andersen, M., Anderson, H., Bailar, III J.C., Boekelheide, K., Brent, R., Charnley, G., Cheung, V.G., Green, Jr. S., Kelsey, K.T., Kerkvliet, N.I., Li A.A., McCray, L., Meyer, O., Patterson, R.D., Pennie, W., Scala, R.A., Solomon, G.A., Stephens, M., Yager, J., and Zeise, L., 2010. Staff of Committee on Toxicity Testing and Assessment of Environmental Agents. Toxicity Testing In The 21st Century: A Vision And A Strategy, *J Toxicol Environ Health B Crit Rev*. February; 13(0): 51–138.

39. Liu, Y., Chen, J., Li, Z., Zhao, T., Ni, M., Li, D., Jiang, C., Shen, F., 2014. High-salt diet enhances hippocampal oxidative stress and cognitive impairment in mice. Neurobiology of Learning and Memory 114, 10–15.

40. Moneim, W.M.A., Yassa, H.A., Makboul, R.A., Mohamed, N.A., 2018. Monosodium glutamate affects cognitive functions in male albino rats Egyptian Journal of Forensic Sciences, 8 (1): 9.

41. Moreira, E.L.G., Farina, M., 2014. An unsolved puzzle: The complex interplay between methylmercury and fish oil derived fatty acids within the cardiovascular system. Toxicol. Res. 3, 300–310.

42. Morland, C., Pettersen, M.N., Hassel, B., 2016. Hyperosmolar sodium chloride is toxic to cultured neurons and causes reduction of glucose metabolism and ATP .levels, an increase in glutamate uptake, and a reduction in cytosolic calcium. Neurotoxicology. 54, 34-43.

43. Nagy, P.M., Aubert, I., 2013. B6eGFPChAT mice over-expressing the vesicular acetylcholine transporter exhibit spontaneous hypo-activity and enhanced exploration in novel environments. Brain Behav. 3, 367–383.

44. Oliveira, C.S., Piccoli, B.C., Aschner, M., Rocha, J.B.T. 2017. Chemical Speciation of Selenium and Mercury as Determinant of their Neurotoxicity of Metals. Advances in Neurobiology 18,53-83.

45. Paul, M.V., Abhilash, M., Varghese, M.V., Alex, M., Nair, R.H., 2012. Protective effects of alpha-tocopherol against oxidative stress related to nephrotoxicity by monosodium glutamate in rats. Toxicol Mech. Methods. 22(8), 625–30.

46. Pavlovic, V., Pavlovic, D., Kocic, G., Sokolovic, D., Sarac, M., Jovic, Z., 2009. Ascorbic acid modulates monosodium glutamate-induced cytotoxicity in rat thymus. Bratisl. Lek. Listy. 110(4), 2059.

47. Peterson, R.T., Nass, R., Boyd, W.A., Freedman, J.H., Dong, K., Narahashi, T., 2008. Use of non-mammalian alternative models for neurotoxicological study, Neurotoxicol. 29, 546–555.

48. Puntel, R.L., Roos, D.H., Grotto, D., Garcia, S.C., Nogueira, C.W., Rocha, J.B., 2007. Antioxidant properties of Krebs cycle intermediates against malonate pro-oxidant activity in vitro: a comparative study using the colorimetric method and HPLC analysis to determine malondialdehyde in rat brain homogenates, Life Sci. 81, 51–62.

49. Ramalho, J.B., Izaguirry, A.P., Soares, M.B., Spiazzi, C.C., Pavin, N.F., Affeldt, R.F., Lüdtke, D.S., Pinton, S., Santos, F.W., Prigol, M., 2018. Selenofuranoside improves long-term memory deficits in rats after exposure to monosodium glutamate: Involvement of Na+, K+-ATPase activity Physiology and Behavior, 184, pp. 27-33.

50. Riemann, B.L., Lephart, S. M., 2002. The sensorimotor system, Part I: the physiologic basis of functional joint stability. J. Athl. Train. 37, 71-79.

51. Rose, E.M., Koo, J.C., Antflick, J.E., Ahmed, S.M., Angers, S. Hampson, D.R., 2009 Glutamate transporter coupling to Na,K-ATPase, J. Neurosci. 29 8143–8155.

52. Sanfeliu, C., Sebastia, J., Ki, S.U., 2001. Methylmercury neurotoxicity in cultures of human neurons, astrocytes, neuroblastoma cells. NeuroToxicol. 22(3), 317–327.

53. Saniova, B., Drobny, M., Lehotsky, J., Sulaj, M., Schudichova, J., 2006. Biochemical and clinical improvement of cytotoxic state by amantadine sulphate. Cell. M ol. Neurobiol., 26, 1475–1482.

54. Spink, A.J., Tegelenbosch, R.A.J., Buma, M.O., Noldus, L.P., 2007. The ethovision vídeo tracking system-a tool for the behavioral phenotyping of transgenic mice. Physiol. Behav. 73,731– 744.

55. Stankiewicz, M., Dąbrowski, M., de Lima, M.E., 2012. Nervous system of *Periplaneta americana* cockroach as a model in toxicological studies: A short historical and actual view. J. Toxicol. 143740.

56. Syversen, T., Kaur, P., 2012. The toxicology of mercury and its compounds. J. Trace Elem. Med. Biol. 26 (4), 215–226.

57. Takaoka, S., Fujino, T. Kawakami, Y., Shigeoka, S., and Yorifuji, T., 2018. Survey of the Extent of the Persisting Effects of Methylmercury Pollution on the Inhabitants around the Shiranui Sea, Japan. Toxics, 6(3), 39.

58. Umukoro, S., Oluwole, G.O., Olamijowon, H.E., Omogbiya, A.I., Eduviere, A.T., 2015. Effect of Monosodium Glutamate on Behavioral Phenotypes, Biomarkers of Oxidative

Stress in Brain Tissues and Liver Enzymes in Mice. World Journal of Neuroscience. 5, 339-349.

59. Unoki, T., Akiyama, M., Kumagai, Y., Gonçalves, F.M., Farina, M., Rocha, J.B., Aschner, M., 2018. Molecular pathways associated with methylmercury-induced Nrf2 modulation. Frontiers in Genetics (Toxicogenomics), in press, doi: 10.3389/fgene.2018.00373.

60. Vorojeikina, D., Broberg, K., Love, T.M., Davidson, P.W., Wijngaarden, EV., Rand, M.D., 2017. Glutathione s-transferase activity moderate's methylmercury toxicity during development in drosophila. Toxicological Sciences, 157(1), 211–221.

61. Xia, S., Liu, L., Feng, C., and Guo, A. 1997. Drug disruption of short-term memory in *Drosophila melanogaster*. Pharmacology biochemistry and behavior, Vol. 58 No. 3, pp. 727-735.

62. Xin, W., Kwan, C.L., Zhao, X., Xu, J., Ellen, R.P., McCulloch, C.A.G., Yu, X. 2005. A Functional Interaction of Sodium and Calcium in the Regulation of NMDA Receptor Activity by Remote NMDA Receptors. The Journal of Neuroscience. 5; 25(1):139 –148.

Table 1: A table showing the composition of the dog feed given to the cockroaches during rearing and/or adaptation.

NUTRIENT	COMPOSITION	NUTRIENT	COMPOSITION (%)	OTHER NUTRIENTS
Vitamin A Acetate	5.000 I.U	Crude Protein (min)	18	Poultry by-product meal
Vitamin B12	22 mcg	Crude Fat (min)	7	Meat and bone meal
Cholecalciferol	500 I.U	Crude Fiber (max)	6	Ground yellow corn
α-Tocopherol Acetate	50 I.U	Moisture (max)	12	Defatted corn germ
Sodium Selenite	0.1 mg	Ash (max)	10	Corn gluten meal
Folic Acid	0.1mg	Calcium (max)	2.4	Rice Meal
Calcium Pantothenate	10 mg	Calcium (min)	1	Wheat mill run
Copper Sulfate	7 mg	Phosphorous (min)	1	Poultry fat (preserved with BHA, BHT)
Ferrous Sulphate	40 mg	Sodium (min)	0.3	Powder hemoglobin
Calcium Iodate	1 mg			Salt
Manganese Oxide	5 mg			Choline Chloride
Thiamine Mononitrate	1 mg			Di-calcium phosphate
Riboflavin Supplement	2 mg			Calcium propionate
Pyridoxine HCL	1 mg			Hydrolyzed deboned chicken,
Biotin	0.2 mg			vitamin mineral premix
Menadione Sodium Bisulfite	1mg			
Niacin Supplement	11 mg			
Zinc Oxide	120 mg			

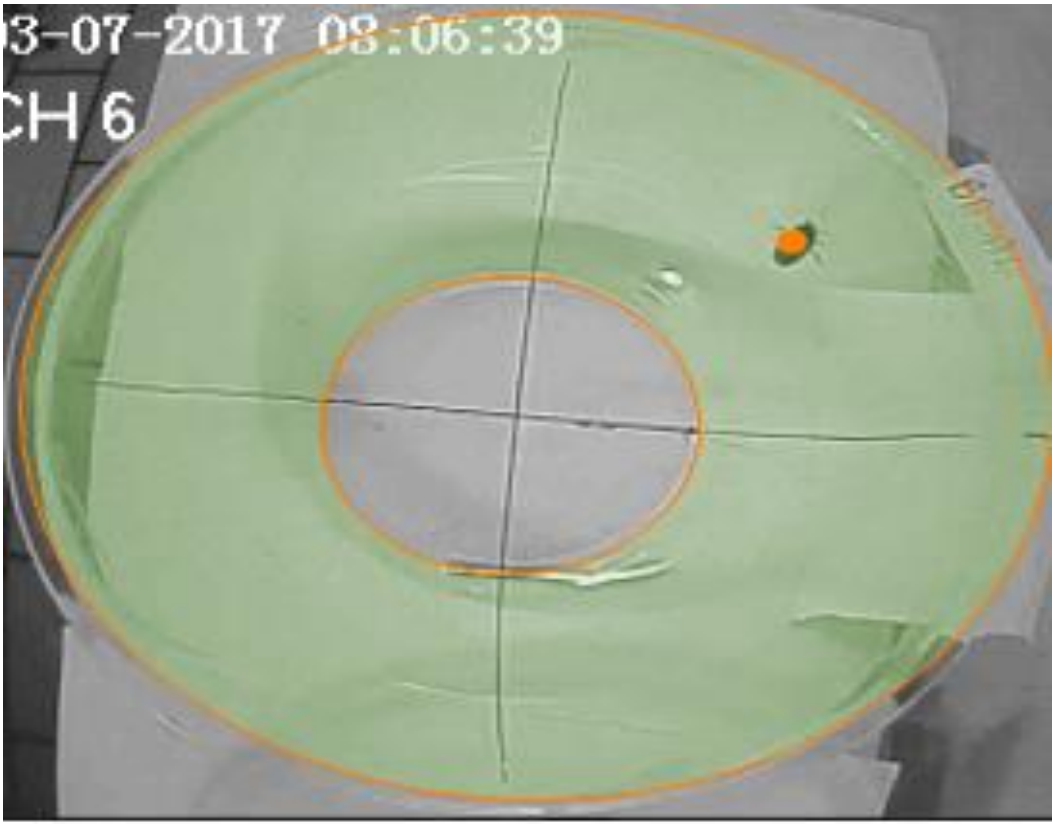


Fig 1: The apparatus for analyzing the behavior of cockroaches, showing the periphery zone (the green area) and the bottom zone (clear bottom). Colours should be used in Print.

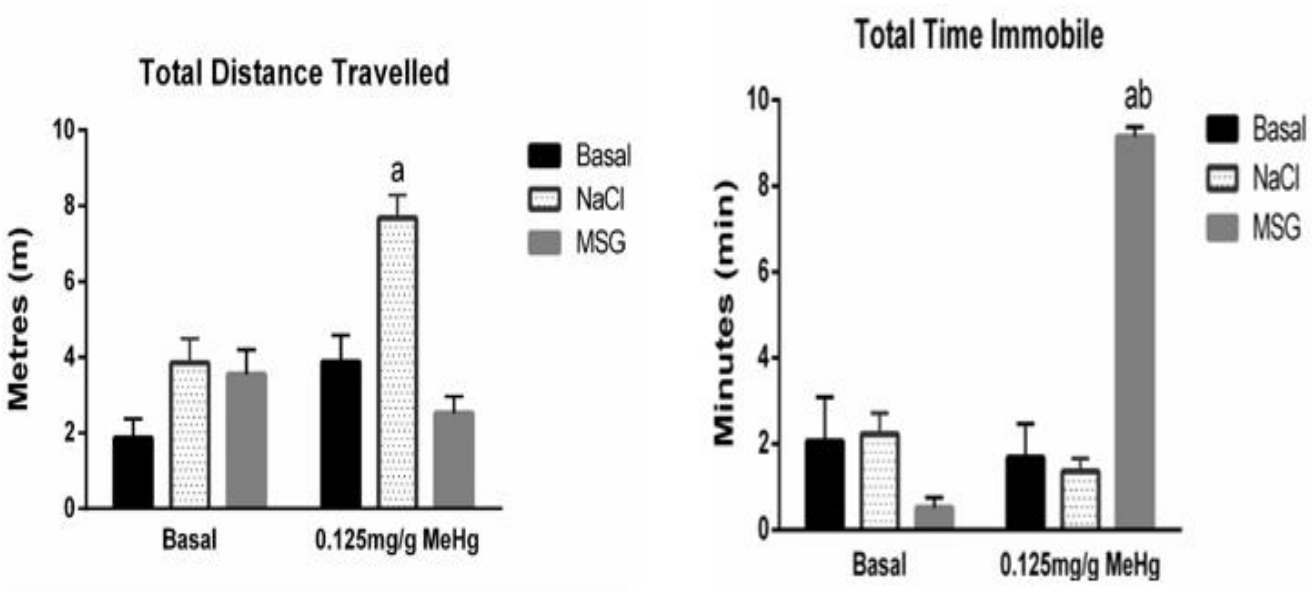


Fig 2: Locomotor activity after treatment for 21 days. The total distance travelled varied due to the interaction between MeHg and the Diet. The distance travelled was due to the effect of NaCl. **MeHg** F (1,53) =10.803: P=0.00180, **Diet** F(2,53)=14.437: P< 0.00001, **MeHg x Diet** F(2,53)=8.8289: P=0.00049. [a]P<0.05 vs NaCl.

Fig 3: The total time immobile varied due to the interaction between MeHg and the Diet. The immobility was due to the effect of MSG. There was a significant difference in the total time immobile between the cockroaches in the groups treated. **MeHg** F(1,54)=29.928: P=0.00000, **Diet** F(2,54)=20.851: P< 0.00000, **MeHg x Diet** F(2,54)=49.693: P=0.00000 **B**. [a]P<0.05 vs MSG.

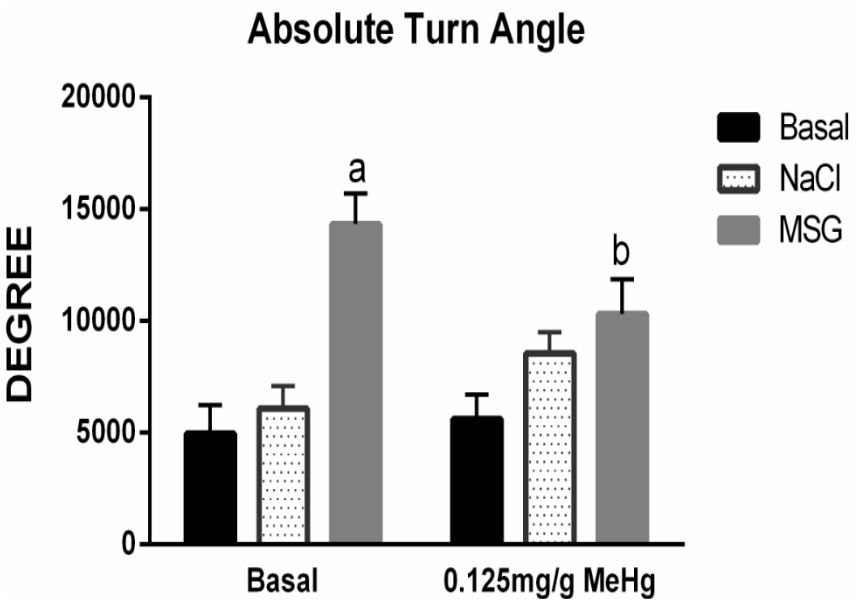


Fig 4: Locomotor activity after treatment for 21 days. The turn angle varied due to the significant interaction between MeHg and MSG, and also main effect of MSG. **MeHg** F (1,54)= 0.08215 : P=0.77551, **Diet** F(2,54)=17.024: P< 0.00000, **MeHg x Diet** F(2,54)=3.7461: P=0.02996 C. [a]P<0.05 vs MSG.

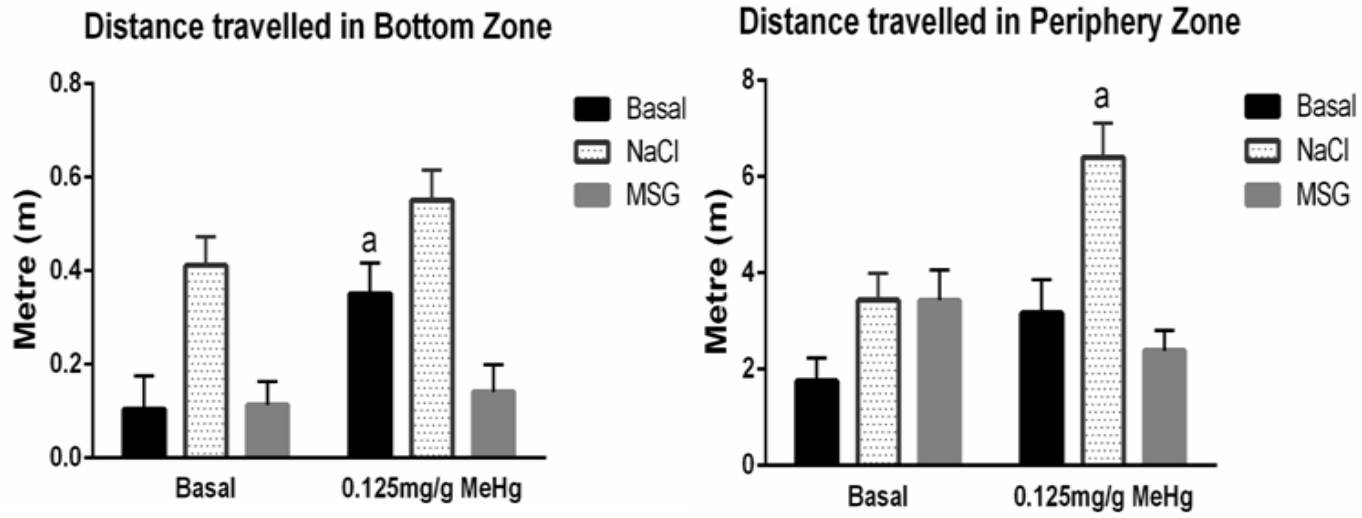


Fig 5: Locomotor activity after treatment for 21 days. There was main effect of diet and MeHg in the distance travelled in bottom zone. **MeHg** F (1, 55) = 7.4734: P=0.00841, **Diet** F(2,55)=18.382: P< 0.00000, **MeHg x Diet** F(2,55)=1.5411: P=0.22325. [a]P<0.05 vs Basal.

