

Effects of Hg(II) Exposure on MAPK Phosphorylation and Antioxidant System in *D. melanogaster*

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ABSTRACT: The heavy metal mercury is a known toxin, but while the mechanisms involved in mercury toxicity have been well demonstrated in vertebrates, little is known about toxicological effects of this metal in invertebrates. Here, we present the results of our study investigating the effects associated with exposure of fruit fly *Drosophila melanogaster* to inorganic mercury (HgCl₂). We quantify survival and locomotor performance as well as a variety of biochemical parameters including antioxidant status, MAPK phosphorylation and gene expression following mercury treatment. Our results demonstrate that exposure to Hg(II) through diet induced mortality and affected locomotor performance as evaluated by negative geotaxis, in *D. melanogaster*. We also saw a significant impact on the antioxidant system including an inhibition of acetylcholinesterase (Ache), glutathione S-transferase (GST) and superoxide dismutase (SOD) activities. We found no significant alteration in the levels of mRNA of antioxidant enzymes or NRF-2 transcriptional factor, but did detect a significant up regulation of the *HSP83* gene. Mercury exposure also induced the phosphorylation of JNK and ERK, without altering p38^{MAPK} and the concentration of these kinases. In parallel, Hg(II) induced PARP cleavage in a 89 kDa fragment, suggesting the triggering of apoptotic cell death in response to the treatment. Taken together, this data clarifies and extends our understanding of the molecular mechanisms mediating Hg(II) toxicity in an invertebrate model. © 2012 Wiley Periodicals, Inc. *Environ Toxicol* 00: 000–000, 2012.

Keywords: mercury; *Drosophila melanogaster*; antioxidant defenses; MAPKs; PARP

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INTRODUCTION

Heavy metals, such as mercury (Hg), have been mined, purified, and used by humans for thousands of years and, although their toxicity has been well reported, exposure to these elements has continued and even increased, mainly in less developed countries (Jarup, 2003). Additionally, human activities can mobilize mercury from natural sources inadvertently causing contamination of water and leading to absorption by organisms along the food chain (Walters et al., 2011). High levels of mercury are associated with hepatotoxicity, nephrotoxicity, and neurological damage in rodents and humans (Ceccatelli et al., 2010; Jan et al., 2011). Exposure of rodents to organic or inorganic forms of mercury has been associated with permanent damage to the central nervous system and psychological symptoms, as well as damage to kidney, immune system, and other organ systems (Jarup, 2003; Zahir et al., 2005). While there is extensive literature on mercury's effects in vertebrates, there is a pronounced lack of studies addressing its effects in invertebrates, although research has demonstrated that invertebrates are susceptible to chemical contaminants, including heavy metals (Balamurugan et al., 2009; Posgai et al., 2011).

The toxicity of mercury exposure is, in part, a function of increased oxidative stress (OS). The increase in OS is possibly from the depletion of thiol compounds (mainly GSH), inhibition of antioxidant enzymes, or both (Franco et al., 2007) leading to cell injury, damage to biomolecules, and lipid peroxidation (Leonard et al., 2004). Metal-induced oxidative stress modulates protein activity along signaling pathways (Leonard et al., 2004). Affected proteins include the mitogen activated protein kinases (MAPKS), a family of serine/threonine protein kinases that are implicated in the regulation of multiple cellular events including differentiation, proliferation, cell death, adaptive and immune responses (Johnson and Lapadat, 2002). The MAPK family includes the extracellular activated protein kinase (ERK 1/2), c-Jun N-terminal kinase (JNK1/2), and 38 kDa kinase (p38^{MAPK}), proteins whose function and regulation are well conserved from unicellular to complex organisms (Johnson and Lapadat, 2002). Activation of these kinases may occur in response to hyperosmotic stress, cytokine exposure, and toxic injury, including OS (Harper and Lograsso, 2001; Kaminska, 2005) and metals, such as mercury, lead, and manganese, induce phosphorylation of MAPK in vertebrates (Turney et al., 1999; Posser et al., 2007, 2010). In *Drosophila melanogaster*, the MAPK pathway is known to be involved in numerous processes during normal development and in the regulation of immune response (Stronach and Perrimon, 1999), but until now, little has been known about the modulation of MAPK by toxins.