Fig 6: There was an interaction between MeHg and diet, as MeHg and NaCl increased the distance travelled in the periphery zone. **MeHg** F(1,54)=5.3792: P=0.02419, **Diet** F(2,54)=9.8571: P=0.00022, **MeHg x Diet** F(2,54)= 6.3341: P=0.00338 D. [a]P<0.05 vs NaCl.

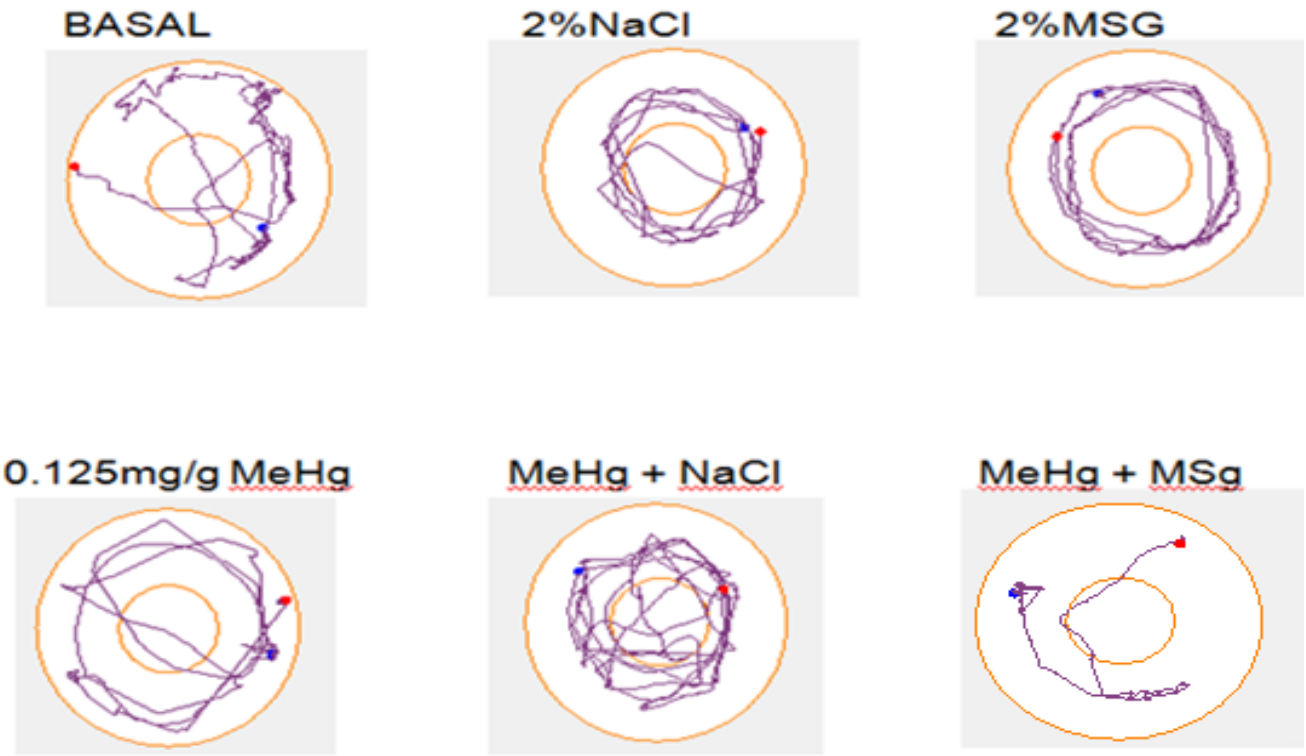


Fig 7: The track plots of a representative of the treated groups. These plots correlate with the results in Figs 5 and 6 which are the distance travelled in the bottom zone and periphery zone. Colours should be used in Print.

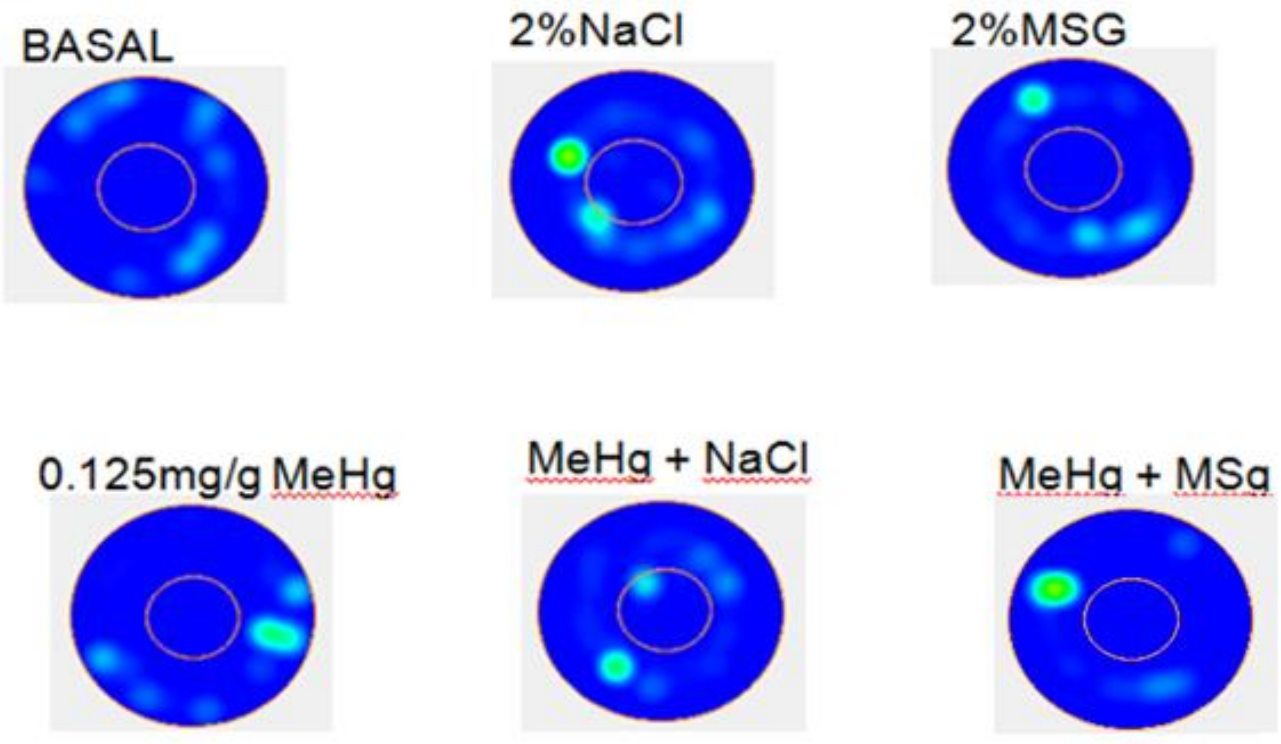
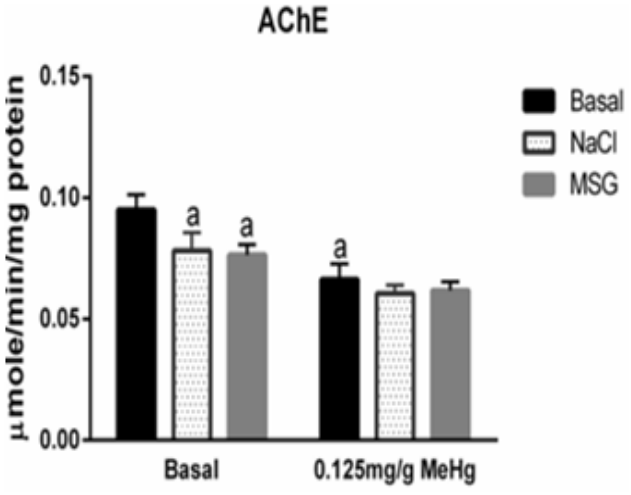


Fig 8: The exploratory profile of the representative of the treated groups. The heat map plots of a cockroach from each group. These plots also correlate with the results in Figs 5 and 6 which are the distance travelled in the bottom zone and periphery zone. Colours should be used in Print.



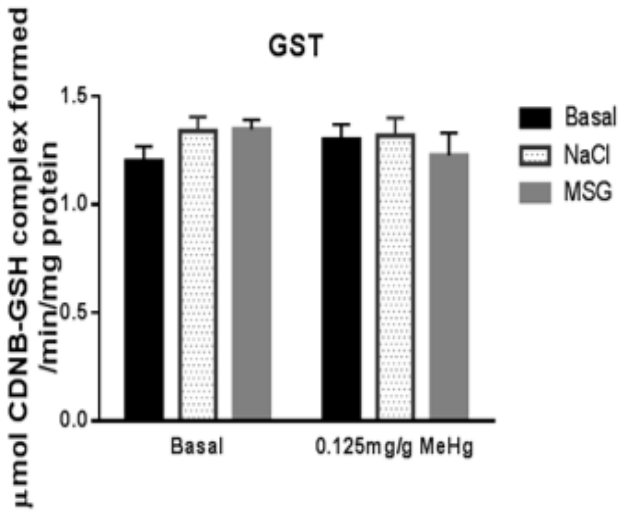


Fig 9: Acetylcholinesterase activity at the end of the 21 day treatment. There was a main effect of MeHg and diet. **MeHg** F (1,76)=21.211: P=0.00002, **Diet** F(2,76)=3.0163: P=0.05473, **MeHg x Diet** F(2,76)=0.93939: P=0.39524 . [a]P<0.05 vs Basal only

Fig 10: Glutathione-S- transferase activity at the end of 21 day treatment. There was no significant interaction between the groups. **MeHg** F(1,76)=0.05346: P=0.81776, **Diet** F(2,76)=0.57797: P=0.56342, **MeHg x Diet** F(2,76)=1.1174: P=0.33230.

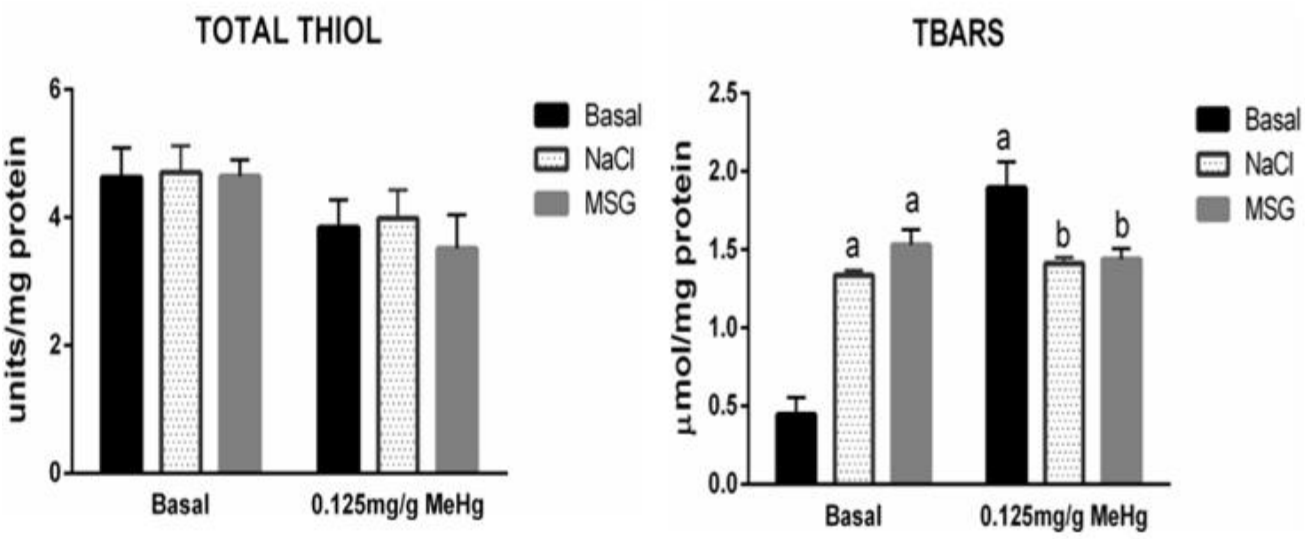


Fig 11: The level of total thiol at the end of the 21day treatment. There was a main effect of MeHg. **MeHg** F (1,36) = 6.2319: P=0.01726, **Diet** F(2,36)=0.19125: P=0.82676, **MeHg x Diet** F(2,36)=0.13272: P=0.87614 .

Fig 12: The level of TBARS at the end of the 21day treatment. There was a main effect of MeHg, diet and an interaction between diet and MeHg. **MeHg** F (1,29) = 40.830: P< 0.00000, **Diet** F(2,29)=5.9047: P=0.00706, **MeHg x Diet** F(2,29)=41.224: P<0.00001. P<0.05 vs Basal only.

CHAPTER 2

Modified expression of antioxidant genes in lobster cockroach, *Nauphoeta cinerea* exposed to methylmercury and monosodium glutamate.

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1. Abstract

Methylmercury (MeHg) is a neurotoxicant that poses risk to human health and the environment, while glutamate homeostasis is necessary for the proper functioning of the brain. We have previously shown an increase in oxidative stress after cockroach exposure to a diet containing monosodium glutamate (MSG), both separately and combined with a low dose of methylmercury. We herein seek to corroborate these findings by quantifying the expression levels of certain antioxidant genes in *Nauphoeta cinerea* exposed to MeHg and MSG. Cockroaches were fed with the basal diet alone, basal diet + 2% NaCl, basal diet + 2% MSG; basal diet + 0.125mg/g MeHg, basal diet + 0.125mg/g MeHg + 2% NaCl; and basal diet + 0.125mg/g MeHg + 2% MSG for 21 days and mRNA from head homogenate was used to quantify the expression of antioxidant genes such as glutathione-s-transferase (*GstS*, *GstT*, *GstD*), thioredoxin (*Trx1*, *Trx2*, *Trx5*), peroxiredoxin (*prx4*), superoxide dismutase (*Sod*), catalase (*Cat*). MeHg, NaCl and MSG alone downregulated mRNA levels of *GstS* and *Trx5*, in contrast, co-exposure of MeHg + MSG, upregulated these genes. MeHg + NaCl upregulated the mRNA levels of C*at* and S*od* but these genes were downregulated by NaCl alone. MeHg + NaCl and MeHg + MSG upregulated G*stD* and *Gst*T. MeHg alone upregulated the transcription levels of T*rx1*, *Trx2* and *Prx4*. The disruptions in the transcription levels of different genes by MeHg and MSG reinforces the toxicity of these neurotoxicants. In general, the data suggest their additive effects and supports the use of *N. cinerea* as a model for toxicological studies.

KEYWORDS: *Nauphoeta cinerea;* Monosodium glutamate; Methylmercury; Antioxidant; *Glutathione-S-transferase*.

2. Introduction

Methylmercury (MeHg) is a neurotoxicant that preferentially affects the developing brain. Sulfate-reducing bacteria generate MeHg by the bio-methylation of inorganic mercury with consequent bioaccumulation along the food chain, and exposure to humans via frequent fish consumption. [1-3]. Numerous studies have demonstrated impairments in learning [4], memory [5,6] and motor activity [7] of rodents exposed to MeHg. Our laboratory has illustrated the neurobehavioral toxicity of MeHg [8], GST enzyme inhibition, increase in reactive oxygen species (ROS) and its amelioration by luteolin [9], as well as alterations in exploratory profile and locomotor activity in *N. cinerea* when co-administered with glutamate suggesting the additive effect of both compounds [10]. MeHg neural toxicity is mediated by the inactivation of sulfhydryl and selenohydryl-containing proteins that are involved in the maintenance of cellular redox state [11], thereby disrupting the homeostasis of different neurotransmitter systems, including the glutamatergic, Gabaminergic [12], and cathecholaminergic systems [13].

Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system (CNS) and at the neuromuscular synapses of insects [14], though glutamatergic excitotoxicity can increase intracellular Ca^{2+} [Ca], disrupt mitochondrial functioning, and generate oxidants downstream the glutamatergic system, which could cause necrotic or apoptotic cell death in rodents [15]. These alterations can be modulated by antioxidant systems (antioxidant enzymes and low molecular weight antioxidants) [16]. Monosodium glutamate (MSG) is a naturally occurring sodium salt of L-glutamic acid commonly utilized as a food enhancer [17]. It can be cytotoxic to tissues such as liver and brain possibly via induction of oxidative stress [18]. MSG has been shown to reduce lifespan and induce an adaptive response to oxidative stress in

Drosophila melanogaster indicating the relevance of insects as models in MSG neurotoxicity [19].

Glutathione–S-transferases (GSTs) are involved in the detoxification of endogenous or exogenous electrophile compounds [20]; they facilitate the conjugation of MeHg [21-22]. Insects possess evolutionarily and structurally different microsomal and cytosolic GSTs. Cytosolic GSTs are more common and are classified as delta, epsilon, sigma, theta, omega, and zeta; furthermore, insects possess varying isoforms of these classes of GST [20]. GST theta is involved in the detoxification of lipid peroxidation products [23] and immune protection after bacterial and viral exposure in *Macrobrachium rosenbergii* [24], while delta and sigma class GST are known to be involved in the adaptive response to oxidative stress in *Bombus ignitus* [25]. Thioredoxins (Trxs) are disulfide reductases that regulate the redox status of target proteins via thiol-disulfide exchange reactions. Mammals possess *Trx1* and *Trx2*, located in the cytosol and mitochondria respectively [26-28]. *Trx1*, *Trx2* and *TrxT* have been identified in *Drosophila melanogaster* [29], while an ortholog of *Trx2*, *AMTRX2* involved in redox homeostasis has been identified in *Apis melifera* [30-31].

Peroxiredoxin (Prx) also known as thioredoxin peroxidase (Tpx), is ubiquitous to biological kingdoms from bacteria to mammals [32]. Prxs in insects compensate for their lack of glutathione peroxidase (GPX) and glutathione reductase (GR) [33]. Five *Prx* genes have been characterized in the silkworm genome [34], one of which is a homolog of the mammalian *Prx* 4 (*BmPrx 4*), and protects *B. mori* from oxidative stress caused by toxicants and external stimuli [35]. Superoxide dismutase (SOD) is a crucial enzyme for the survival of aerobic cells; it catalyzes the dismutation of superoxide radical to form oxygen and hydrogen peroxide, to defend mammalian and insect cells against oxygen toxicity and external stressors [36]. Three SOD

proteins have been discovered in insects: the cytoplasmic copper/zinc form, SOD1; the mitochondrial, Mn-SOD (SOD2); and the extracellular form, EC-SOD (SOD3) found in the hemolymph and secreted fluid during ecdysis [37]. Recently, seven isoforms of SODs have been found in *Bombyx mori*, each possessing different response-ability to stress and external stressors [38]. Catalase is an antioxidant enzyme that scavenges hydrogen peroxide; its combined activity with various peroxidases converts peroxides to water in *Helicoverpa armigera* and *D. melanogaster* [39-42].

The lobster cockroach, *N. cinerea* is a promising model for investigating MeHg toxicity [8-10]. We previously demonstrated the modulation of antioxidant and locomotor activity of cockroaches exposed to diets containing these two neurotoxicants – MeHg and MSG. We herein estimate Hg levels in the cockroaches and attempt to gain further insight into how the mRNA expression levels of antioxidant proteins vary with MeHg and MSG exposure.

3. Materials and methods

3.1.1 Chemicals

Monosodium glutamate (MSG 99 % purity Ajinomoto®) was obtained from a supermarket in Santa Maria, sodium chloride, methylmercury (MeHg) and ethanol were obtained from Sigma Aldrich (St. Louis, MO, USA). Nitric acid (HNO_3) was obtained from Labimpex industria e comercia de produtos LTD, SP. Brazil. The standard ICP solutions were obtained from Quimlab produtos de química fina LTDA, SP, Brazil.

3.1.2 Cockroach experimental protocol

Nymphs of lobster cockroach (*Nauphoeta cinerea*) were obtained from the Departamento de Bioquímica e Biologia Molecular, Centro de Ciências Naturais e Exatas (CCNE), Universidade Federal de Santa Maria, Brasil. The insects were reared in plastic boxes (45.5 cm x 40.2 cm x 29.5 cm). Nymphs were selected and put in small transparent boxes (10.8 cm x 10.8 cm x 7.9 cm) for the period of the experiment. The nymphs were adapted in the new box for 10 days before being exposed to the treatment. The nymphs were maintained at 24 ± 3ºC and 71% relative humidity with a photoperiod of 12:12 (light: dark) from September to October 2017. The insects had free access to water and standard dog food, procured from (Total Alimentos LTDA. D.Industria- Três corações, Minas Gerais, Indústria Brasileira) during rearing and adaptation. In the course of the experiment, the cockroaches were exposed to a formulated diet containing MSG, NaCl or MeHg dissolved in ethanol. The basal diet contained (per 20g of diet): 10g milled corn flour, 7g wheat flour, 2g granulated sugar (sucrose), 0.5g casein, 0.4g powder milk and 0.1g table salt (NaCl). The components were mixed with a blender. In the case of diets containing MSG and NaCl, they were added to the basal diet to a final concentration of 2%. MeHg was added dissolved in ethanol, and an identical volume of ethanol was added to the diets that did not

contain MeHg. All the diets were left in the open until all the ethanol was evaporated, and then stored at -20^{o}C.

The cockroaches were divided into six (6) groups consisting of 60 nymphs each of length varying between 1.10 and 1.32 cm at the start of the experiment. At the end of the experiment, the length varied between 1.58 and 1.70cm. Cockroaches were fed with the basal diet alone, the basal diet with 2% NaCl, the basal diet with 2% MSG; the basal diet with 0.125mg/g MeHg, the basal diet mixed with 0.125mg/g MeHg and 2% NaCl, and the basal diet mixed with 0.125mg/g MeHg and 2% MSG for 21 days. The sub-toxic dose of MeHg was selected from the study by Adedara *et al*., (2015) and this dose of MeHg has been utilized in one of our previous study [9].

3.1.3 General Sample Preparation

The cockroaches were put on ice for 15 minutes, the heads removed, put in micro-tubes, weighed and kept in ice. For the analysis of gene expression and quantification of mercury contents, the heads were removed as described above, then further processing of tissue samples was carried out.

3.1.4 Sample Preparations for ICP-AES analysis

Evidence has shown the use of laboratory methods to make reliable quantification of elements in the parts per trillion range [43-45], one of such is the Inductively coupled plasma atomic emission spectroscopy (ICP-AES) as approved by the US Environmental Protection Agency [44]. The head samples of the cockroaches were put in a 2 ml cryogenic air tube (to minimize evaporation during and after digestion), digested in concentrated double-distilled Nitric acid (HNO_3, 65% PA) and then put in the oven set to a temperature of about 70^{o}C for about three (3) hours to enable the complete digestion of the head samples. After complete digestion of the

samples (light yellow clear solution), the samples were diluted to a solution of 10% HNO_3. Water-free from impurities (Milli Q model, Millipore) was used for the preparation of the reagents and sample digest dilutions. The elements in the solution were determined using ICP-AES (ICPE-9000 Multitype ICP Shimadzu, Japan). Argon (99% purity, White Martins, Brazil) was used to generate plasma during the process.

3.1.5 Calibration Curve for ICP-AES analysis

The standard solution of mercury was used to make a calibration curve. A solution of 10% Nitric acid was used as blank while the standard solution of Hg (1000 ppm) was prepared to final concentrations of 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100, 250 ppm, measured in the ICP-AES, ICPE-9000 Multitype ICP Shimadzu, and the curve plotted. The wavelength (nm) used for the determination of Hg is 194.227. The plasma was set at a radial view, the gas flow rate and the sample flow rate was set at 10 L min^{-1} and 1.0-1.5 mL min^{-1} respectively. The correlation coefficient (R) of 0.99 or more indicates a strong linear relationship between the concentration and intensity.

3.2 Total RNA Extraction and cDNA synthesis

Total RNA was isolated from 10–30 mg tissues of the head of cockroaches, using TRIzol® reagent (Invitrogen Life Technologies, USA) following the manufacturer's instruction. DNA contamination was removed by DNase 1, amplification grade (Invitrogen Life Technologies, USA). The yield and the purity of RNA were determined by NanoDrop 2000 spectrophotometer (Nanodrop Technologies, USA). The RNA samples with an absorbance ratio OD 260/280 and OD 260/230 between 1.9–2.0 were used for further analysis. RNA integrity was assessed using agarose gel electrophoresis.

The strand of cDNA was synthesized from 1µg of total RNA, using iScript cDNA synthesis kit (BIO-RAD Laboratories. Inc, USA). Briefly, the assay mixture for each tube consists of 10 µl of 1µg RNA template, 1 µl of 5X iScript Reaction mix, 1 µl iScript reverse transcriptase and 5 µl dietyhlpyrocarbonate (DEPC) water incubated at 25°C for 5 min. The tubes were continuously incubated at 42°C for 30 min, inactivated at 85°C for 5 min, and then chilled on ice. The cDNA samples were stored at -20°C for future analysis. For qPCR analysis, each cDNA sample was diluted 10 times with nuclease-free water.

3.3 Quantitative Reverse-Transcription polymerase chain reaction (qRT-PCR)

The Real-time PCRs were conducted in Quant studio^{TM} 3 system. For each reaction, the 20µl mixture contained 10µl of diluted cDNA, 1X PCR buffer, 3mM $MgCl_2$, 0.2 µm each of the forward and reverse primers, 0.1X of SYBR green (Invitrogen USA), 0.2 mM deoxyribonucleotide triphosphate(dNTP's), and 0.25U taq polymerase using the Platinum Taq DNA Polymerase kit (Thermo Fischer Scientific). The amplification protocol used is as follows: 94°C for 5 min (1 cycle), followed by 40 cycles at 94°C for 15 s, 60°C for 10s and 72^{o}C for 15s (fluorescence collection). After the amplification step, a thermal denaturing cycle was included to obtain the dissociation curve of the PCR products (amplicons) to verify the amplification specificity.

The reactions were carried out in two technical duplicates of each of the six biological replicates. The gene *GAPDH* was used as the reference gene for sample normalization and the basal diet alone samples were used for calibrating calculations performed with the $2^{-\Delta\Delta Ct}$ method [46].

3.4 Primer design

The NCBI platform (https://www.ncbi.nlm.nih.gov/) and Fly base were used to procure the nucleotides of genes that code for proteins that function as antioxidant enzymes in the *Drosophila melanogaster, Zootermopsis nevadensis, Cryptotermes secundus* and *Blatella germanica*. The sequence of the obtained nucleotides was procured in the transcriptome of *Nauphoeta cinerea* as published by our research group. The accession numbers of the *N. cinerea* transcriptome are SRR3581673 and SRR3581312 [47] and the sequence of interest was analyzed using BLASTx from the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence of CDS was aligned utilizing the Mega Alignment Explorer version 6 to choose the exons. Primer 3 input platform version 0.4.0 (http://primer3.ut.ee/) was used to design the primer of interest from the Nauphoeta sequences and the quality of the primer sequence was tested with Oligoanalyzer 3.1 (https://www.idtdna.com/pages/tools/oligoanalyzer).

Primers were purchased from Invitrogen (Thermo Fisher Scientific) with their certificates of analysis. The primer features of the genes are listed in Table 1. Thirteen genes were selected, three (3) classes of glutathione-s-transferase (*GstS, GstT, GstD*), three (3) classes of thioredoxin (*Trx1, Trx2, Trx5*), peroxiredoxin (*Prx4*), superoxide dismutase (*Sod*), catalase (*Cat*) of which four of them were analyzed for stability to be used as Housekeeping genes. The Housekeeping genes analyzed include *Gapdh, Ef1a1, Tbp* and *Tubulin*. The identity of the amplified genes was checked by sequencing the PCR products using Sangers method of sequencing conducted in the ABI-Prism 3500 Genetic Analyzer (Applied Biosystems) at Ludwig Biotecnologia Ltda (Alvorada, RS, Brazil).

The qRT-PCR efficiencies in the exponential phase were calculated for each primer pair using standard curves (10 fold serial dilutions of the cDNA), the mean threshold cycle (Ct) values for each serial dilution of each primer were plotted against the logarithm of the cDNA dilutions and calculated according to the equation $E= 10^{(-1/slope)} -1$, where the slope is the gradient of the linear regression line, and the correlation coefficients (R2) shows the linearity of the standard curve for each gene.

3.5 Analysis of stability of the Housekeeping genes.

The stability of the Housekeeping genes was analyzed using two familiar normalization algorithms, Bestkeeper [48] and normfinder [49]. Normfinder calculations were based on Ct values being converted to non-normalized relative quantities according to the formula: $2^{-\Delta Ct}$ (ΔCt = the corresponding Ct value – minimum Ct). Bestkeeper was used to analyze raw Ct values. NormFinder was used to approximate both intra- and inter-group variations of gene expression and the results merged to produce a stability value for all the samples in the groups. The genes with the lowest rank were deemed to be the most stably expressed and were the most appropriate to be selected as the housekeeping gene(s) for the particular experimental setup [49].

BestKeeper was used to calculate the standard deviation (SD) and the power of each Housekeeping gene [48]. The BestKeeper index was calculated from the geometric average of the candidates Ct values for each specific sample. In BestKeeper, Standard deviation of Ct value<1 indicates genes which are stably expressed. The most stable reference genes are the ones with the lowest SD values and the highest coefficients of correlation with the BestKeeper index [50]. Bestkeeper© version 1 was used for the analysis of the Housekeeping genes in the present study. According to Bestkeeper, *Gapdh* and *EF1a1* were the best reference genes, while

Normfinder selected *Tubulin* and *Gapdh* as the best genes, but in the course of the experiment, *EF1a1* and *Tubulin* genes were responsive to the treatment. Therefore, we decided to use only the *Gapdh* gene as the reference gene to normalize the samples utilized in this study as it was clearly not altered by the treatment.

3.6 Statistical analysis

The data from the ICP-AES analysis and qRT-PCR were expressed as the mean ±standard error of the mean (SEM). Statistical analyses were carried out by two-way analysis of variance (ANOVA); [2 (with and without 0.125mg MeHg/g of diet) by 3 (Basal/2%NaCl/2%MSG)] followed by Tukey's *post-hoc* test. Significance was set at $p<0.05$. The statistical analyses were performed using Graph Pad Prism 6 and Statistica software packages

4 Results

4.1 Quantification of Hg levels in cockroaches co-exposed to MeHg and MSG.

Factorial ANOVA showed a significant main effect of MeHg [F (1, 24) = 651.71: P<0.000] and diet [F (2, 24) = 8.0884: P= 0.00206] (Fig. 1) for the mercury (Hg) levels in the heads of cockroaches. Referring to the main effect of MeHg, the mean of the MeHg treated group (0.17±0.007 ng/g) was higher than the mean of the non-MeHg treated group (0.07±0.012 ng/g) (Table 2). In relation to the main effect of diet, the means are as follows: basal groups (0.12±0.009 ng/g n=10); NaCl groups (0.12±0.01 ng/g n=10); MSG groups (0.107±0.011 ng/g n=10) (Table 3). In general, there was an increase in the Hg levels of groups concomitantly exposed to MeHg + NaCl and MeHg + MSG in relation to the general mean of the mercury levels.

4.2 Effects of Dietary co-exposure of MSG and MeHg on Glutathione-S-transferase (*GstD, GstT* and *GstS*) mRNA expression in *N. cinerea*.

Two-way ANOVA indicated a significant main effect of MeHg [F (1,63) = 4.09: P=0.04], for the mRNA levels of *GstD* (Fig. 2a). Additionally, the mean of the MeHg treated groups (1.82±1.57, n= 33) (Table 2) was higher than the non-MeHg treated groups (1.16±0.98 n= 36) (Table 2).

The main effect of MeHg for the mRNA levels of *GstT* was also significant [F (1,60) = 3.85: P=0.05] (Fig. 2b). The mean of the MeHg treated groups (3.2±2.15 n=30) (Table 2) was higher than the non-MeHg treated groups (2.3±1.5 n=36) (Table 2).

Two-way ANOVA showed a significant MeHg x diet interaction [F (2,64) = 3.65: P=0.03] (Fig. 2c) for the mRNA levels of *GstS*. The interaction was significant because the simultaneous exposure to MeHg + MSG upregulated the expression of *GstS*.

4.3 Effects of dietary co-exposure of MSG and MeHg on thioredoxin (*Trx5, Trx2,* and *Trx1*) mRNA expression in *N. cinerea*.

Two-way ANOVA indicated a significant MeHg x diet interaction [F (2,66) = 3.10: P=0.05] (Fig. 3a) for the mRNA levels of *Trx5*. The significant interaction can be accounted for by the decrease in the expression of mRNA levels of *Trx5* in the group exposed to MSG only whereas there was an increase in the mRNA levels in the MeHg + MSG group in relation to the general mean of the *Trx5* expression.

Two-way ANOVA indicated a significant MeHg x diet interaction [F (2,65) = 19.68: P<0.00001] (Fig. 3b) for the mRNA levels of *Trx 2*. The significant interaction can be deciphered by the marked increase in the expression levels of *Trx 2* in the group exposed to MeHg alone when compared to the basal group alone. Also, the significant interaction can be described by the distinct increase in mRNA levels of *Trx 2* in the MSG group alone and its concomitant decrease in the MeHg + MSG group (Fig.3b).

There was a significant main effect of MeHg [F (1,66) = 3.70: P=0.05] and diet [F (2,66) = 3.87: P= 0.03] (Fig. 3c) for the mRNA levels of *Trx1*. Concerning the main effect of MeHg, the mean of *Trx1* expression in the MeHg treated groups (1.41±1.16 n=36) was higher than the non-MeHg treated groups (0.94±0.71 n=36) (Table 2). With reference to the main effect of diet, the means are as follows: Basal groups (1.46±1.1 n=24), NaCl groups (1.36±1.18 n=24) and MSG groups

(0.71±0.53 n=24) (Table 3). The groups exposed to MSG had the lowest means when compared to the other two groups.

4.4 Effects of dietary co-exposure of MSG and MeHg on Superoxide dismutase (*Sod*), Catalase (*Cat*) and Peroxiredoxin (*Prx4*), mRNA expression in *N. cinerea* and Mortality profile.

Two-way ANOVA showed a significant MeHg x diet interaction [F (2,66) = 3.37: P=0.04] for the mRNA levels of *Sod* (Fig. 4a). In the absence of MeHg, the interaction can be described by the low expression level of *Sod* as observed in the group treated with NaCl alone, meanwhile, the MeHg treated groups, MeHg + NaCl and MeHg + MSG upregulated the expression of *Sod*.

For *Cat* (Fig. 4b), two-way ANOVA showed a significant main effect of MeHg [F (1,66) = 6.71: P= 0.01] and diet [(F (2,66) = 7.15: P= 0.002)] for the mRNA levels. Concerning the main effect of MeHg, the mean of *Cat* expression in the MeHg treated groups (1.77±1.27 n=36) was higher than the mean of the non-MeHg treated groups (1.14±0.54 n=36) (Table 2). In relation to the main effect of diet, the basal (1.12±0.55 n=24) and MSG groups (1.15±1.08; n=24) had lower means than the NaCl groups (2.10±1.11 n=24) (Table 3).

Two-way ANOVA indicated no significant interaction, the main effect of diet or MeHg for the mRNA levels of *Prx*4 (Fig. 4c). Also, we found no significant difference in cockroach mortality rates across all the treated groups (data not shown).

5 Discussion

The present study estimated Hg levels in the exposed cockroaches, investigated for the first time the possible alterations in the transcriptional levels of several antioxidant genes in the nymphs of *N. cinerea* exposed to a diet containing MeHg and MSG which are known neurotoxicants. Exposure to these toxicants is reflected in human consumption of fish and seasoned foods [2-3, 51]. There has been substantial support and advocacy for the use of invertebrate animal models in neuroscience research due to their availability and ease of handling [52]. Previous researches have reported the effect of methylmercury on the expression of genes in some model organisms [53]. However, the effect of simultaneous exposure of MeHg and MSG on the transcriptional levels of some antioxidant genes in the brain is yet to be elucidated.

Results showed an increase in the mercury levels in the heads of the exposed cockroaches which corroborates studies that have shown a high affinity of the brain to mercury due to its potential to cross the blood-brain barrier via the neutral amino acid transport, hence its distribution to the brain regions [54]. Our results demonstrated about three-fold increase of Hg in the brain of *N. cinerea*; which is lower than that reported in mice exposed to high levels of MeHg (about 10 times higher) [55]. However, since the cockroach brain is less than 20% of the mass of its' head, the actual concentration of Hg in the brain is possibly greater than the one determined using the entire head. Furthermore, the accumulation of Hg in the different parts of the brain of flies (protocerebrum, deutocerebrum and tritocerebrum) [56] is not homogenous [57]. Thus, if it was possible to work with specific areas of the brain of the cockroaches, we could have observed more pronounced effects of MeHg.

Oxidative stress and impaired antioxidant systems have been described as important mechanisms by which MeHg exerts its neurotoxicity in the central nervous system [58]. There are also reports of oxidative stress in tissues [59], and impaired brain function [60] after MSG exposure. However, the brain is provided with antioxidant systems that mitigate against oxidative stress caused by ROS generation [61].