The fruit fly *D. melanogaster* is one of modern genetics premier model systems, with an extensive literature ranging from classical and modern genetics to biochemistry to

physiology and complex phenotypes. Although humans and *D. melanogaster* are only distantly evolutionarily related, almost 75% of disease-related genes in humans have functional orthologs in the fly (Pandey and Nichols, 2011), making the fly a reasonable model system for humans and other vertebrate. *D. melanogaster* have a rapid reproductive cycle and are easily maintained and handled in the lab making them ideal organisms for the use on *in vivo* bioassays. They have been also proving to be a powerful model system for the study the development and functioning of nervous system and for the study of fundamental cellular pathways responsible for metal and insecticide toxicity (Ahamed et al., 2010; Bonilla-Ramirez et al., 2011).

Here we present the results of our study of the biochemical responses in *D. melanogaster* to exposure to inorganic mercury, Hg(II), focusing on gene expression and MAPK phosphorylation/expression and oxidative stress induction. This work contributes to the understanding of the molecular targets of Hg(II) in organisms beyond vertebrates and reinforces the usefulness of the *D. melanogaster* insect model for toxicological studies.

MATERIAL AND METHODS

Materials

Mercury chloride, quercetin (Q4951), 5,5'-dithiobis(2-nitrobenzoic acid) (D8130), acetylthiocholine iodide (A5751), 1-chloro-2,4-dinitrobenzene (237329), 2',7'-dichlorofluorescein diacetate (DCHF-DA, 35845), β -mercaptoethanol (M6250), and anti-rabbit immunoglobulin (alkaline phosphatase-linked antibody, A-3687) were obtained from Sigma Aldrich (St Louis, MO). Anti-phospho-p38 (Thr180/Tyr182) and anti-total-p38, anti-phospho JNK1/2 (Thr183/Tyr185) and anti-total-JNK1/2, anti-phospho ERK1/2 (Thr202/Tyr204) and anti-total-ERK1/2 and β -actin antibodies were purchased from cell signaling technology. SDS, acrylamide, bis-acrylamide, hybond nitrocellulose were obtained from GE Healthcare Life Division. Poly (ADP)-ribose polymerase (PARP) antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were commercial products of the highest purity grade available.

Drosophila Stock and Culture

D. melanogaster (Harwich strain) was obtained from the National Species Stock Center, Bowling Green, OH. The flies were reared in 2.5×6.5 cm² glass bottles containing 5 mL of standard medium (1% w/v brewer's yeast; 2% w/v sucrose; 1% w/v powdered milk; 1% w/v agar; 0.08% v/w nepagin) at constant temperature and humidity ($25 \pm 1^\circ\text{C}$; 60% relative humidity, respectively). All experiments were performed with the same strain.

Mercury Chloride Exposure

Adult flies, 5- to 7-day post eclosion, were exposed to HgCl₂ using the following protocol. One hundred and twenty adult flies (both genders) were starved in empty vials for 3 h at 25°C, starvation promotes feeding and was used to maximize feeding rate once flies were transferred to HgCl₂ solutions. Following starvation, groups of 30 flies were placed for 48 h in vials containing a filter paper saturated with 500 µL of one of three HgCl₂ solutions diluted in 2 M sucrose. The HgCl₂ solutions were 0, 10, 30, and 100 µM, the 0 µM solution, that is sucrose only, served as control. Filters were changed daily. The HgCl₂ concentrations were based on previous studies focused in the evaluation of *in vivo* Mercury toxicity in *D. melanogaster* model (Carmona et al, 2008; Rand et al., 2009).

Survival Rate Analysis

To quantify fly survival rates across the HgCl₂ concentrations, three replicates of groups of 30 adult flies were exposed to different concentrations of HgCl₂ for 48 h and the number of live and dead flies counted every 24 h. Results were analyzed and plotted as percent live flies.

Sample Preparation and Enzyme Assays

For enzymes activity, groups of 20 flies were homogenized in 1 mL 0.1 M phosphate buffer, pH 7.0, and centrifuged at 1000 g for 5 min at 4°C. The supernatant was isolated and a 200 µL aliquot separated for determination of Acetylcholinesterase (Ache) activity based on protocols previously described (Franco et al., 2009). The remaining supernatant was then centrifuged at 20,000 g for 30 min. The resulted supernatant was used for determination of glutathione S-transferase (GST), catalase (CAT), and superoxide dismutase (SOD) according to methods described earlier (Franco et al., 2009).