Glutathione S-Transferase is a family of highly polymorphic enzymes that catalyzes the conjugation of xenobiotics with glutathione, detoxifying them in the process [62]. Here, it was observed that the co-exposed group, MeHg +MSG, upregulated the mRNA levels of *GstD*, *GstT* and *GstS*. The significant elevation in *GstS* underscores the widely held belief that it is crucial for xenobiotic detoxification and our observation is similar to a previous study where the expression of *GST sigma* significantly increased more than those of other GSTs in response to H_2O_2 exposure in *Tigriopus japonicas* [63].

Furthermore, changes in glutathione homeostasis have been correlated with impaired motor function [64], similar to our earlier findings of a significant increase in total time spent immobile by the cockroach nymphs exposed to MeHg + MSG where GST activity decreased [10]. Our result also corroborates a study that demonstrated an increase in the expression of *GstS* and *GstT* in *Crassostrea ariakensis* in response to Prorocentrum lima and associated diarrhetic shellfish poisoning (DSP) toxins [65]. Similarly, an increase in expression in of *GstD* has been observed in the midgut of *D. melanogaster* exposed to dietary H_2O_2 [66]. This data further indicates that GSTs might play roles in the detoxification of xenobiotics and modulation of oxidative stress, probably by conjugating the cytotoxic aldehyde products of ROS with glutathione [67]. GST catalyzes the conjugation of GSH with electrophiles. MeHg is a soft electrophile which forms a RS-CH_3 complex with the –SH group of GSH and could also interact with proteins and nucleic

acid [68]. We hypothesize that the complex interaction of MeHg + MSG with GST sigma could induce its synthesis to compensate pro-oxidative effects of the toxicants. Although the existence of real differences between *GstS* and other GSTs are scanty in the literature, *GstS* has been shown to be most abundant in *P. americana* [69] and also can play roles as a biomarker for metal exposure in arthropods [63]. We emphasize that only these three *GST* genes were identified and classified with reliability in our *N. cinerea* transcriptome [47].

We identified and classified three thioredoxins in the transcriptome of *N. cinerea* [47]. Thioredoxins confer protection on cells due to their ability to scavenge ROS [70]; they have been known to promote neural cell growth in neurodegenerative conditions [71]. In the present study, upregulation of *Trx5* was observed in the cockroaches simultaneously co-exposed to MeHg and MSG while *Trx2* was downregulated. This observation is instructive as *Trx2* is the major thioredoxin found in the mitochondria which is a major source of ROS in the brain [72]. Studies have implicated reduced *Trx2* in neurodegenerative diseases [70,73] which is usually characterized by oxidative stress [74].

We also found upregulated expression levels of *Trx1* in the group exposed to MeHg, in line with increased *Trx1* levels in *C. elegans* after exposure to MeHg [75], thus suggesting that increased levels of *Trx1* might participate in MeHg resistance via antioxidant protection. Consequently, thioredoxins are very sensitive to mercury both in-vitro [76] and in-vivo [77], because one of the mechanisms of mercury compound toxicity is the interaction with nucleophilic compounds such as thiols and selenols, therefore, affecting the activity of antioxidant enzymes [78].

Superoxide dismutase and catalase are antioxidant enzymes that respond to oxidative stress as they have roles in preventing oxidative damage caused by toxicants or oxidative stress. These

two enzymes scavenge superoxide and hydrogen peroxide in the cells. During oxidative stress, cells increase the expression of genes whose products protect the cell either by repairing stress-related damage or by inactivating ROS [79]. Our present study showed MeHg + MSG and MeHg + NaCl upregulated the expression levels of superoxide dismutase and catalase in the heads of *N. cinerea*. This observation is supported by earlier researches that reported that the promoter of *SOD1* has a metal response element (MRE) that upregulates the gene in response to heavy metals [80] while *SOD2* gene is highly inducible by oxidants [81], the generation of which has been linked with both MeHg [82] and MSG [83] in biological systems, especially in the brain. Surprisingly, MeHg + NaCl upregulated the expression of catalase in *N. cinerea*. Although, the upregulated expression of catalase by NaCl has been earlier reported [84], the effect of MeHg + NaCl in this regard is still a matter of conjecture. Our laboratory once showed a decrease in the mRNA levels of *Cat* gene in *D. melanogaster* exposed to 4-vinyvlcyclohexene 1,2-monoepoxide (VCM) and 4-vinyvlcyclohexene diepoxide (VCD), metabolites of an ovotoxicant, 4-vinyvlcyclohexene (VCH) [85].

Peroxiredoxins belong to a family of proteins that scavenge H_2O_2 and also play an important role in the response of the cell to oxidative stress. *PRX*4 is copiously expressed in several tissues and to a lesser extent in the brain [86]. We observed that there was no alteration in the expression level of *Prx4* among the various cockroaches treated in this study probably due to the lower expression level of *Prx4* in the brain than other tissues. Based on the results obtained from this study, there is a high chance that the changes in mRNA expression levels that we recorded are reflected in levels of protein production across treatment groups, as this has been demonstrated in yeasts exposed to an environmental form of oxidative stress [87,88].

Altogether, in the case of genes (*GstD*, *GstT*, *Trx1*, *Cat*) that showed a significant main effect of MeHg only, the relative mean of the expression of these genes in the MeHg treated groups (MeHg; MeHg + NaCl; MeHg + MSG) was greater than their relative mean expression in the non-MeHg treated groups (Basal; NaCl; MSG) suggesting that MeHg influenced the mRNA levels of these genes. We propose that the changes in the expression of GSTs, Trxs, Sod and Cat might play significant roles in protecting cockroaches from oxidative stress induced by the potent toxicants (MeHg and MSG). This study further supports the use of *Nauphoeta cinerea* as a valid alternative model for basic toxicological and molecular studies. *N. cinerea* can also be utilized as biomarkers of bio-accumulated environmental contaminants such as pesticides, insecticides and trace or heavy metals, this enhances environmental monitoring and might help minimize public risk to such contaminants.

Conflicts of interest

The authors have no conflict of interest to declare.

Acknowledgements

B.A. Afolabi and O.C. Olagoke are indebted to TWAS-CNPq, with fellowship number 2015 (FR number: 3240286510) and 2017 (FR number: 3240299312) respectively.

This work was supported by FAPERGS-CNPQ, 12/2014 PROEX (23038.004173/2019-93 0493/2019), CNPq (INCT 465671/20144). M. Aschner was supported by grants from the National Institute of Environmental Health Sciences NIEHS R01ES07331, NIEHS R01ES10563, and NIEHS R01ES020852.

References

1. C.S. Oliveira, P.A. Nogara, D.M.P. Ardisson-Araújo, M. Aschner, J.B.T. Rocha, J.G. Dórea, Neurodevelopmental Effects of Mercury, Advances in Neurotoxicology 2 (2018) 27-86.

2. P.A. Nogara, C.S. Oliveira, G.L. Schmitz, C.P. Paulo, M. Farina, M. Aschner, J.B.T. Rocha, 2019. Methylmercury's chemistry: From the environment to the mammalian brain. BBA-General Subjects 1863, 129284. https://doi10.1016/j.bbagen.2019.01.006

3. P.A. Nogara, M. Farina, M. Aschner, J.B.T. Rocha, Mercury in our food, Chem. Res. Toxicol. 32 (2019) 1459-1461.

4. E.M. Paletz, M.C. Craig-Schmidt, M.C., Newland, Gestational exposure to methylmercury and n-3 fatty acids: Effects on high- and low-rate operant behaviour in adulthood, Neurotoxicol. Teratol. 28 (2006) 59–73.

5. M.R. Carratu, P. Borracci, A. Coluccia, A. Giustino, G. Renna, M.C. Tomasini, E. Raisi, T. Antonelli, V. Cuomo, E. Mazzoni, L. Ferraro, Acute exposure to methylmercury at two developmental windows: Focus on neurobehavioral and neurochemical effects in rat offspring, Neuroscience 141 (2006) 1619–1629.

6. E. Dare, S. Fetissov, T. Hokfelt, H. Hall, S.O. Ogren, S. Ceccatelli, Effects of prenatal exposure to methylmercury on dopamine-mediated locomotor activity and dopamine D2 receptor binding, Naunyn Schmiedebergs, Arch. Pharmacol. 367 (2003) 500–508.

7. S. Sakamoto, A. Kakita, K. Wakabayashi, H. Takahashi, A. Nakano, H. Akagi, Evaluation of changes in methylmercury accumulation in the developing rat brain and its effects: A study with consecutive and moderate dose exposure throughout gestation and lactation periods, Brain Res. 949 (2002) 51–59.

8. I.A. Adedara, D.B. Rosemberg, D.O. Souza, J.P. Kamdem, E.O. Farombi, M. Aschner, J.B.T Rocha, Biochemical and behavioural deficits in the lobster cockroach *Nauphoeta cinerea* model of methylmercury exposure, Toxicol. Res. 4 (2015) 442-451.

9. I.A. Adedara, D.B. Rosemberg, D.O. Souza, E.O. Farombi, M. Aschner, J.B.T Rocha, Neuroprotection of luteolin against methylmercury-induced toxicity in lobster cockroach *Nauphoeta cinerea*, Environ Toxicol Pharmacol. 42 (2016) 243-51.

10. B.A. Afolabi, I.A. Adedara, D.O. Souza, J.B.T. Rocha, Dietary co-exposure to methylmercury and monosodium glutamate disrupts cellular and behavioural responses in the lobster cockroach, *Nauphoeta cinerea* model, Environmental toxicology and pharmacology 64 (2018) 70-77.

11. M. Farina, M. Aschner, Methylmercury-Induced Neurotoxicity: Focus on Pro-oxidative Events and Related Consequences, Adv. Neurobiol. 18 (2017) 267-286.

12. W.D. Atchison, Is chemical neurotransmission altered specifically during methylmercury-induced cerebellar dysfunction? Trends Pharmacol Sci. 26 (2005) 549-57.

13. M. Farina, M. Aschner, J.B.T. Rocha, 2017. Chapter Two- The Catecholaminergic Neurotransmitter System in Methylmercury-Induced Neurotoxicity, Advances in Neurotoxicology 2017 pp. 47-81.
14. R.F. Chapman, S.J. Simpson, A.E. Douglas, The Insects: Structure and Function, fourth ed., Cambridge University Press, 2013.
15. H. Prentice, J. Pravinchandra, J. Wu, 2015. Mechanisms of Neuronal Protection against Excitotoxicity, Endoplasmic Reticulum Stress, and Mitochondrial Dysfunction in Stroke and Neurodegenerative Diseases, Oxidative medicine and cellular longevity 964518. http://dx.doi.org/10.1155/2015/964518.
16. S. Samina, Oxidative Stress and the Central Nervous System. The Journal of pharmacology and experimental therapeutics 360 (2017) 201-205.
17. S.G. Dixit, P. Rani, A. Anand, K. Khatri, R. Chauhan, V. Bharihoke, To study the effect of monosodium glutamate on histomorphometry of cortex of kidney in adult albino rats, Renal Failure 36 (2014) 266-270.
18. M.V. Paul, M. Abhilash, M.V. Varghese, M. Alex, R.H. Nair, Protective effects of alpha-tocopherol against oxidative stress related to nephrotoxicity by monosodium glutamate in rats, Toxicol Mech. Methods 22 (2012) 625–30.
19. A.O. Abolaji, A.O. Olaiya, O.J. Oluwadahunsi, E.O. Farombi, Dietary consumption of monosodium L-glutamate induces adaptive response and reduction in the lifespan of *Drosophila Melanogaster* Cell. Biochem. Funct. 35 (2017) 164-170.
20. J.D. Hayes, J.U. Flanagan, I.R. Jowsey, Glutathione transferases. Annu. Rev. Pharmacol. Toxicol. 45 (2005) 51–88.
21. D. Vorojeikina, K. Broberg, T.M. Love, P.W. Davidson, E.V. Wijngaarden, M.D. Rand, Glutathione S-Transferase Activity Moderates Methylmercury Toxicity During Development in Drosophila, Toxicol sci. 157 (2017) 211–221.
22. P. Espitia-Pérez, S. Marin Albino, H. Tais da Rosa, A.K. Silveira, L. Espitia-Pérez, H. Brango, D.P. Moraes, P.R.S. Hermann, M. Mingori, F. Barreto, A. Kunzler, D.P Gelain, E.S Schnorr, J.C.F Moreira, Effects of methylmercury and retinol palmitate co-administration in rats during pregnancy and breastfeeding: Metabolic and redox parameters in dams and their offspring. Ecotoxicology and Environmental Safety 162 (2018) 603–615.
23. K. Yamamoto, P. Zhang, F. Miake, N. Kashige, Y. Aso, Y. Banno, H. Fujii, Cloning, expression and characterization of theta-class glutathione S-transferase from the silkworm, Bombyx mori, Comp Biochem Physiol B Biochem Mol Biol. 141 (2005) 340–346.
24. J. Arockiaraj, A.J. Gnanam, R. Palanisamy, P. Bhatt, V. Kumaresan, M.K. Chaurasia, M. Pasupuleti, H. Ramaswamy, A. Arasu, A. Sathyamoorthi, A cytosolic glutathione s-transferase, GST-theta from

freshwater prawn Macrobrachium rosenbergii: molecular and biochemical properties, Gene 546 (2014) 437–442.

25. B.Y. Kim, W.L. Hui, K.S. Lee, H. Wan, H.J. Yoon, Z.Z. Gui, S. Chen, B.R. Jin, Molecular cloning and oxidative stress response of a sigma class glutathione S-transferase of the bumblebee Bombus ignites, Comp Biochem Physiol B Biochem Mol Biol. 158 (2011) 83–89.
26. J.F. Collet, J. Messens, Structure, function, and mechanism of thioredoxin proteins, Antioxid. Redox Signal 13 (2010) 1205–1216.
27. S.J. Welsh, W.T. Bellamy, M.M. Briehl, G. Powis, The redox protein thioredoxin-1 (Trx-1) increases hypoxia-inducible factor 1alpha protein expression: Trx-1 overexpression results in increased vascular endothelial growth factor production and enhanced tumor angiogenesis, Cancer Res. 62 (2002) 5089–5095.
28. T. Maruyama, Y. Sachi, K. Furuke, Y. Kitaoka, H. Kanzaki, Y. Yoshimura, J. Yodoi, Induction of thioredoxin, a redox-active protein, by ovarian steroid hormones during growth and differentiation of endometrial stromal cells in vitro, Endocrinology 140 (1999) 365–372.
29. H. Bauer, S.M. Kanzok, R.H. Schirmer, Thioredoxin-2 but not thioredoxin-1 is a substrate of thioredoxin peroxidase-1 from Drosophila melanogaster: isolation and characterization of a second thioredoxin in Drosophila melanogaster and evidence for distinct biological functions of Trx-1 and Trx-2, J. Biol. Chem. 277 (2002) 17457–17463.
30. M. Corona, G.E. Robinson, Genes of the antioxidant system of the honey bee: annotation and phylogeny, Insect Mol. Biol. 15 (2006) 687–701.
31. P. Yao, L. Hao, F. Wang, X. Chen, Y. Yan, X. Guo, B. Xu, Molecular cloning, expression and antioxidant characterisation of a typical thioredoxin gene (AccTrx2) in Apis cerana cerana, Gene 527 (2013) 33–41.
32. H.Z. Chae, T.B. Uhm, S.G Rhee, Dimerization of thiol specific antioxidant and the essential role of cysteine 47, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 7022–7026.
33. S.N. Radyuk, R.S. Sohal, W.C. Orr, Thioredoxin peroxidases can foster cytoprotection or cell death in response to different stressors: over- and underexpression of thioredoxin peroxidase in Drosophila cells, Biochemical Journal 371 (2003) 743–752.
34. G.Q. Shi, Q.Y. Yu, Z. Zhang, Annotation and evolution of the antioxidant genes in the silkworm, Bombyx mori, Arch Insect Biochem Physiol. 79 (2012) 87–103.
35. G.Q. Shi, Q.Y. Yu, L. Shi, Z. Zhang, Molecular cloning and characterization of peroxiredoxin 4 involved in protection against oxidative stress in the silkworm Bombyx mori, Insect Molecular Biology 21 (2012) 581–592.

36. J.M. McCord, I. Fridovich, Superoxide Dismutase: An enzymic function for erythrocuprein (hemocuprein), The Journal of Biological Chemistry Fed. Proc. 244 (1969) 6049-6055.

37. J. Zhang, A. Lu, L. Kong, Q. Zhang, E. Ling, Functional analysis of insect molting fluid proteins on the protection and regulation of ecdysis, J Biol Chem. 289 (2014) 35891–35906.

38. Y. Kobayashi, Y. Nojima, T. Sakamoto, K. Iwabuchi, T, Nakazato, H. Bono, A. Toyoda, A. Fujiyama, M.R. Kanost, H. Tabunoki, 2019. Comparative analysis of seven types of superoxide dismutases for their ability to respond to oxidative stress in Bombyx mori, Scientific reports 9 2170. https://doi: 10.1038/s41598-018-38384-8.

39. X. Zhang, Y. Li, J. Wang, T. Zhang, T. Li, W. Dong, E. Ma, J. Zhang, Identification and characteristic analysis of the catalase gene from Locusta migratoria, Pestic. Biochem. Phys. 132 (2016) 125–131.

40. D.P. Jones, Radical-free biology of oxidative stress, Am. J. Physiol. Cell Physiol. 295 (2008) C849–C868.

41. Y. Wang, L. Wang, Z. Zhu, W. Ma, C. Lei, The molecular characterization of antioxidant enzyme genes in Helicoverpa armigera adults and their involvement in response to ultraviolet—a stress, J. Insect Physiol. 58 (2012) 1250–1258.

42. W.C. Orr, R.J. Mockett, J.J. Benes, R.S. Sohal, Effects of overexpression of copper-zinc and manganese superoxide dismutases, catalase, and thioredoxin reductase genes on longevity in Drosophila melanogaster, J. Biol. Chem. 278 (2003) 26418–26422.

43. P. Kurup, C. Sullivan, R. Hannagan, S. Yu, H. Azimi, S. Robertson, D. Ryan, R. Nagarajan, T. Ponrathnam, G. Howe, A Review of Technologies for Characterization of Heavy Metal Contaminants, Indian Geotechnical Journal 47 (2017) 421–436.

44. S. Jignesh, K. Vineeta, S. Abhay, K. Vilasrao, Analytical methods for estimation of metals, Int. J. Res. Pharm. Chem. 2 (2012) 146–163.

45. R. Komiya, T. Wada, F. Tsushima, K. Sakamoto, T. Ikeda, A. Yamaguchi, H. Harada, M. Uo, Quantitation and distribution of metallic elements in sequestra of medication-related osteonecrosis of jaw (MRONJ) using inductively coupled plasma atomic emission spectroscopy and synchrotron radiation X-ray fluorescence analysis. Journal of Bone and Mineral Metabolism, J Bone Miner Metab.37 (2019) 676-684.

46. K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real time quantitative PCR and the 2(-delta delta C(T)) method, methods 25 (2001) 402-408.

47. A.L.A. Segatto, J.F. Diesel, E.L.S. Loreto, J.B.T. Rocha, De novo transcriptome of the lobster cockroach *Nauphoeta cinerea* (Blaberidae), Genetics and Molecular Biology, 41 (2018) 713-721.

48. M.W. Pfaf, A. Tichopad, C. Prgomet, T.P. Neuvians, Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations, Biotechnol Lett. 26 (2004) 509–515.

49. C.L. Andersen, J.L. Jensen, T.F. Orntof, Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets, Cancer Res. 64 (2004) 5245–5250.

50. H. Gong, L. Sun, B. Chen, Y. Han, J. Pang, W. Wu, R. Qi, T. Zhang, 2016. Evaluation of candidate reference genes for RT-qPCR studies in three metabolism related tissues of mice after caloric restriction, Scientific Reports 6, 38513. https://DOI: 10.1038/srep38513.

51. S. Ekino, M. Susa, T. Ninomiya, K. Imamura, T. Kitamura, Minamata disease revisited: an update on the acute and chronic manifestations of methyl mercury poisoning. J Neurol Sci. 262 (2007) 131–44.

52. W.G. Balogun, A.E. Cobham, A. Amin, A. Seeni, Using invertebrate model organisms for neuroscience research and training an opportunity for Africa, Metabolic Brain Disease 33 (2018) 1431–1441.

53. M.R.C. Schetinger, T.V. Peres, L.P. Arantes, F. Carvalho, V. Dressler G. Heidrich, A.B. Bowman, M. Aschner, Combined exposure to methylmercury and manganese during L1 larval stage causes motor dysfunction, cholinergic and monoaminergic up-regulation and oxidative stress in L4 Caenorhabditis elegans, Toxicology 411 (2019) 154–162.

54. T. Syversen, P.J. Kaur, The toxicology of mercury and its compounds, J Trace Elem Med Biol. 26 (2012) 215-26.

55. L.F. Jeferson, A. Teixeira, F.C. Meotti, C.M. Ribas, J. Stringari, S.C.G. Pomblum, A.M. Moro, D. Bohrer, A.V, Bairros, A.L. Dafre, A.R.S. Santos, M. Farina, Cerebellar thiol status and motor deficit after lactational exposure to methylmercury, Environmental Research 102 (2006) 22-28.

56. L.N James. Sr., Insect physiology and Biochemistry, third ed., CRC Press, Taylor & Francis group, 2016.

57. A.C. Niehoff, O.B. Bauer, S. Kroger, S. Fingerhut, Jacqueline Schulz, S. Meyer, M. Sperling, A. Jeibmann, T. Schwerdtle, U. Karst, Quantitative Bioimaging to Investigate the Uptake of Mercury Species in Drosophila Melanogaster, Anal. Chem. 2015 (87) 10392-10396.

58. M. Farina, M. Aschner, J.B. Rocha, Oxidative stress in MeHg-induced neurotoxicity, Toxicol Appl Pharmacol. 256 (2011) 405-417.

59. M.V. Paul, M. Abhilash, M.V. Varghese, M. Alex, R.H. Nair, Protective effects of alpha-tocopherol against oxidative stress related to nephrotoxicity by monosodium glutamate in rats, Toxicol Mech Methods 22 (2012) 625–30.

60. B.D. Shivasharan, P. Nagakannan, B.S. Thippeswamy, V.P. Veerapur, Protective Effect of Calendula officinalis L. Flowers Against Monosodium Glutamate Induced Oxidative Stress and Excitotoxic Brain Damage in Rats, Indian J Clin Biochem. 28 (2013) 292–8.

61. R. Quintana-Cabrera, S. Fernandez-Fernandez, V. Bobo-Jimenez, J. Escobar, J. Sastre, A. Almeida, J.P. Bolaños, γ-Glutamylcysteine detoxifies reactive oxygen species by acting as glutathione peroxidase-1 cofactor, Nat. Commun. 3 (2012) 718.

62. S.M. Fang, Insect glutathione S-transferase: a review of comparative genomic studies and response to xenobiotics, Bull. Insectol. 65 (2012) 265–271.

63. K.W. Lee, S. Raisuddin, J.S. Rhee, D.S. Hwang, I.T. Yu, Y.M. Lee, H.G. Park, J.S. Lee, Expression of glutathione S-transferase (GST) genes in the marine copepod, *Tigriopus japonicas* exposed to trace metals, Aquatic Toxicology 89 (2008) 158-166.

64. B.L. Tan, M.E. Norhaizan, W.P. Liew, H.S. Rahman, Antioxidant and oxidative stress: A mutual interplay in Age-related diseases, Front. Pharmacol 9 (2018) 21-28. https:// doi: 10.3389/fphar.2018.01162.

65. Y. Zou, X.M. Wei, H.W. Weng, H.Y. Li, J.S. Liu, W.D. Yang, Expression profile of eight glutathione S-transferase genes in Crassostrea ariakensis after exposure to DSP toxins producing dinoflagellate Prorocentrum lima, Toxicon 105 (2015) 45- 55.

66. H.M. Li, G. Buczkowski, O. Mittapalli, J. Xie, J. Wu, R. Westerman, B.J. Schemerhorn, L.L. Murdock, B.R. Pittendrigh, Transcriptomic profiles of Drosophila melanogaster third instar larval midgut and responses to oxidative stress, Insect Mol. Biol. 17 (2008) 325–339.

67. B. Halliwell, J.M.C. Gutteridge, Free Radicals in Biology and Medicine, fourth ed., Oxford University Press, New York 1999.

68. G. Ginsberg, S. Smolenski, D. Hattis, K.Z. Guyton, D.O. Johns, B. Sonawane, Genetic Polymorphism in Glutathione Transferases (GST); Population distribution of GST M1, T1 and P1 Conjugating Activity. Journal of Toxicology and Environmental Health Part B, 12 (2009) 389-439.

69. N. Sookrung, O. Reamtong, R. Poolphol, N. Indrawattana, W. Seesuay, N. Saelim, P. Tantilipikorn, C. Bunnag, W. Chaicumpa, A. Tungtrongchitr 2018. Glutathione-S-transferase (GST) of American cockroach, *Periplaneta Americana:* Classes, Isoforms and Allergenicity 8:484 |DOI:10.1038/s41598-017-18759-z.

70. M. Koharyova, M. Kollarova, Oxidative stress and thioredoxin system, Gen. Physiol. Biophys. 27 (2008) 71–84.

71. H. Masutani, J. Bai, Y.C. Kim, J. Yodoi, Thioredoxin as a neurothrophic cofactor and an important regulator of neuroprotection, Mol. Neurobiol. 29 (2004) 229–242.

72. E. Rybnikova, A.E. Damdimopulos, J.A. Gustaffson, G. Spyrou, M. Pelto-Huikko, Expression of novel antioxidant thioredoxin 2 in the rat brain, European Journal of Neuroscience 12 (2000) 1669-1678.

73. E. Holzerova, K. Danhauser, T.B. Haack, L.S. Kremer, M. Melcher, I. Ingold, S. Kobayashi, C. Terrile, P. Wolf, J. Schaper, J. Mayatepek, F. Baertling, A.J.I. Friedmann, M. Conrad, T.M. Stroj, T. Meitinger, H. Prokisch, M. Distelmaier, Human thioredoxin 2 deficiency impairs mitochondrial redox homeostasis and causes early-onset neurodegeneration, Brain 139 (2016) 346–354.

74. B. Uttara, A.V. Singh, P. Zamboni, R.T. Mahajan, Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options, Current Neuropharmacology 7 (2009) 65-74.

75. J.A. Ruszkiewicz, G.T. Macedo, A.M. Vizuete, A.B. Bowman., J. Bornhorst, T. Schwerdtle, F.A.A Soares, M. Aschner, Sex-Specific Response of Caenorhabditis elegans to Methylmercury Toxicity, Neurotoxicity Research 35 (2019) 208–216.

76. C.M.L. Carvalho, E. Chew, S.I. Hashemy, J. Lu, A. Holmgren, Inhibition of human thioredoxin system: a molecular mechanism of mercury toxicity, J. Biol. Chem. 283 (2008) 11913–11923.

77. V. Branco, J. Canário, J. Lu, A. Holmgren, C. Carvalho, Mercury and selenium interaction in vivo: effects on thioredoxin reductase and glutathione peroxidase, Free Radic. Biol. Med. 52 (2012) 781–793.

78. J.P.K. Rooney, The role of thiols, dithiols, nutritional factors and interacting ligands in the toxicology of mercury, Toxicology 234 (2007) 145–156.

79. E.A. Veal, W.M. Toone, N. Jones, B.A. Morgan, Distinct roles for glutathione S-transferases in the oxidative stress response in *Schizosaccharomyces pombe*, J Biol Chem. 277 (2002) 35523-31.

80. H.Y. Yoo, M.S. Chang, H.M. Rho, Heavy metal-mediated activation of the rat Cu/Zn superoxide dismutase gene via a metal-responsive element, Mol. Gen. Genet. 262 (1999): 310–313.

81. Y. Soini, R. Kaarteenaho-Wiik, P. Paakko, V. Kinnula, Expression of antioxidant enzymes in bronchial metaplastic and dysplastic epithelium, Lung Cancer 39 (2003) 15–22.

82. G. Shanker, L.A. Mutkus, S.J. Walker, M. Aschner, Methylmercury enhances arachidonic acid release and cytosolic phospholipase A2 expression in primary cultures of neonatal astrocytes, Brain Res Mol Brain Res. *106* (2002) 1-11.

83. E.O. Farombi, O.O. Onyema, Monosodium Glutamate Induced Oxidative Damage and Genotoxicity in the Rat: Modulatory Role of Vitamin C, Vitamin E and Quercetin, Human Experimental Toxicology 25 (2006) 251-259.

84. L. Chen, F. Mao, G.C. Kirumba, C. Jiang, M. Manefield, Y. He, Changes in metabolites, antioxidant system and gene expression in *Microcystis aeruginosa* under sodium chloride stress, Ecotoxicology and Environmental safety 122 (2015) 126-135.

85. A.O. Abolaji, J.P. Kamdem, T.H. Lugokenski, E.O. Farombi, D.O. Souza, E.L.S. Loreto, J.B.T. Rocha, Ovotoxicants 4-vinylcyclohexene 1,2-monoepoxide and 4-vinylcyclohexene diepoxide disrupt redox status

and modify different electrophile sensitive target enzymes and genes in *Drosophila melanogaster*, Redox Biology 5 (2015) 328–339.

86. D.Y. Jin, H.Z. Chae, S.G. Rhee, K.T. Jeang, Regulatory role for a novel human thioredoxin peroxidase in NF-kB activation, J Biol Chem 272 (1997) 30952-30961.
87. D.H. Lackner, S. Schmidt, D.A Wu, J. Wolf, Bahler, 2012. Regulation of transcriptome, translation and proteome in response to environmental stress in fission yeast, Genome Biol 13, 4. https//doi: 10.1186/gb-2012-13-4-r25.
88. M.V. Lee, S.E. Topper, S.L. Hubler, J. Hose, C.D. Wenger, J.J. Coon, A.P. Gasch, A dynamic model of proteome changes reveals new roles for transcript alteration in yeast, Mol Syst Biol. 7 514 (2011) 1-12.

Target Gene	**Primer Sequence (5'>3')**	**Annealing Temperature**
Gapdh	F- CCGTGTCCCTGTTCCTAATG R-GTCCAAGATGCCCTTCAGAG	62
EF1a1	F- CGTGTCTGTTAAGGAACTGC R-CAAGCAATGTGAGCTGTATG	61
Tbp	F- GGTGCGAATGTGGAGTACAG R-TAGTGGCTCCAGTGCAAGTC	64
Tubulin	F- TTGCCAGTGATGAGTTGCTC R-AGCATGTACCAAGGGCAGTT	63
GstT	F- AAGAAATGGGTTGCAGGAGA R-CATTGGGATGCTTGCTTACA	61
GstS	F- GGGACCTCTGAATGACGAAA R-CATGCCGTCCAAATAATCAA	61
GstD	F- AATCGGCGTTGATCTGAATC R- CGGCAAATAGCTCGACTTTC	61
Sod	F- GTATTCTGGTGGCTGCGAAA R- TAAACCCAACACAGAGCCTTG	63
Prx4	F- GCTGTTCTATCCGCTGGACT R-CACTGAGCAAGCAACGACTT	63
Cat	F- ACGAGATCCAGCACTGACC R- CTCCACGGTTATCCACAGGT	63
Trx1	F- AGTATCCACGCGCCGTATT R- TGGGGTCTGCTCCTTGTATC	63
Trx2	F- TTATCAGCATTGGCACCAGA R- GCTGAATATCCGGCTGTCAT	61
TRX5	F- TCCTACCTGGGATGCTCTTG R- CCGAGCCTAGCATTTCACTT	62

Table 1: The primer features of the selected genes. F-Forward Primer; R- Reverse Primer

Table 2: A summary of the significant main effect of MeHg for all the genes analysed and Hg quantification.

	Without MeHg	**With MeHg**	**Main Effect F value**
Genes	**Basal + Basal**	**MeHg + Basal**	
GstD	1.16±0.98	1.82±1.57	F (1,63) = 4.09: P=0.04
GstT	2.30±1.5	3.20±2.15	F (1,60) = 3.85: P=0.05
Trx1	0.94±0.71	1.41±1.16	F (1,66) = 3.70: P=0.05
Cat	1.14±0.54	1.77±1.27	F (1,66) = 6.71: P= 0.01
Hg (ng/g)	0.07±0.012	0.17±0.008	F (1,24)= 651.71: P<0.000

Table 3: A summary of the significant main effect of Diet for all the genes analysed and Hg quantification.

	Diet			**Main Effect F value**
Genes	**Basal**	**NaCl**	**MSG**	
Trx1	1.46±1.1	1.36±1.18	0.71±0.53	F (2,66) = 3.87: P= 0.03
Cat	1.12±0.55	2.10±1.11	1.15±1.08	F (2,66) = 7.15: P= 0.002
Hg (ng/g)	0.121±0.009	0.124±0.010	0.107±0.011	F(2,24) = 8.09: P= 0.002

1

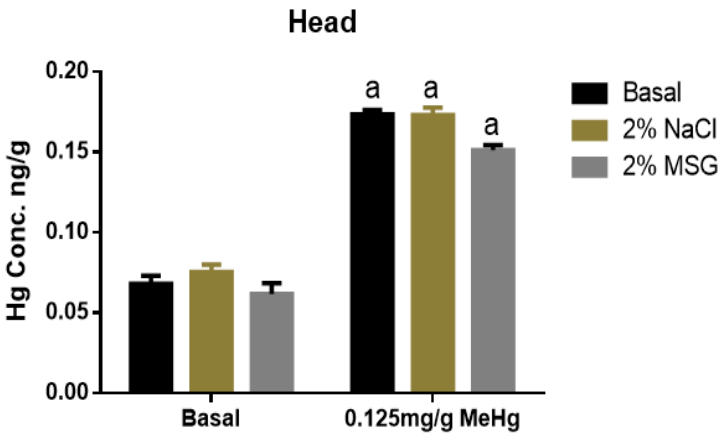

Fig 1: The concentration of mercury (Hg) in the heads of cockroaches co-exposed to diet containing MeHg and MSG for 21 days. There was a significant main effect of MeHg and diet for the mercury levels in the cockroaches. **MeHg** = F (1,24)= 651.71: P<0.000; **Diet**= F(2,24) = 8.0884: P= 0.00206; **MeHg*Diet** =F(2,24)= 1.4593, P =0.25229.

[a]P<0.05 vs **Basal**

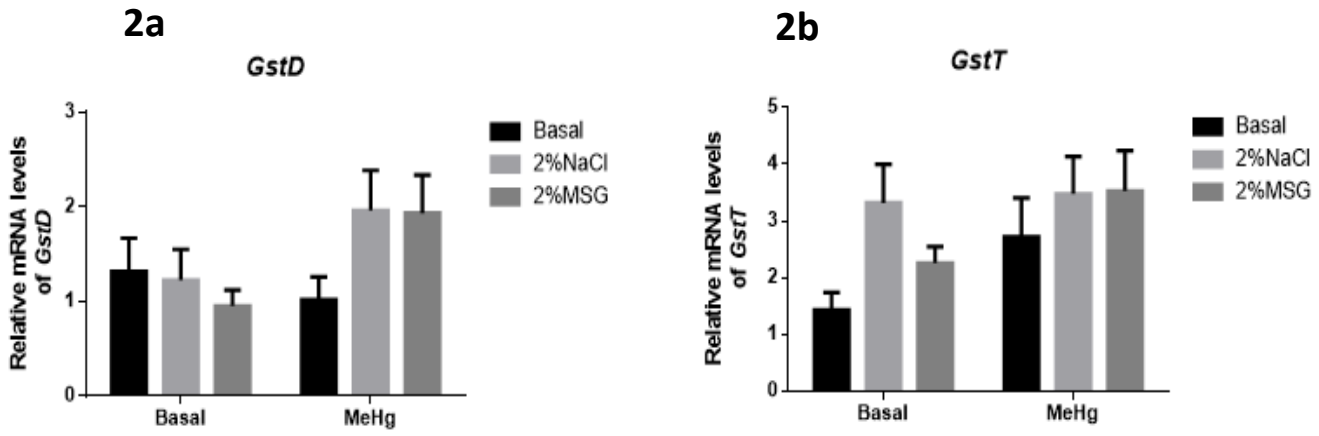

Figure 2a: The relative mRNA expression levels of *GstD* in *N. cinerea* after exposure for 21 days. There was a main effect of MeHg. **MeHg** = F (1,63) = 4.09: P=0.04; **Diet**= F (2,63) = 0.10: P= 0.91; **MeHg*Diet**=F (2,63) = 0.45: P=0.64.

Figure 2b: The relative mRNA expression levels of *GstT* in *N. cinerea* after exposure for 21 days. There was a main effect MeHg. **MeHg** = F (1,60) = 3.85: P=0.05; **Diet**= F (2,60) = 2.73: P= 0.07; **MeHg*Diet** = F (2,60) = 0.67: P=0.52.

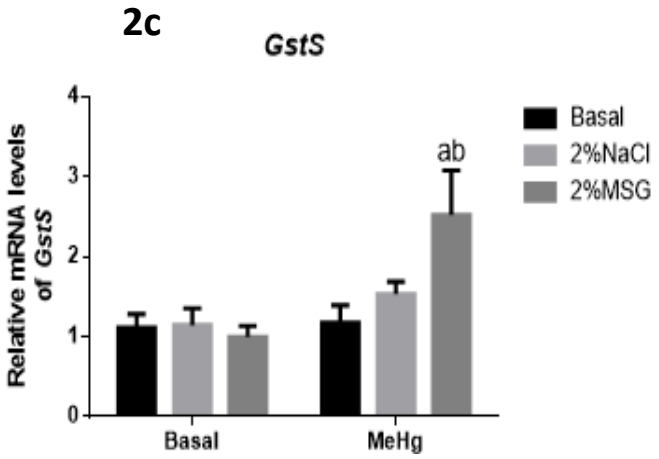

Figure 2c: The relative mRNA expression levels of *GstS* in *N. cinerea* after exposure for 21 days. There was an interaction between MeHg and diet, as MeHg and MSG increased expression levels of *GstS*. **MeHg** =F (1,64) = 7.99: P=0.006; **Diet**= F (2,64) = 2.40: P= 0.09; **MeHg*Diet**=F (2,64) = 3.65: P=0.03.