Quantification of MAPKs phosphorylation and PARP cleavage was performed using Western blotting according with Posser et al. (2009) with minor modifications. Thirty flies were homogenized at 4°C in 300 µL of buffer (pH 7.0) containing 50 mM Tris, 1 mM EDTA, 0.1 mM phenylmethyl sulfonyl fluoride, 20 mM Na₃VO₄, 100 mM sodium fluoride and phosphatase inhibitor cocktail (Sigma, MO). The homogenates were centrifuged at 1000 × g for 10 min at 4°C and the supernatants (S1) collected. After protein determination (following Bradford, 1976) using bovine serum albumin as standard, β-mercaptoethanol and glycerol was added to samples to a final concentration of 8 and 25%, respectively, and the samples frozen until further analysis. Proteins were separated using SDS–PAGE with 10% gels, and then electrotransferred to nitrocellulose membranes as previously described (Posser et al., 2009). Membranes were washed in tris-buffered saline with Tween (100 mM tris–

HCl, 0.9% NaCl and 0.1% Tween-20, pH 7.5) and incubated overnight (4°C) with different primary antibodies, all produced in rabbit (anti-ERK1/2, anti-p38, anti-JNK1/2 total and phosphorylated forms, anti-β-actin and anti-PARP). Following incubation, membranes were washed in tris-buffered saline with Tween and incubated for 1 h at 25°C with alkaline phosphatase-linked anti rabbit-IgG secondary specific antibodies. Antibody binding was visualized using the NBT-BCIP kit (KPL, MD). Band staining density was quantified using the Scion Image software and expressed as a fold change of the mean relative to control group (treated only with sucrose). The loading controls were performed by analysis of total proteins (total MAPKs) and β-actin using specific antibodies.

Locomotor Assay

Locomotor ability was determined using the negative geotaxis assay as described by Coulom and Birman (2004). Briefly, for each assay, 20 flies (7 days old; both genders) were immobilized on ice for 1–2 min and placed separately in vertical glass columns (length, 25 cm; diameter, 1.5 cm). After 30 min recovery, flies were gently tapped to the bottom of the column and the time required for flies to climb up to 8 cm in the columns was registered. The assays were repeated five times at 1-min intervals. Results are presented as mean time (s) ± SE obtained in three independent experiments. In parallel to individual performance, in a second experiment, a group of 20 flies were gently tapped to the bottom of a glass column and after 1 min, the number of flies that reach 8 cm of the column (top) and flies that remained below this mark (bottom) were counted.

Determination of Lipid Peroxidation and DCF-DA Oxidation

Lipid peroxidation products were quantified as thiobarbituric acid reactive substance (TBARS) following the method of Ohkawa et al. (1979) with minor modifications. Briefly, groups of 20 flies from each treatment were homogenized in 1 mL 0.1 M phosphate buffer pH 7.0 and centrifuged at 1000 g for 5 min (4°C). After centrifugation, the supernatant was incubated in 0.45 M acetic acid/HCl buffer pH 3.4, 0.28% thiobarbituric acid, 1.2% SDS, at 95°C for 60 min and absorbance then measured at 532 nm. Malondialdehyde (0–3 nmol) was used as a standard. Results represent the mean of five independent experiments. In each experiment, each treatment was done in duplicate. The TBARS values were normalized by protein concentration. The results were expressed as nmol TBARS/min/mg tissue at 37°C using molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$. We also quantified 2',7'-dichlorofluorescein diacetate (DCF-DA) oxidation as a general index of oxidative stress following Pérez-Severiano et al. (2004). The fluorescence

emission of DCF resulting from DCF-DA oxidation was monitored at regular intervals at an excitation wavelength of 485 nm and an emission wavelength of 530 nm in a EnsPire[®] multimode plate reader (PerkinElmer, USA). The amount of DCF formed was calculated based on a calibration curve constructed using a commercial DCF standard. The rate of DCF formation was calculated as a percentage of the DCF formation in relation to the sucrose-treated control group. Results represent the mean of five independent experiments. In each experiment, each treatment was done in duplicate.

Enzyme Assays

Glutathione transferase (GST; EC 2.5.1.18), activity was assayed following the procedure of Jakoby and Habig (1981) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The assay is based on the formation of the conjugated complex of CDNB and GSH at 340 nm. The reaction was conducted in a mix consisting of 100 mM phosphate buffer pH 7.0, 1 mM EDTA, 1 mM GSH, and 2.5 mM CDNB. Catalase (CAT; EC 1.11.1.6), activity was assayed following the clearance of H₂O₂ at 240 nm in a reaction media containing 50 mM phosphate buffer pH 7.0, 0.5 mM EDTA, 10 mM H₂O₂, 0.012% TRITON X100 according to the procedure of Aebi (1984). SOD, EC 1.15.1.1, activity assayed following the procedure of Kostyuk and Potapovich (1989). The assay consists in the inhibition of superoxide-driven oxidation of quercetin by SOD at 406 nm. The complete reaction system consisted of 25 mM phosphate buffer, pH 10, 0.25 mM EDTA, 0.8 mM TEMED and 0.05 μ M quercetin. Acetylcholinesterase activity was assayed following the procedure of Ellman et al. (1961). The system consisted of 100 mM phosphate buffer pH 8.0, 0.5 mM DTNB, and 0.35 mM acetylthiocholine as initiator. The reaction was monitored for 2 min at 412 nm. All enzyme activities were performed at room temperature ($25 \pm 1^\circ\text{C}$) using a Thermo Scientific Evolution 60s UV-Vis spectrophotometer.

Metal Content Determination

We quantified mercury levels in flies exposed to 100 μ M Hg(II) for 48 h. Two hundred flies per group (control and treated groups) were weighed, washed in deionized water and then placed on a filter paper in the incubator (37°C) for 60 min to dry. Dry flies were digested with HNO₃ using a model Multiwave 3000 microwave oven equipped with high-pressure quartz vessel (max 80 bar, 280°C, Anton Paar, Graz, Austria). After digestion, samples were diluted with water to 25 mL and transferred to graduated polypropylene vials. Mercury was determined using a cold vapor (CV) continuous flow (CF) system, following established protocols (Kaercher et al., 2005), coupled to Inductively

TABLE I. Genes tested by quantitative real-time RT-PCR analysis and used forward and reverse primers

Genes	Primer sequences
Actin	LEFT 5' TACCCCATGAGCACGGTAT 3' RIGHT 5' GGGTCATCTTCTCACGGTTG 3'
GPDH	LEFT 5' ATGGAGATGATTCGCTTCGT 3' RIGHT 5' GCTCCTCAATGGTTTTTCCA 3'
Catalase	LEFT 5' ACCAGGGCATCAAGAATCTG 3' RIGHT 5' AACTTCTTGGCCTGCTCGTA 3'
Superoxide dismutase	LEFT 5' GGAGTCGGTGATGTTGACCT 3' RIGHT 5' GTTCGGTGACAACACCAATG 3'
HSP83	LEFT 5' CAAATCCCTGACCAACGACT 3' RIGHT 5' CGCACGTACAGCTTGATGTT 3'
NRF2	LEFT 5' CCAACTTCCTCAAGGAGCAG 3' RIGHT 5' CGGCGACAAATATCATCCTT 3'
MPK2	LEFT 5' GGCCACATAGCCTGTCATCT 3' RIGHT 5' ACCAGATACTCCGTGGCTTG 3'

Coupled Plasma Mass Spectrometry (ICP-MS) using a CF instead of flow injection that consists of a CV system, composed by a peristaltic pump (Gilson, Miniplus, France), a manual injector and a U-type gas-liquid separator. Tygon[®] pump tubings with 1.14 mm i.d. were used to transport 0.1% (w/v) NaBH₄ and sample solutions and 1.69 mm i.d. tubings were used to transport 1.0 mol L⁻¹ HCl solution. Water was used as sample carrier and samples were on-line mixed with 1.0 mol L⁻¹ HCl and then NaBH₄ (0.1%, w/v) solution. The mixture was pumped to the gas-liquid separator and Hg was measured by ICP-MS. The results were expressed in μ g of Hg/g of dried weight tissue.