[a]P<0.05 vs **Basal**; [b]P<0.05 vs **MeHg + Basal.**

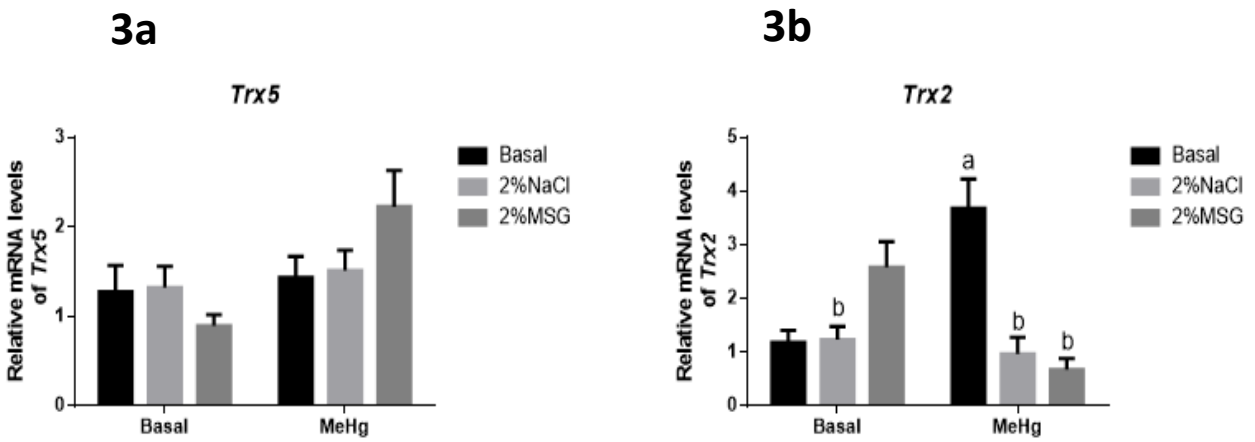

Figure 3a: The relative mRNA expression levels of *Trx5* in *N. cinerea* after exposure for 21 days. There was an interaction between MeHg and diet. **MeHg** = F (1,66) = 6.57: P=0.01; **Diet** = F (2,66) = 0.31: P= 0.73; **MeHg*Diet**=F (2,66) = 3.10: P=0.05.

Figure 3b: The relative mRNA expression levels of *Trx2* in *N. cinerea* after exposure for 21 days. There was an interaction between MeHg and diet, as MeHg + NaCl and MeHg + MSG decreased expression levels of *Trx2* in relation to MeHg alone. **MeHg** = F (1,65) = 0.15: P=0.70; **Diet** = F (2,65) = 7.08: P= 0.002; **MeHg*Diet** = F (2,65) = 19.68: P<0.0000

[a]P<0.05 vs **Basal**; [b]P<0.05 vs **MeHg + Basal**.

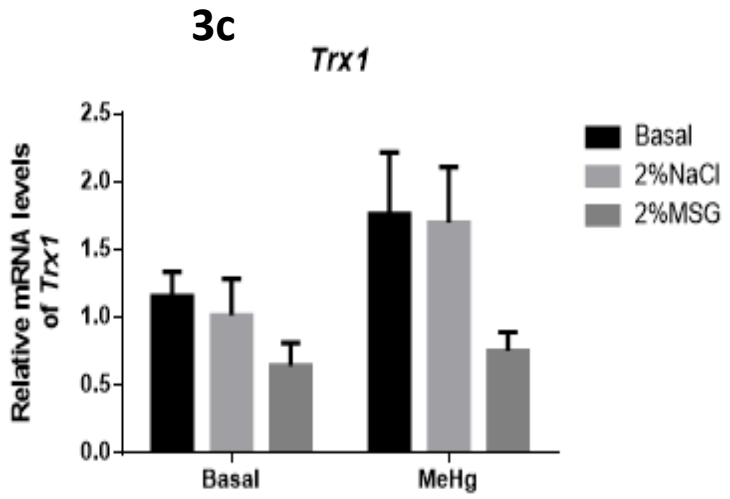

Figure 3c: The relative mRNA expression levels of *Trx1* in *N. cinerea* after exposure for 21 days. There was a main effect of MeHg and diet. **MeHg** = F (1,66) = 3.70: P=0.05883; **Diet**= F (2,66) = 3.87: P= 0.03; **MeHg*Diet**=F (2,66) = 0.54: P=0.58.

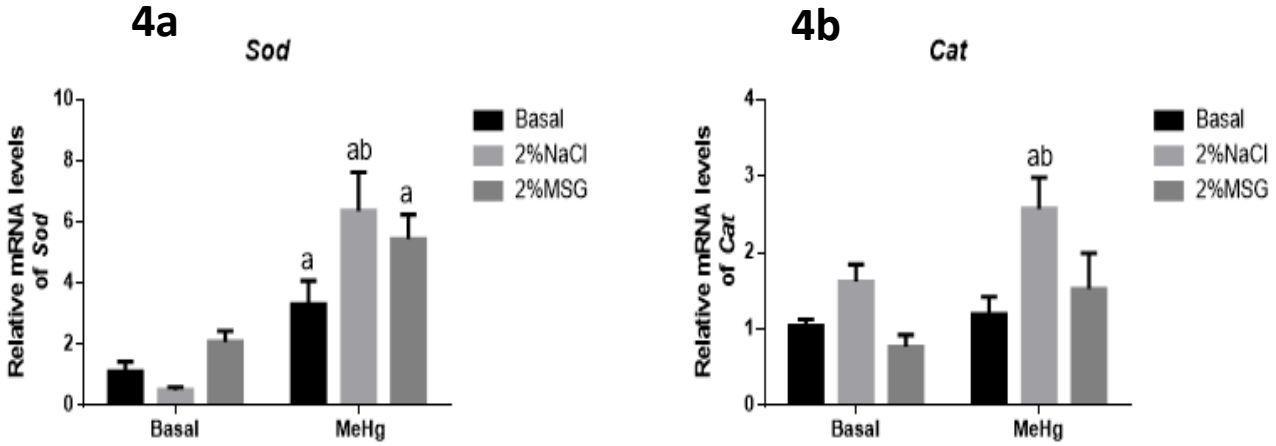

Figure 4a: The relative mRNA expression levels of *Sod* in *N. cinerea* after exposure for 21 days. There was an interaction between MeHg and diet, as MeHg + NaCl and MeHg + MSG increased expression levels of *Sod*. **MeHg** = F (1,66) = 36.06: P<0.00000; **Diet**= F (2,66) = 2.00: P= 0.14; **MeHg*Diet**=F (2,66) = 3.37: P=0.04.

Figure 4b: The relative mRNA expression levels of *Cat* in *N. cinerea* after exposure for 21 days. There was a main effect of MeHg and diet. **MeHg** = F (1,66) = 6.71: P= 0.01; **Diet**= F (2,66) = 7.14: P= 0.002; **MeHg*Diet** = F (2,66) = 0.99: P=0.38.

[a]P<0.05 vs **Basal**; [b]P<0.05 vs **MeHg + Basal**.

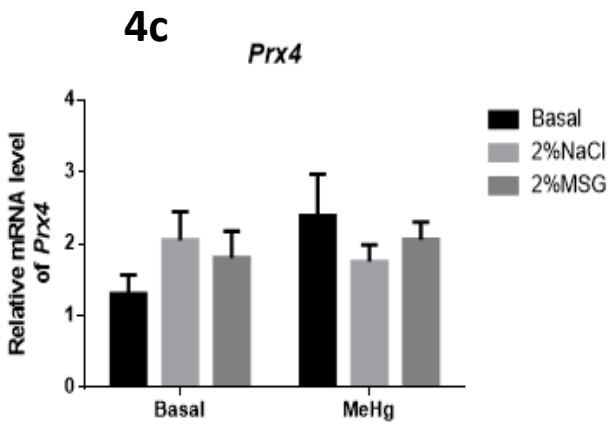

Figure 4c: The relative mRNA expression levels of *Prx4* in *N. cinerea* after exposure for 21 days. **MeHg** = F (1,66) = 1.34: P=0.25; **Diet**= F (2,66) = 0.02: P= 0.98; **MeHg*Diet** = F (2,66) = 1.80: P=0.17.

P<0.05 vs Basal; [b]P<0.05 vs MeHg + Basal.

PART III

This part contains the Discussion, Conclusion, Perspectives and References.

Discussion

Insects are suitable alternative models to provide insight into toxicology, pharmacology, and molecular biology research. *Nauphoeta cinerea* (lobster cockroach) is a promising insect being utilized to investigate the toxicity of environmental toxicants such as MeHg and VCH (Afolabi *et al.*, 2018; Adedara *et al.*, 2016; Waczuk *et al.*, 2019). Recently, studies have shown the additive, synergistic, antagonistic and/or potentiation effects of combined exposure of metals or compounds which could reflect real-life experiences (Gombeau *et al.*, 2019; Steevens and Benson, 1999), hence increasing the concerns about the potential toxicity of chemical mixtures. MeHg is a known neurotoxicant that crosses the blood-brain barrier and has deleterious effects on the developing brain (Paletz *et al.*, 2006). MSG also poses toxic effects on the central nervous system (Husarova and Ostatnikov, 2013).

This study showed a probable additive interaction between MeHg and MSG and an alteration in the redox homeostasis of the cockroach *Nauphoeta cinerea*. This correlates to the alteration observed in the behavior of the cockroaches (immobility), acetylcholinesterase activity, and total thiol levels. Consequently, MeHg + MSG caused an increase in the mRNA levels of *GstS, GstT, GstD, Trx2* and *Trx1* but a decrease in the mRNA levels of *Trx5*.

Due to the susceptibility of the brain to oxidative stress, the heads of the nymphs of cockroach were used to evaluate the effects of 4% MSG and 1% MSG on oxidative stress indices for 30 days. These concentrations were chosen based on the most consumed concentration derived from a preliminary food preference study using 1, 2, 3, 4% of MSG and NaCl respectively. The cockroaches preferred 4% MSG and 3% NaCl (data not shown). The results obtained from this study showed that 4% MSG caused an increase in the levels of RONS and a

decrease in AChE, GST, CAT in the cockroaches (data not shown), hence 4% MSG was used in the next study.

Cockroach nymphs were then co-exposed to diets containing 4% MSG and 0.125 mg of MeHg /g diet (Basal; 4% NaCl; 4% MSG; 0.125 mg/g MeHg; 0.125 mg/g MeHg + 4%NaCl; 0.125 mg/g MeHg + 4% MSG) for 30 days and some oxidative stress parameters, food and water consumption were evaluated (data not shown), but it was observed that the MeHg treated groups showed a significant reduction in food consumption (aversion), probably because of the taste, eventually 2% MSG was used for subsequent studies to avoid false-positive results.

In the next study, cockroaches were co-exposed to diets containing 2% MSG and 0.125 mg of MeHg /g diet (Basal; 2% NaCl; 2% MSG; 0.125 mg/g MeHg; 0.125 mg/g MeHg + 2%NaCl; 0.125 mg/g MeHg + 2% MSG) for 21 days, oxidative stress and behavioural analysis were evaluated. Results from the behavioural analysis showed cockroaches co-exposed to MeHg treated groups (MeHg + NaCl; MeHg + MSG) revealed impairments in motor activity as depicted by increased turn angle and immobility as well as reduced distance travelled (Afolabi *et al.,* 2018). Turn angle indicates motor coordination in animals (Spink et *al.,* 2002), it can be deduced as a whirling movement in cockroaches according to our results. Distance travelled and immobility could be used as biomarkers for motor coordination which can be pointers to neurotoxicity (Cory-Slechta., 2001). These behavioral parameters could be directly or indirectly related to alterations in the cholinergic and or monoaminergic system, therefore they could be responsible for the decrease in AChE activity observed in the MeHg treated groups (MeHg; MeHg + NaCl; MeHg + MSG) of nymphs since MeHg can bind to the thiol groups of cholinesterases (Piccoli *et al.,* 2020).

In the same vein, nymphs in the MeHg treated groups (MeHg; MeHg + NaCl; MeHg + MSG) showed reduced total thiol levels (Afolabi *et al.,* 2018). This could be due to the formation of an excretable RS-$HgCH_3$ complex (interaction of Hg atoms with –SH groups of GSH). This interaction has been proposed to decrease the levels of GSH which could be attributed to oxidative stress, as the complex formed can lead to the generation of reactive oxygen species and cause oxidative damage to biomolecules (Farina *et al.,* 2010). Our results showed no significant alteration in GST activity probably due to the duration of the exposure. This can be explained by the hypothesis that MeHg (contingent on the concentration) can cause oxidative stress without altering some antioxidant levels. MeHg alone caused an increase in TBARS level, though MeHg + NaCl and MeHg + MSG increased TBARS levels more than the Basal alone but decreased TBARS levels than MeHg alone, the reason for this is unknown and requires further investigation. Lipid peroxidation involves the reaction of oxygen-derived free radicals with polyunsaturated fatty acids.

The expression levels of genes responsible for detoxification of xenobiotics and certain antioxidant genes (glutathione-S-transferase, superoxide dismutase, catalase, thioredoxin, peroxiredoxin) - utilizing the head and fat-body transcriptome of *N. cinerea* sequenced in our laboratory were evaluated. Also, quantification of Hg content was evaluated using the heads of the nymphs co-exposed to 2% MSG and 0.125 mg of MeHg /g diet (Basal; 2% NaCl; 2% MSG; 0.125 mg/g MeHg; 0.125 mg/g MeHg + 2%NaCl; 0.125 mg/g MeHg + 2% MSG). MeHg treated groups (MeHg; MeHg + NaCl; MeHg + MSG) presented an increase in Hg levels confirming the accumulation of Hg content in the heads of the nymphs (Manuscript 2).

MeHg treated groups (MeHg + NaCl; MeHg + MSG) showed an upregulation in the expression of *GstT* and *GstD*. MeHg + MSG upregulated the expression of *GstS* and increased

immobility (published article) corroborating studies that showed alteration in glutathione homeostasis correlates with impaired motor function (Tan *et al.,* 2018). GSTs are known to be involved in detoxification of xenobiotics and adaptation to oxidative stress in insects in response to environmental toxicants (Houxia *et al.,* 2012). The increase in the expression of the GSTs suggests that MeHg binds to GSH forming an excretable complex by the bile in rodents, however, it is unknown if this mechanism exists in insects. Nevertheless, our results showed no correlation between the enzymatic activity of GST and its expression levels, propounding that the level of mRNA expression might not directly explain the biological activity of the corresponding protein (Abolaji *et al,* 2015).

Our study showed that MeHg + MSG caused a significant increase in mRNA levels of *Trx*5 but caused a down-regulation of *Trx2* and *Trx1* (manuscript 2), this could be because *Trx2* plays roles as the main thioredoxin in the brain (Rybnikova et al., 2000). Also, *Trx2* and *Trx1* are sensitive to oxidation reactions by mercury (Holmgren *et al.,* 2012). Furthermore, the upregulation of *Trx*5 could be a form of compensatory mechanism to preserve thioredoxin homeostasis. Thioredoxins play essential roles in redox homeostasis and confer protection against oxidative stress (Schetinger *et al.,* 2019).

MeHg treated groups (MeHg + NaCl; MeHg + MSG) showed an upregulation of *Sod* while MeHg + NaCl caused an upregulation of *Cat* (manuscript 2). This suggests that SOD and CAT play roles against oxidative stress induced by heavy metals (Yoo *et al.,* 1999). Although, studies have shown that NaCl upregulates the expression of *Cat* (Chen *et al.,* 2015) however, the effect of MeHg + NaCl in the present study is unknown and requires further investigation. The present study showed no significant difference with respect to mRNA levels of *Prx4*, this could be due to the low expression of *Prx4* in the brain than the other tissues.

Based on these considerations, it is worthy to note that in rodents, the brain glutamate does not cross the blood-brain barrier (BBB) in appreciable amounts compared to their brain concentrations but the BBB can be modified by inflammation (Brusilow and Pointe 2005). Furthermore, studies have shown that MeHg can cause inflammation of cells (Farina *et al.*, 2010), we then propose that the inflammation could have been caused by MeHg rendering the BBB receptive to glutamate hence causing damage to the brain. We suggest that this mechanism is also possible in insects since they have hemolymph-brain (CNS) barrier which has similar functions to the BBB in mammals. The hemolymph-brain (CNS) barrier is made up of neurolemma (outer layer) and perineurium (inner layer) that protect the brain, ventral connectives, ventral ganglia and large nerves from direct contact with the hemolymph (Scharrer, 1939; Strausfield 1976; Treherne 1985).

The present study contributed towards a better understanding of the interaction between MeHg and glutamate, their effects on antioxidant genes and oxidative stress indices in an alternative organism *N. cinerea*. This corroborates other studies that support the use of *N. cinerea* as a valid organism for basic toxicological and molecular studies. Moreover, *N. cinerea* can also be used as a biomarker for environmental contaminants such as insecticides, heavy or trace metals that could help minimize public risk to such pollutants. However, further studies are required to understand the mechanism of interaction between MSG and MeHg as well as create a better understanding of the interaction between NaCl and MeHg as it seemed to alter some biochemical parameters as well.

Conclusion

Based on the results obtained from the present study, evaluating the effect of the interaction of both MeHg and MSG in the nymphs of *N. cinerea*, we infer the following:

Chapter 1

- MeHg + MSG altered locomotor activity (time immobile) suggesting an alteration in the cholinergic and or monoaminergic system
- MeHg + MSG decreased acetylcholinesterase activity suggesting an alteration in the cholinergic synaptic transmission.
- MeHg; MeHg + NaCl; MeHg + MSG reduced total thiol levels indicating an adaptive response to oxidative stress suggesting a probable overwhelming of the antioxidant system containing thiol proteins.
- MeHg; MeHg + NaCl; MeHg + MSG increased TBARS levels indicating disruption of redox homeostasis.

Chapter 2

- MeHg; MeHg + NaCl; MeHg + MSG caused an increase in Hg content in the nymphs' head indicating accumulation of mercury.
- MeHg + MSG caused an upregulation of *GstS, GstT, GstD* and *Trx5* suggesting these genes play essential roles in protection against oxidative stress.
- MeHg + NaCl caused an upregulation of *GstT, GstD, Trx1, Sod, Cat* suggesting these genes play essential roles in protection against oxidative stress.
- Altogether, it can be concluded that MeHg and MSG showed more additive interaction than synergistic or antagonistic interaction.