Quantitative Real-Time RT-PCR and Gene Expression Analysis

Approximately 2 μ g of total RNA from 20 young flies was extracted using the Trizol Reagent (Invitrogen) accordingly to the manufacturer's suggested protocol. After quantification, total RNA was treated with DNase I (Invitrogen, NY) and cDNA was synthesized with M-MLV reverse transcriptase enzyme and random primers again accordingly to the manufacturer's suggested protocol (Invitrogen, NY). Quantitative real-time polymerase chain reaction was performed in 20 μ L reaction volumes containing 1x PCR Buffer, 25 μ M dNTPs, 0.2 μ M of each primer (described in Table I), 3 mM MgCl₂, 0.1 x SYBR Green I (molecular probes) and 0.5 U platinum Taq DNA polymerase (Invitrogen, NY) using a StepOnePlus real time PCR systems (Applied Biosystems, NY). The qPCR protocol was the following: activation of the Taq DNA polymerase at 95°C for 5 min, followed by 40 cycles of 15 s at 95°C, 15 s at 60°C, and 25 s at 72°C. All samples were analyzed as technical and biological triplicates with a negative control. Threshold and

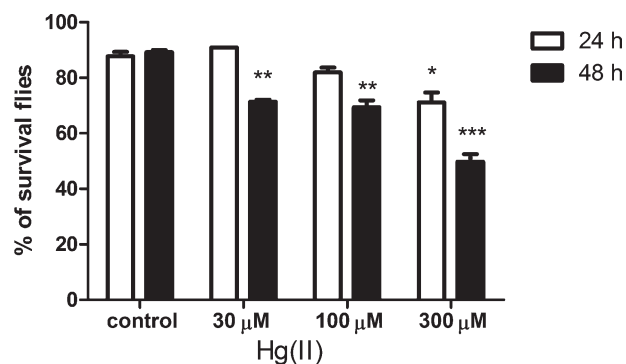


Fig. 1. Effects of exposure to Hg(II) on fly survival. We calculated percent survival after flies were exposed to four concentrations of HgCl₂, 0, 30, 100, and 300 μM, for 24 or 48 h. Bars represent the mean ± SEM of experiments performed individually and are expressed as percent live flies in relation to control group (absence of Hg). **p* < 0.05 in comparison to control; ***p* < 0.01 in comparison to control; ****p* < 0.001 in comparison to control.

baselines were manually determined using the StepOne Software v2.0 (Applied Biosystems, NY). SYBR fluorescence was analyzed by StepOne software version 2.0 (Applied Biosystems, NY), and the CT (cycle threshold) value for each sample was calculated and reported using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). The Actin and GPDH genes were used as endogenous reference genes presenting no alteration in response to the treatment. For each well, analyzed in quadruplicate, a ΔC_T value was obtained by subtracting the Actin and GPDH C_T value from the C_T value of the interest gene (sequences of tested genes are represented in Table I). The ΔC_T mean value obtained from the control group of each gene was used to calculate the $\Delta\Delta C_T$ of the respective gene ($2^{-\Delta\Delta C_T}$).

Statistical Analysis

Statistical analysis was performed using a one- or two-way ANOVA followed by Tukey's post hoc test. Differences were considered to be significant at the *p* < 0.05 level.

RESULTS

In this study, flies (*D. melanogaster*) were exposed to Hg(II) toxicity for 24 or 48 h using three concentrations (30, 100 or 300 μM) of HgCl₂ dissolved in a sucrose solution and survival, locomotor activity, and biochemical/physiological responses quantified. Hg(II) exposure resulted in significant fly mortality. Twenty-four hour exposure resulted in a significant decrease (25%) in fly survival only at highest concentration, while exposure for 48 h resulted in significant decrease in fly survival at 30, 100, and 300 μM (30, 30, and 50%, respectively; Fig. 1). Hg(II)

exposure also had a significantly deleterious impact on locomotor behavior. Flies treated with 100 μM Hg(II) for 48 h took longer to achieve 8 cm in the vials [Fig. 2(A)] and significantly more flies remained at the base of the flasks when tapped to the bottom of tubes when compared to control groups [Fig. 2(B)]. Flies treated with 300 μM of HgCl₂ took more than the maximum time of observation (2 min) to climb 8 cm over the flask or were unable to climb, thus this group was not represented in the graph. All three assays indicated that mercury exposure significantly impacts complex phenotypes, such as survivorship and locomotor behavior.