Perspectives

Based on the results obtained from the present study, the following can be further investigated:

- Glutamate quantification in the heads and fat-bodies of *N. cinerea*.
- The type(s) of glutamate receptor encoding in the *N. cinerea* transcriptome.
- Standardize histology of the head and fat-body tissues of *N. cinerea*.
- The activity of ATPase utilized by *N. cinerea*.
- Evaluate the activity of enzymes such Peroxiredoxin, Cytochrome p450 in *N. cinerea*.
- The mechanisms of interaction between MeHg and NaCl in *N. cinerea.*

REFERENCES

Adedara, I.A., Rosemberg, D.B., Souza, D.O., Farombi, E.O., Aschner, M., Rocha, J.B. Neuroprotection of luteolin against methylmercury-induced toxicity in lobster cockroach *Nauphoeta cinerea*. **Environ Toxicol Pharmacol.** 42:243-51, 2016.

Afolabi, B.A., Adedara, I.A., Souza, D.O., Rocha J.B.T. Dietary co-exposure to methylmercury and monosodium glutamate disrupts cellular and behavioral responses in the lobster cockroach, *Nauphoeta cinerea* model. **Environmental toxicology and pharmacology**, 64, 70-77, 2018.

Agarwal, M., Giannoni Guzman, M., Morales-Matos, C., Del Valle Diaz, R.A., Abramson, C.I., Giray, T. Dopamine and octopamine influence avoidance learning of honey bees in a place preference assay. **PLoS One.** 6: e25371, 2011.

Arnér, E.S.J., Holmgren, A. Physiological functions of thioredoxin and thioredoxin reductase. **Eur. J. Biochem**. 267:6102–6109, 2000.

Atchison, W.D. Is chemical neurotransmission altered specifically during methylmercury-induced cerebellar dysfunction? **Trends Pharmacol Sci**. 26(11):549-57, 2005.

Ballinger, E.C., Ananth, M., Talmage, D.A., Role, L.W. Basal forebrain cholinergic circuits and signaling in cognition and cognitive decline. **Neuron** 91 (6), 1199–1218, 2016.

Beadle, D.J., Horseman, G., Pichon, Y., Amar, M., Shimahara, T. Acetylcholine-activated ion channels in embryonic cockroach neurones growing in culture. **J. exp. Biol**. 142: 337.-355, 1989.

Bell, W.J., Roth, L.M., Nalepa, C.A. Cockroaches: Ecology, Behavior, And Natural History. **The Johns Hopkins University Press** 2715 North Charles Street Baltimore, Maryland 21218-4363, 2007.

Bi, Z., Yang, X., Yu, W., Shu, J., Zhang, Y. Diapause-associated protein3 functions as Cu/Zn superoxide dismutase in the Chinese oak silkworm (Antheraea pernyi). **PLoS One** 9: e90435. 2014.

Birben, E., Sahiner, U.M., Sackesen, C., Erzurum, S., Kalayci, O. Oxidative Stress and Antioxidant Defense. **World Allergy Organization**: 9-19, 2012.

Bridges, R., Lutgen, V., Lobner, D., Baker, D.A. Thinking outside the cleft to understand synaptic activity: contribution of the cystine-glutamate antiporter (System xc-) to normal and pathological glutamatergic signaling. **Pharmacol Rev**. 64(3):780–802, 2012.

Britton, G., Goodwin, T.W. Carotenoids in insects. In (eds.), Carotenoid chemistry and biochemistry. Proceedings of the 6th International Symposium on Carotenoids. **Pergamon Press Ltd.,** Liverpool, United Kingdom, pp. 195–211, 1982.

Brusilow, W.S., Pointe, G. Decreasing Brain neuronal glutamate levels using alpha keto branched chain amino acids. **United states patent.** ROTHWELL, FIGG, ERNST & MANBECK. 1425 KStreet, NW, Suite 800, 2005.

Burdo, J., Dargusch, R., Schubert, D. Distribution of the cystine/glutamate antiporter system xc- in the brain, kidney, and duodenum. **J Histochem Cytochem** 54(5):549–57, 2006.

Brosnan, J.T., Drewnowski, A., Friedman, M.I. Is there a relationship between dietary MSG and obesity in animals or humans? **Amino Acids**. 46(9):2075–2078. 2014.

Bubak, A.N., Grace, J.L., Watt, M.J., Renner, K.J., Swallow, J.G. Neurochemistry as a bridge between morphology and behavior: perspectives on aggression in insects. **Curr Zool** 60:778–790, 2014.

Burton, G.J., Jauniaux, E. Oxidative stress. **Best Practice & Research Clinical Obstetrics and Gynaecology** 25: 287–299, 2011.

Canavoso, L.E., Jouni, Z.E., Karnas, K.J., Pennington, J.E., Wells, M.A. Fat metabolism in insects. **Annu. Rev. Nutr**. 21:23–46, 2001.

Cardoso, C.C., Lobato, K.R., Binfaré, R.W., Ferreira, P.K., Rosa, A.O., Santos, A.R.S., Rodrigues, A.L.S. Evidence for the involvement of the monoaminergic system in the antidepressant-like effect of magnesium. **Progress in Neuro-Psychopharmacology & Biological Psychiatry** 33:235–242, 2009.

Chan, T.Y. Inorganic mercury poisoning associated with skin lightening cosmetic products. **Clin Toxicol (Phila)** 49(10): 886-891, 2011.

Chandrasekaran, A., Idelchik, M.D.P.S., Melendez, J.A. Redox control of senescence and age-related disease. **Redox Biol.** 11:91–102, 2017.

Cheng, J., Wang, C.Y., Lyu, Z.H., Chen, J.X., Lin, T. Identification and characterization of the catalase gene involved in resistance to thermal stress in Heortia vitessoides using RNA interference. **Journal of Thermal Biology** 78:114–121, 2018.

Chunyan, Z., Dongyu, Y., Zhiqiang, L., Yijuan, X. Toxicity of flavor enhancers to the oriental fruit fly, Bactrocera dorsalis (Hendel) (Diptera: Tephritidae). **Ecotoxicology** 27:619–626, 2018.

Clarke, P.B., Hamill, G.S., Nadi, N.S., Jacobowitz, D.M., Pert, A. 3H-nicotine- and 125I-alpha-bungarotoxin-labeled nicotinic receptors in the interpeduncular nucleus of rats. II. Effects of habenular deafferentation. **J. Comp. Neurol.** 251, 407-413, 1986.

Corley, L.S., Blankenship, J.R., Moore, A.J., Moore, P.J. Genetic variation and asexual reproduction in the facultatively parthenogenetic cockroach Nauphoeta cinerea: implications for the evolution of sex. **J. EVOL. BIOL.** 1 4; 68-74**,** 2001.

Corona, M., Robinson, G.E. Genes of the antioxidant system of the honey bee: annotation and phylogeny. **Insect Mol. Biol**. 15:687–701, 2006.

Laher, I. (Ed). Systems Biology of Free radicals and Antioxidants. **Springer-Reference** 2014.

Dare, E., Fetissov, S., Hokfelt, T., Hall, H., Ogren, S.O., Ceccatelli, S. Effects of prenatal exposure to methylmercury on dopamine-mediated locomotor activity and dopamine D2 receptor binding. **Naunyn Schmiedebergs Arch. Pharmacol**. 367: 500–508, 2003.

David, J.A., Sattelle, D.B. Ionic basis of membrane potential and of acetylcholine-induced currents in the cell body of the cockroach fast coxal depressor motor neurone. **J. exp. Biol**. 151: 21-39, 1990.

Dhinaut, J., Balourdet, A., Teixeira, M., Chogne, M., Moret, Y. A dietary carotenoid reduces immunopathology and enhances longevity through an immune depressive effect in an insect model. **Sci. Rep**. 7: 12429, 2017.

Donly C. Jevnikar J. McLean H. Caveney S. Substrate-stereoselectivity of a high-affinity glutamate transporter cloned from the CNS of the cockroach Diploptera punctata**.** ***Insect Biochemistry and Molecular Biology*** *30: 369-376, 2000.*

Donly, B.C., Richman, A., Hawkins, E., McLean, H., Caveney, S. Molecular cloning and functional expression of an insect high-affinity Na^+ dependent glutamate transporter. **Eur. J. Biochem.** 248, 535-542, 1997.

Draper, I., Kurshan, P.T., McBride, E., Jackson, F.R., Kopin, A.S. Locomotor activity is regulated by D2-like receptors in Drosophila: an anatomic and functional analysis. **Dev Neurobiol**. 67:378–93, 2007.

Dupuis, J., Louis, T., Gauthier, M., Raymond, V. Insights from honeybee (Apis mellifera) and fly (Drosophila melanogaster) nicotinic acetylcholine receptors: from genes to behavioral functions. **Neurosci Biobehav Rev.** 36:1553–64, 2012.

El Hassani, A.K., Schuster, S., Dyck, Y., Demares, F., Leboulle, G., Armengaud, C. Identification, localization and function of glutamate gated chloride channel receptors in the honeybee brain. **Eur J Neurosci**. 36:2409–20, 2012.

El-Agamey, A., Lowe, G.M., McGarvey, D.J., Mortensen, A., Phillip, D.M., Truscott, T.G. Carotenoid radical chemistry and antioxidant/pro-oxidant properties. **Arch Biochem Biophys**. 430:37–48. 97, 2004.

Engler, D.E. Mercury "bleaching" creams. **J Am Acad Dermatol** 52(6):1113-1114, 2005.

Espitia-Pérez, P., Marin Albino, S., Tais da Rosa, H., Silveira, A.K., Espitia-Pérez, L., Brango, H., Moraes, D.P., Hermann, P.R.S., Mingori, M., Barreto, F., Kunzler, A., Gelain, D.P., Schnorr, E.S., Moreira, J.C.F. Effects of methylmercury and retinol palmitate co-administration in rats during pregnancy and breastfeeding: Metabolic and redox parameters in dams and their offspring. **Ecotoxicology and Environmental Safety** 162: 603–615, 2018.

Evans, P.D. The uptake of L-glutamate by the central nervous system of the cockroach, Periplaneta Americana. **J. exp. Biol**. 62, 55-67, 1957.

Eweka, A.O., Igbigbi, P.S., Ucheya, R.E. Histochemical studies of the effects of monosodium glutamate on the liver of adult Wistar rats. **Ann Med Health Sci Res**. 1(1):21–29, 2011.

Farina, M., Aschner, M., Rocha, J.B.T. Chapter Two- The Catecholaminergic Neurotransmitter System in Methylmercury-Induced Neurotoxicity. **Advances in Neurotoxicology**. 1:47-81, 2017.

Farina, M., Rocha, J.B.T. Aschner, M. Oxidative stress and methylmercury-induced neurotoxicity. **Indianapolis: John Wiley & Sons**. p. 357-385, 2010.

Farina, M., Rocha J.B.T., Aschner, M. Mechanisms of Methylmercury induced neurotoxicity: evidence from experimental studies. Life Sci. 89: 555–563, 2011.

Felton, G.W., Summers, C.B. Antioxidant systems in insects. **Arch. Insect Biochem**. 29:187–197, 1995.

Fenton, G.W., Macflynn, G., Montgomery, E.A., Rutherford W. Measurement of reaction time following minor head injury. **Journal of Neurology, Neurosurgery, and Psychiatry** 47: 1326-1331, 1984.

Fox, D. L. Animal biochromes and structural colours. **University of California Press,** Los Angeles, CA, 1976.

Underwood, D.L.A. Biology 316. **Insect anatomy and physiology**. General entomology. Spring 2009.

Frank, P.B., David, L.M., Christian, C.F., Jürgen, W. Use of M1–M5 Muscarinic Receptor Knockout Mice as Novel Tools to Delineate the Physiological Roles of the Muscarinic Cholinergic System. **Neurochemical Research**, 28: 437–442, 2003.

Gary, W.F., Clinton, B.S. Antioxidant Systems in insects. **Archives of Insect Biochemistry and Physiology** 29:187-I97, 1995.

Gazzola, G.C., Dall'Asta, V., Bussolati, O., Makowske, M., Christensen, H.N. A stereo-selective anomaly in dicarboxylic amino acid transport. **J. Biol.Chem**. 256, 6054-6059, 1981.

Gegelashvili, G., Schousboe, A. Cellular distribution and kinetic properties of high-affinity glutamate transporters. **Brain Res. Bull**. 45, 233-238, 1998.

Genestra, M. Oxyl radicals, redox-sensitive signalling cascades and antioxidants. **Cell Signal.** 19(9):1807–1819, 2007.

Giordana, B, Sacchi, V.F., Parenti, P., Hanozet, G.M., 1989. Amino acid transport systems in intestinal brush-border membranes from lepidopteran larvae. **Am. J. Physiol** 257: R494–R500, 1989.

Gombeau, K., de Oliveira, R.B., Sarrazin, S.L.F., Mourão, R.H.V., Bourdineaud, J.P. Protective Effects of *Plathymenia reticulata* and *Connarus favosus* Aqueous Extracts against Cadmium- and Mercury-Induced Toxicities. **Toxicol Res.** 35(1):25-35, 2019.

Greer, C.L., Grygoruk, A., Patton, D.E., Ley, B., Romero-Calderon, R., Chang, H.Y., Houshyar R., Bainton, R.J., Diantonio, A., Krantz, D.E. A splice variant of the Drosophila vesicular monoamine

transporter contains a conserved trafficking domain and functions in the storage of dopamine, serotonin, and octopamine. **J Neurobiol**.64:239–58, 2005.

Gromer, S., Urig, S., Becker, K. The thioredoxin system-from science to clinic. **Med Res Rev**. 24:40–89, 2004.

Haber, F., Weiss, J.J. The catalytic decomposition of hydrogen peroxide by iron salts**. Proc R Soc Lond Ser A**. 147:332–351. 27, 1934.

Habig, W.H., Pabst, M.J., Jakoby, W.B. Glutathione S-transferases the first enzymatic step in mercapturic acid formation. **J. Biol. Chem.** 249, 7130–7139, 1974.

Halliwell, B., and Gutteridge, J.M.C. **Free radicals in biology and medicine**. 3rd ed. Oxford: Oxford Science Publications, 1999.

Harada, M. Minamata Disease: Methylmercury Poisoning in Japan Caused by Environmental Pollution. **Critical Reviews in Toxicology**, 25 (1): 1-24, 1995.

Harris, W.E., Moore, P.J. Sperm competition and male ejaculate investment in *Nauphoeta cinerea*: effects of social environment during development. **J. Evol. Biol** 18, 474–480, 2005.

Hastings, F.L., Lucier, G.W., Klein, R. Methylmercury-cholinesterase interactions in rats. **Environ Health Perspect.** 12:127–130, 1975.

Hayes, J.D., Flanagan, J.U., Jowsey, I.R. Glutathione transferases. **Annu. Rev. Pharmacol. Toxicol**. 45:51–88, 2005.

He, K., Du, S., Xun, P., Sharma, S., Wang, H., Zhai, F., Popkin, B. Consumption of monosodium glutamate in relation to incidence of overweight in Chinese adults. China health and nutrition survey (CHNS). **Am J Clin Nutr.** 93:1328-36, 2011.

Heuer, C.M., Binzer, M., Schachtner, J. SIFamide in the brain of the sphinx moth, *Manduca sexta*. **Acta Biol. Hung** 63 (Suppl 2): 48-57, 2012.

Hildebrand, J.G. Analysis of chemical signals by nervous systems. ***Proc. Natl. Acad. Sci. USA*** 92: 67–74, 1995.

Hildebrand, J.G. 1996. Olfactory control of behavior in moths: Central processing of odor information and the functional significance of olfactory glomeruli. ***J. Comp. Physiol A*** 178: 5–19, 1996.

Homberg, U, Christensen, T.A., Hildebrand, J.G. Structure and function of the deutocerebrum in insects. ***Annu. Rev. Entomol*** 34: 477–501, 1989.

Houxia, S., Lianghong, P., Shasha, G., Shicheng, Z., Yanyun, W., Yi, Z., Bin, L. Glutathione S-transferase (GST) genes in the redflour beetle,Tribolium castaneum,and comparative analysis withfive additional insects. **Genomics** 100:327-335, 2012.

Huang, J., Hult, E.F., Marchal, E., Tobe, S.S. Identification and characterization of the NMDA receptor and its role in regulating reproduction in the cockroach *Diploptera punctata*. **The Journal of Experimental Biology** 218, 983-990, 2015.

Husarova, V., Ostatnikov, D. Monosodium Glutamate Toxic Effects and Their Implications for Human Intake: **A Review. JMED Research,** Article ID: 608765, 2013.

Ikeda, K. New seasonings. **Chem Senses**. 27:847–849, 2002.

James, L.N., Sr. Insect physiology and Biochemistry. 3rd edition. University of Florida, Gainsville. **CRC Press; Taylor & Francis group LLC**. ISBN 13: 978-1-4822-47602, 2016.

Jubaidi, F.F., Mathialagan, R.D., Noor, M.M., Taib, I.S., Budin, S.B. Monosodium glutamate daily oral supplementation: study of its effects on male reproductive system on rat model. **Syst Biol Reprod Med.** 65(3):194-204, 2019.

Kahsai, L., Martin, J.R., Winther, A.M.E. Neuropeptides in the Drosophila central complex in modulation of locomotor behavior**. J. Exp. Biol.** 213: 2256–2265, 2010.

Kahsai, L., Winther, A.M.E. Chemical neuroanatomy of the Drosophila central complex: Distribution of multiple neuropeptides in relation to neurotransmitters. **J Comp. Neurol.** 519: 290–315, 2011.

Kamhi, J.F., Traniello, J.F.A. Biogenic amines and collective organization in a superorganism: neuromodulation of social behavior in ants. **Bra Behav Evol** 82:220–236, 2013.

Katoh, K., Iwasaki, M., Hosono, S., Yoritsune, A., Ochiai, M., Mizunami, M., Nishino, H. Group-housed females promote production of asexual ootheca in American cockroaches. **Zoological Letters** 3:3, 2017.

Kim, B.Y., Hui, W.L., Lee, K.S., Wan. H., Yoon, H.J., Gui, Z.Z., Chen, S., Jin, B.R. Molecular cloning and oxidative stress response of a sigma class glutathione S-transferase of the bumblebee Bombus ignitus. **Comp Biochem Physiol B Biochem Mol Biol** 158 (1):83–89, 2011.

Kim, K., Müller, L.T.M., Bohnen, N.I., Sarter, M., Lustig, C. The cortical cholinergic system contributes to the top-down control of distraction: Evidence from patients with Parkinson's disease. **NeuroImage**. 190: 107–117, 2019.

Kirkman, H.N., Rolfo, M., Ferraris, A.M., Gaetani, G.F. Mechanisms of protection of catalase by NADPH. Kinetics and stoichiometry. **J Biol Chem**. 274:13908–13914, 1999.

Kunst, M., Pförtner, R., Aschenbrenner, K., Heinrich, R. Neurochemical architecture of the central complex related to its function in the control of grasshopper acoustic communication. **PLoS One** 6: c25613, 2011.