Oxidative stress is an important mechanism implicated in Mercury toxicity, however there is a lack of studies addressing the mechanisms of toxicity of mercury in insects. Here we quantify DCFDA oxidation as a general indicator of oxidative stress and TBARS as an indicator of lipid oxidation in flies exposed to Hg(II) stress (Fig. 3). To test for concentration dependence, we exposed flies to three concentrations, 10, 20, and 100 μM, of HgCl₂ for 48 h. DCF and TBARS were both significantly elevated in the

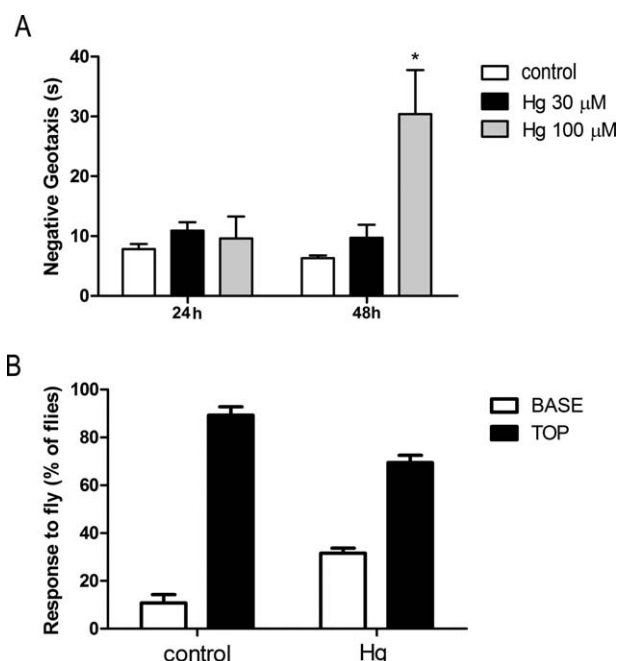


Fig. 2. Effects of exposure to Hg(II) on locomotor performance in *D. melanogaster*. We quantified negative geotaxis after flies were treated with 100 μM HgCl₂ for 24 or 48 h (A). Results are expressed as mean ± SEM of three independent experiments and represent the number of seconds needed to climb up to 8 cm in a glass tube. (B) It represents the number of flies that reached the top of a column and those that remained at the bottom after 1 min. The results represent a mean ± SEM of number of flies at the top and bottom of at least three individual experiments and are expressed as percentage of total number of flies submitted to the treatment. **p* < 0.05 in comparison to control.

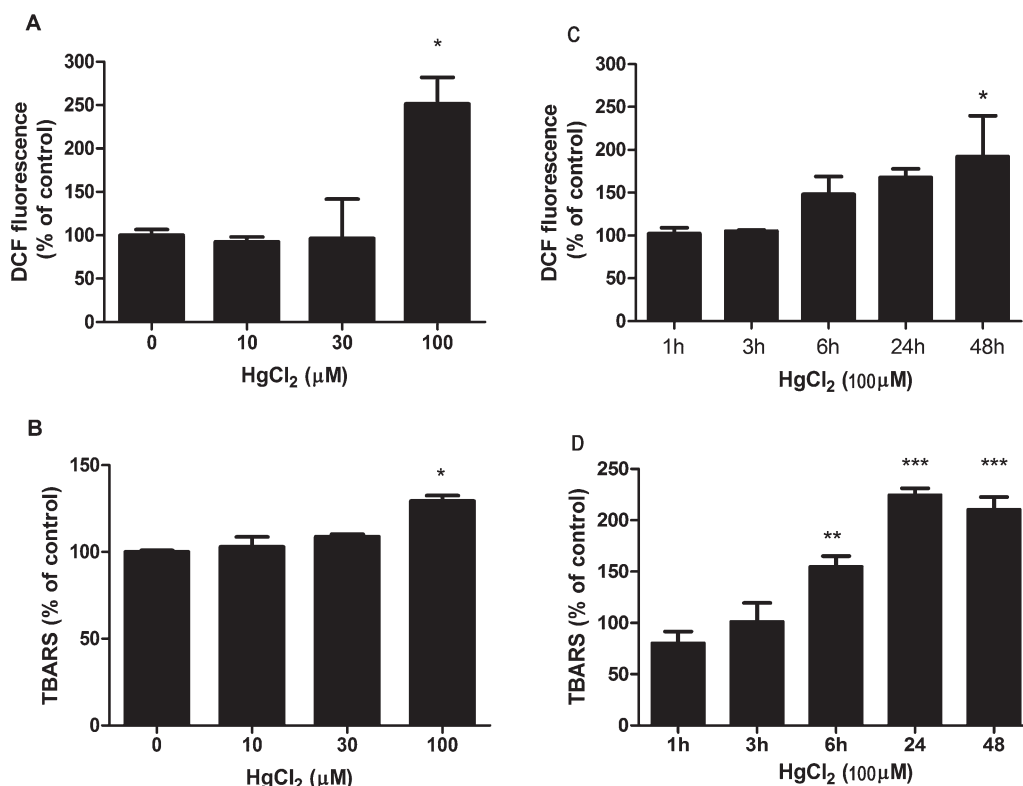


Fig. 3. Analysis of ROS production and lipid peroxidation in response to Hg(II) exposure in *D. melanogaster*. Flies were subjected to treatment with increasing concentrations of Hg(II) for 48 h (A,B) or with 100 μM of Hg(II) over a time course (C,D). After treatments, flies were homogenized and the supernatant was used for analysis of DCF-DA fluorescence as an index of ROS production and lipid peroxidation by TBARS assay. A and C show the DCF-DA intensity in total flies homogenate, expressed as percentage of control. B and D show the end products of lipid peroxidation determined by TBARS assay, expressed as percentage of TBARS production in relation to control. The data represent a mean ± SEM of five experiments performed individually. * $p < 0.05$ in comparison to control; *** $p < 0.001$ in comparison to control.

100 μM treatment, but not in the two lower concentration treatments [Fig. 3(A,B)]. To test for time dependence, we exposed flies to 100 μM HgCl₂ for 1, 3, 6, 24, and 48 h. TBARS concentration was significantly elevated after 6 h with concentration continuing to increase through 24 h and remaining elevated at 48 h [Fig. 3(D)]. DCF fluorescence was significantly elevated only after 48 h of exposure, but the increasing trend is apparent after 6 h [Fig. 3(C)].

We also quantified the levels of three enzymes known to reflect oxidative stress status in other systems, catalase, SOD, and glutathione-*S*-transferase (GST), following 48 h of exposure to 100 μM mercury. Additionally, we also determined the activity of acetylcholinesterase (Ache). Mercury exposure resulted in significantly lower levels of GST, SOD and Ache activity (82.46 ± 2.96 to 69.99 ± 2.40 , 90.06 ± 4.27 to 56.38 ± 5.15 , and 172.5 ± 4.51 to 121.31 ± 21.08 mU/mg protein, respectively, Table II) while catalase activity was not altered (5.67 ± 0.19 to 5.80 ± 0.24 mU/mg protein). We also quantified fly uptake and

accumulation of mercury. Previous research has demonstrated that adult *D. melanogaster* can accumulate lead (Pb) when this metal is present in the diet (Hirsch et al., 2003). Here, we show that mercury levels increase approximately 100× with 48 h of exposure to 100 μM HgCl₂ (0.17 to 13.5 μg/g of dry weight tissue, Table II).

TABLE II. Summary of the effects of HgCl₂ 100 μM exposure in *D. melanogaster* on metal content determination and enzymatic activities

	Control group	Treated group
Hg levels	0.17 μg/g	13.5 μg/g
Catalase	5.67 ± 0.19	5.80 ± 0.24
GST	82.46 ± 2.96	69.99 ± 2.40^a
SOD	90.06 ± 4.27	56.38 ± 5.15^a
Ache	172.5 ± 4.51	121.31 ± 21.08^a

Values are expressed as mean (mU/mg protein) ± SEM, $n = 5 - 6$.

^a $p < 0.05$.