Kurylas, A.E., Ott, S.R. Schachtner, J. Elphick, M.R., Williams, L., Homberg, U. Localization of nitric oxide synthase in the central complex and surrounding midbrain neuropils of the locust Schistocerca gregaria. **J. Comp. Neurol**. 484: 206–223, 2005.

Ladizinski, B., Mistry, N., Kundu, R.V. Widespread use of toxic skin lightening compounds: medical and psychosocial aspects. **Dermatologic Clinics**, 29:111–123, 2011.

Laura, S.C., Jill, R., Allen, J.M., Patricia, J.M. Developmental constraints on the mode of reproduction in the facultatively parthenogenetic cockroach Nauphoeta cinerea. **EVOLUTION & DEVELOPMENT** 1:2, 90–99, 1999.

Lee, M.C., Ting, K.K., Adams, S., Brew, B.J., Chung, R., Guillemin, G.J. Characterisation of the expression of NMDA receptors in human astrocytes. **PLoS One** 5(11): e14123, 2010.

Lee, S., Kim, S.M., Lee, R.T. Thioredoxin and thioredoxin target proteins: from molecular mechanisms to functional significance. **Antioxid. Redox Signaling** 18:1165–1207; 2013.

Lemon, W.C., Getz, W.M. **Ann. Entomol. Soc**. Am., 92,861,1999.

Lesage, A., and Steckler, T. Metabotropic glutamate mGlu1 receptor stimulation and blockade: Therapeutic opportunities in psychiatric illness. **Eur J Pharmacol** 639(1–3): 2–16, 2010.

Li, H.B., Dai C.G., He, Y.F., Hu, Y. Characterization and Expression of Genes Encoding Superoxide Dismutase in the Oriental Armyworm, *Mythimna separata* (Lepidoptera: Noctuidae). **J Econ Entomol.** 112 (5): 2381-2388, 2019.

Lingtian, X., Jennifer, L.F., Nigel, D., David H.F., David, A.D., David, B.B. Mercury(II) Bioaccumulation and Antioxidant Physiology in Four Aquatic Insects. **Sci. Technol**. 43: 934–940, 2009.

Liochev, S.I., Fridovich, I. The Haber–Weiss cycled 70 years later: an alternative view. **Redox Rep.** 7:55–57, 2002.

Lobo, V., Patil, A., Phatak, A., Chandra, N. Free radicals, antioxidants and functional foods: impact on human health. **Pharmacogn Rev.** 4(8):118–126, 2010.

Lu, J., Holmgren, A. The thioredoxin antioxidant system. **Free Radical Biology and Medicine**, 66:75–87,2014.

Lucas, B.S., Navdeep, S.C. Mitochondrial reactive oxygen species and cancer. **Cancer & Metabolism** 2:17, 2014.

Malgorzata, S., Jan, L., Wieslawa, J., Grzegor, R. Evidences for an ATP-sensitive potassium channel (K_{ATP}) in muscle and fat body mitochondria of insect. **Journal of Insect Physiology** 59: 1125–1132, 2013.

Marella, S., Mann, K., Scott, K. Dopaminergic modulation of sucrose acceptance behavior in Drosophila. **Neuron.** 73:941–50, 2012.

Marion-Poll, F., Tobin, T.R. Temporal coding of pheromone pulses and trains in *Manduca sexta* J. Comp. Physiol. 171: 505-512.

Masella, R., Di Benedetto, R., Vari, R., Filesi, C., Giovannini, C. Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes. **J Nutr Biochem**. 16:577–586, 2005.

McCarthy, E.V., Wu, Y., Decarvalho, T., Brandt, C., Cao, G., Nitabach, M.N. Synchronized bilateral synaptic inputs to Drosophila melanogaster neuropeptidergic rest/arousal neurons. **J Neurosci.** 31:8181–93, 2011.

Mondal, M., Sarkar, K., Nath, P.P., Paul. G. Monosodium glutamate suppresses the female reproductive function by impairing the functions of ovary and uterus in rat. **Environmental toxicology**. 1–11, 2017.

Müller, U. The nitric oxide system in insects. **Prog. Neurobiol**. 51: 363–381, 1997.

Nässel, D.R. Neuropeptides in the insect brain: A review **Cell Tissue Res**. 273: 1–29, 1993.

Nässel, D.R. Histamine in the brain of insects: A review ***Microsc. Res. Tech.*** 44: 121–136. 1999.

Nässel, D.R. Neuropeptides in the nervous system of Drosophila and other insects: Multiple roles as neuromodulators and neurohormones. ***Prog. Neurobiol.*** 68: 1–84, 2002.

Nässel, D.R., Homberg, U. Neuropeptides in interneurons of the insect brain. **Cell Tissue Res**. 326: 1–24, 2006.

Nogara, P.A., Oliveira, C.S., Schmitz, G.L., Paulo, C.P., Farina, M., Aschner, M., Rocha J.B.T. Methylmercury's chemistry: From the environment to the mammalian brain. **BBA - General Subjects**. 0304-4165, 2019.

Oliveira, C.S., Nogara, P.A., Ardisson-Araújo, D.M.P., Aschner, M., Rocha J.B.T., Dórea, J.G. Neurodevelopmental Effects of Mercury. **Advances in Neurotoxicology**, Volume 2, 27-86, 2018.

Paletz, E.M., Craig-Schmidt, M.C., Newland, M.C. Gestational exposure to methylmercury and n-3 fatty acids: Effects on high- and low-rate operant behavior in adulthood. **Neurotoxicol. Teratol** 28:59–73, 2006.

Panek, I., Torkkeli, P.H. Inhibitory glutamate receptors in spider peripheral mechanosensory neurons. **Eur J Neurosci**. 22:636–46, 2005.

Park, J.P., Zheng, W. Human Exposure and Health Effects of Inorganic and Elemental Mercury. **J Prev Med Public Health**. 45:344-352, 2012.

Perkins, A., Nelson, K.J., Parsonage, D., Poole, L.B., Karplus, P.A. Peroxiredoxins: Guardians against oxidative stress and modulators of peroxide signaling. **Trends in Biochemical Sciences**, 40:435–445, 2015.

Perry, E., Walker, M., Grace, J., Perry, R. Acetylcholine in mind: a neurotransmitter correlates of consciousness? **Trends Neurosci**. 22 (6), 273–280, 1999.

Pfeiffer, K, Homberg, U. Organisation and functional roles of the central complex in the insect brain. ***Annu. Rev. Entomol*** 59: 165–184, 2014.

Picciotto, M.R., Higley, M.J., Mineur, Y.S. Acetylcholine as a neuromodulator: cholinergic signaling shapes nervous system function and behavior. **Neuron** 76, 116–129 2012.

Pisoschi, A.M., Pop, A. The role of antioxidants in the chemistry of oxidative stress: a review. **Eur J Med Chem**. 97:55–74, 2015.

Radyuk, S.N., Sohal, R.S., Orr, W.C. Thioredoxin peroxidases can foster cytoprotection or cell death in response to different stressors: over- and under-expression of thioredoxin peroxidase in Drosophila cells. **Biochemical Journal** 371:743–752, 2003.

Ranganatha, N., Kuppast, I.J. A review on alternatives to animal testing methods in drug development. **Int. J. Pharm. Pharm. Sci**. 4, 28–32, 2012.

Rival, T. Soustelle, L. Strambi, C. Besson, M.T. Iche´, M. Birman, S. Decreasing Glutamate Buffering Capacity Triggers Oxidative Stress and Neuropil Degeneration in the Drosophila Brain. **Current Biology**, Vol. 14, 599–605, 2004.

Robinson, A., Huttley, G.A., Booth, H.S., Board, P.G. Modelling and bioinformatics studies of the human kappa class glutathione transferase predict a novel third transferase family with homology to prokaryotic 2-hydroxychromene-2-carboxylate isomerases. **Biochem** J. 379:541–552, 2004.

Rodrigues, N.R., Nunes, M.E.M., Silva, D.G.C., Zemolin, A.P.P., Meinerz, D.F., Cruz, L.C., Pereira, A.B., Rocha, J.B.T., Posser, T., Franco, J.L. Is the lobster cockroach Nauphoeta cinerea a valuable model for evaluating mercury induced oxidative stress? **Chemosphere** 92:1177–1182, 2013.

Roth, L. M., Willis, E. R. **Smithsonian Miscellaneous Collections.** 122, 1–49, 1954.

Ruszkiewicz, J.A., Teixeira de Macedo, G., Miranda-Vizuete, A, Bowman, A.B., Bornhorst, J., Schwerdtle, T., Antunes Soares, F.A., Aschner, M. Sex-Specific Response of Caenorhabditis elegans to Methylmercury Toxicity. **Neurotox Res**. 5(1):208-216, 2016.

Rybnikova, E., Damdimopulos, A.E., Gustaffson, J., Spyrou, G., Pelto-Huikko, M. Expression of novel antioxidant thioredoxin 2 in the rat brain. **European Journal of Neuroscience**, Vol 12, pp. 1669-1678, 2000.

Sakamoto, M., Kakita, A., Wakabayashi, K., Takahashi, H., Nakano, A., Akagi, H. Evaluation of changes in methylmercury accumulation in the developing rat brain and its effects: A study with consecutive and moderate dose exposure throughout gestation and lactation periods. **Brain Res.** 949: 51–59, 2002.

Salisbury, D., Bronas, U. Reactive oxygen and nitrogen species: impact on endothelial dysfunction. **Nurs Res.** 64(1):53–66, 2015.

Sattelle, D.B. Receptors for l-glutamate and gaba in the nervous system of an insect (periplaneta americana). **Camp. Biochem. Physiol**. Vol. 103C, No. 3, pp. 429438, 1992.

Scharrer, B.C.J. The differentiation between neuroglia and connective tissue sheath in the cockroach (Periplaneta americana**) *J. Comp. Neurol*** 70: 77–88, 1939.

Seal, R.P., Daniels, G.M., Wolfgang, W.J., Forte, M., Amara, S. Identification and characterization of a cDNA encoding a neuronal glutamate transporter from Drosophila melanogaster. **Receptors and Channels** 6, 51-64, 1998.

Segatto, A.L.A., Diesel, J.F., Loreto, E.L.S., Rocha, J.B.T. De novo transcriptome of the lobster cockroach *Nauphoeta cinerea* (Blaberidae). **Genetics and Molecular Biology**, 41(3) 713-721, 2018.

Sharma. Monosodium glutamate induced oxidative kidney damage and possible mechanisms: a mini review. **Journal of Biomedical Science**. 22:93, 2015.

Sharma, A., Wongkham, C., Prasongwattana, V., Boonnate, P., Thanan, R., Reungjui, S., Cha'on., U. Proteomic analysis of kidney in rats chronically exposed to monosodium glutamate. **PLoS One.** 9(12): 2014.

Shamim, G., Ranjan, S.K., Pandey, D. M., Ramani. R. Biochemistry and biosynthesis of insect pigments. **Eur. J. Entomol**. 111: 149–164, 2014.

Siegl, T., Schachtner, J., Holstein, G.R., Homberg, U. NO/cGMP signalling: l-citrulline and cGMP immunostaining in the central complex of the desert locust *Schistocerca gregaria*. **Cell Tissue** Res. 337: 327–340, 2009.

Stankiewicz, M., Dąbrowski, M., de Lima, M.E. Nervous system of *Periplaneta americana* cockroach as a model in toxicological studies: A short historical and actual view. **J. Toxicol.** 143740, 2012.

Steevens, .J.A., Benson, W.H. Toxicological interactions of chlorpyrifos and methyl mercury in the amphipod, Hyalella Azteca. **Toxicol Sci**. 52(2):168-77, 1999.

Stocker, R.F. The organization of the chemosensory system in Drosophila melanogaster*:* A review: **Cell Tissue Res.** 275: 3-26, 1994.

Strausfeld, N.J. 1976. Atlas of an Insect Brain. **Springer-Verlag**, New York.

Susanne, E.T., Arthur, R.Z., May R.B. Ascorbic and Uric Acid Responses to Xanthotoxin Ingestion in a Generalist and a Specialist Caterpillar. **Archives of Insect Biochemistry and Physiology** 42:26–36, 1999.

Swallow, J.G., Bubak, A.N., and Grace J.L. The role of monoamines in modulating behavior. **Current Zoology**. 62(3), 253–255, 2016.

Tawfik, M.S., Al-Badr, N. Adverse Effects of Monosodium Glutamate on Liver and Kidney Functions in Adult Rats and Potential Protective Effect of Vitamins C and E. **Food and Nutrition Sciences** 3, 651-659, 2012.

Treherne, J.E. Blood-brain barrier, in G.A. Kerkut and L.I. Gilbert (eds.), *Comprehensive Insect Physiology Biochemistry and Pharmacology,* vol 5 **Pergamon Press, Oxford**, U.K., pp. 115–137.

Valko, M., Rhodes, C.J., Moncol, J., Izakovic, M., Mazur, M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. **Chem Biol Interact**. 160:1–40, 2006.

VanDuyn, N., Raja, S., Garry, W., Richard, N. SKN-1/Nrf2 Inhibits Dopamine Neuron Degeneration in a Caenorhabditis elegans Model of Methylmercury Toxicity. **TOXICOLOGICAL SCIENCES** 118(2): 613–624, 2010.

Veerman, A. Photoperiodic time measurement in insects and mites: a critical evaluation of the oscillator-clock hypothesis. **J. Insect Physiol**. 47: 1097–1109, 2001.

Ullrich, V., Kissner, R. Redox signaling: Bioinorganic chemistry at its best. **Journal of Inorganic Biochemistry** 100: 2079–2086, 2006.

Umukoro, S., Oluwole, G.O., Olamijowon, H.E., Omogbiya, A.I., Eduviere, A.T. Effect of Monosodium Glutamate on Behavioral Phenotypes, Biomarkers of Oxidative Stress in Brain Tissues and Liver Enzymes in Mice. **World Journal of Neuroscience**, 5, 339-349, 2015.

Tan, B.L. Norhaizan, M.E. Liew, W.P. Rahman, H.S. Antioxidant and oxidative stress: A mutual interplay in Age-related diseases, Front. Pharmacol 9: 21-28, 2018.

Walter, M. H., Strack, D. Carotenoids and their cleavage products: biosynthesis and functions. **Nat. Prod. Rep**. 28: 663–692, 2011.

Wang, H.D., Shi, Y., Wang, L., Liu, S., Wu, S.W., Yang, Y.H., René F., Yidong W. CYP6AE gene cluster knockout in Helicoverpa armigera reveals role in detoxification of phytochemicals and insecticides. **Nat. Commun**. 9:4820, 2018.

Wang, Y., Wang, L.J., Zhu, Z.H., Ma, W.H., Lei, C.L. The molecular characterization of antioxidant enzyme genes in Helicoverpa armigera adults and their involvement in response to ultraviolet-A stress. **J. Insect Physiol**. 58: 1250–1258, 2012.

Washio, H. Glutamate Receptors on the Somata of Dorsal Unpaired Median Neurons in Cockroach, Periplaneta americana Thoracic Ganglia. **ZOOLOGICAL SCIENCE** 19: 153–162, 2002.

Wenzel, B., Kunst, M., Günther, C., Ganter, G.K., Lakes-Harlan, R., Elsner, N., Heinrich, R. Nitric oxide/cyclic guanosine monophosphate signaling in the central complex of the grasshopper brain inhibits singing behavior. **J. Comp. Neurol.** 488: 129–139, 2005.

Willard, S.S., Koochekpour S. Glutamate, glutamate receptors, and downstream signaling pathways. Int J Biol Sci. 9(9): 948–59, 2013.

Wilson-Sanders, S.E. Invertebrate models for biomedical research, testing, and education. **ILAR** J. 52, 126–152, 2011.

Wolstenholme, A.J. Glutamate-gated Chloride Channels. **J Biol Chem**. 287:40232–8, 2012.

Wu, H.H., Liu, Y.M., Zhang, X.Y., Zhang, J.Z., Man, E.B. Antioxidant defenses at enzymatic and transcriptional levels in response to acute lead administration in Oxya chinensis. **Ecotoxicol. Environ. Saf.** 168: 27–34, 2019.

Wu, J.Q., Kosten, T.R., Zhang, X.Y. Free radicals, antioxidant defense systems, and schizophrenia. **Prog Neuropsychopharmacol Biol Psychiatry**. 46:200–206, 2013.

Xu, G., Wu, S.F., Wu, Y.S., Gu, G.X., Fang, Q., Ye, G.Y. De novo assembly and characterization of central nervous system transcriptome reveals neurotransmitter signaling systems in the rice striped stem borer, Chilo suppressalis. **BMC Genomics**. 16:525, 2015.

Xu, J., Lu, M.X., Huang, D.L., Du, Y.Z. Molecular cloning, characterization, genomic structure and functional analysis of catalase in Chilo suppressalis. **J. Asia-Pac. Entomol**. 20: 331–336, 2017.

Yamamoto, K., Banno, Y., Fujii, H., Miake, F., Kashige, N., Aso, Y. Catalase from the silkworm, Bombyx mori: gene sequence, distribution, and overexpression. **Insect Biochem. Mol. Biol.** 35: 277–283, 2005.

Yamamoto. K., Zhang. P., Miake. F., Kashige. N., Aso. Y., Banno. Y., Fujii. H. Cloning, expression and characterization of theta-class glutathione S-transferase from the silkworm, Bombyx mori. **Comp Biochem Physiol B Biochem Mol Biol** 141(3):340–346, 2005.

Yan, Y., Zhang, Y., Huaxia, Y., Wang, X., Yao, P., Guo, X., Xu, B. Identification and characterisation of a novel 1-Cys thioredoxin peroxidase gene (AccTpx5) from Apis cerana cerana. **Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,** 172–173, 39–48, 2014.

Yao, P., Hao, L., Wang, F., Chen, X., Yan, Y., Guo, X., Xu, B. Molecular cloning, expression and antioxidant characterisation of a typical thioredoxin gene (AccTrx2) in Apis cerana cerana. **Gene** 527:33–41, 2013.

Zhang, H., Jia, J., Wang, W., Chen, X., Wu, S., Wang, H., Wang, J. Current status and future trends of food additives research in China. **J Food Sci Biotechnol** 35:225–233, 2016.

Zhang, J., Lu, A., Kong, L., Zhang, Q., Ling, E. Functional analysis of insect molting fluid proteins on the protection and regulation of ecdysis**. J Biol Chem**. 289:35891–35906, 2014.

Zhang, X.Y., Li, Y.H., Wang, J.X., Zhang, T.T., Li, T., Dong, W., Ma, E.B., Zhang, J.Z. Identification and characteristic analysis of the catalase gene from Locusta migratoria. **Pestic. Biochem. Phys.** 132:125–131, 2016.

Zundorf, G., Kahlert, S., Bunik, V.I., Reiser, G. Alpha-Ketoglutarate dehydrogenase contributes to production of reactive oxygen species in glutamate stimulated hippocampal neurons in situ. **Neuroscience** 158(2):610–6, 2009.

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