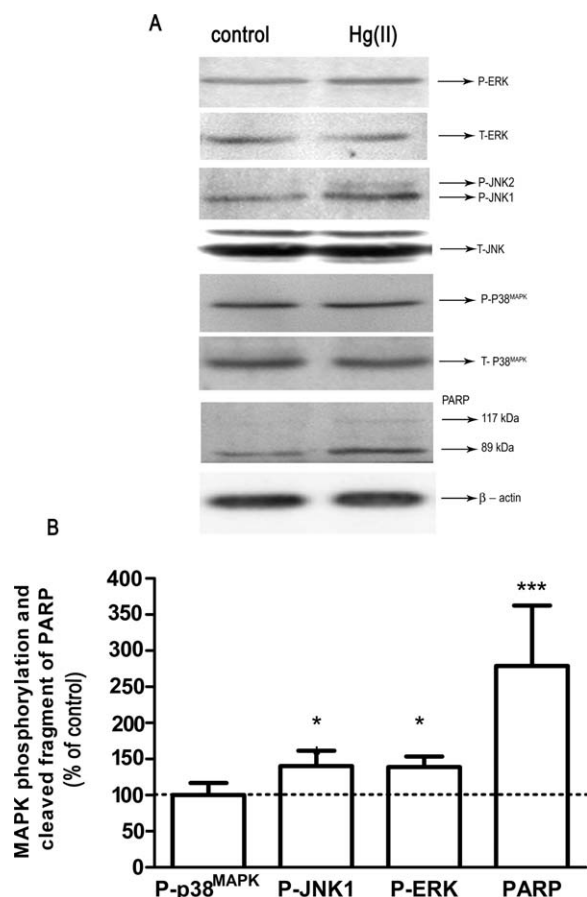


Fig. 4. Modulation of MAPKs phosphorylation and PARP cleavage in response to treatment of *D. melanogaster* with Hg(II) (100 μ M) for 48 h. Flies were treated for 48 h with 100 μ M Hg(II), proteins separated by SDS-PAGE, and transferred to nitrocellulose membrane. We quantified total content and phosphorylation of proteins using specific antibodies. The upper panel is a Western blot showing phosphorylated and total forms of ERK, JNK, p38MAPK, PARP, and β -actin content. Both cleavage products of PARP are visible. The lower panel is a graph showing the quantification (percentage of control O.D.) of immunoreactive bands, each bar represents the mean \pm SEM of three independent experiments. The levels of phosphorylation of MAPKs are expressed as a ratio of phosphorylated to total form of these kinases. The quantification of smaller PARP fragment in relation to control group is expressed as % of control. * p < 0.05 in comparison to control; *** p < 0.001 in comparison to control.

MAPKs proteins are well conserved across eukaryotic organisms in which they mediate several cellular responses (Tibbles and Woodgett, 1999). Metals have been demonstrated to modulate the phosphorylation of members of MAPK family in vertebrates (Leal et al., 2006; Posser et al., 2007; Rigon et al., 2008; Lu et al., 2011). Prior to this study, however, there has been no published examination of the impact of heavy metal exposure on MAPK proteins in invertebrates. Here, we demonstrate that acute

in vivo exposure to mercury can induce stimulation in JNK and ERK phosphorylation by 50% in relation to the control group without altering p38^{MAPK} phosphorylation levels (Fig. 4). Further, using specific antibody against cleaved form of PARP we detect a smaller fragment of 89 kDa, indicating the activation of apoptotic cascade leading to cell death in response to mercury exposure (Fig. 4).

Finally, we used qPCR to quantify gene expression following 48 h exposure to 100 μ M Hg(II). We specifically examined five genes known to be involved in metal response in other organisms: SOD, catalase, NF-E2-related factor2 (Nrf2), HSP83 (which encodes *D. melanogaster* HSP90), and MPK2 (which encodes *D. melanogaster* p38^{MAPK}) using specific primers listed in Table I. HSP83 expression was 400% higher in treated flies than controls, while the expression levels of the other four genes were not significantly different between treated and control flies (Fig. 5).

DISCUSSION

The study of a variety of systems, invertebrate and vertebrate, mammalian and non-mammalian, has broadened and improved our understanding of the biochemical mechanism of chemical toxicity, advancing our knowledge of the susceptibility and molecular targets. Considering the wide spectrum of organisms that may be affected by mercury exposure, this study helps to expand and clarify knowledge about molecular targets of mercury in insects, using *D. melanogaster*. High levels of mercury have been associated with hepatotoxicity, nephrotoxicity, and neurological damage in rodents and humans (Ceccatelli et al., 2010; Jan et al., 2011). Studies carried out in mercury-polluted areas have highlighted the toxic effects of this metal on wildlife,

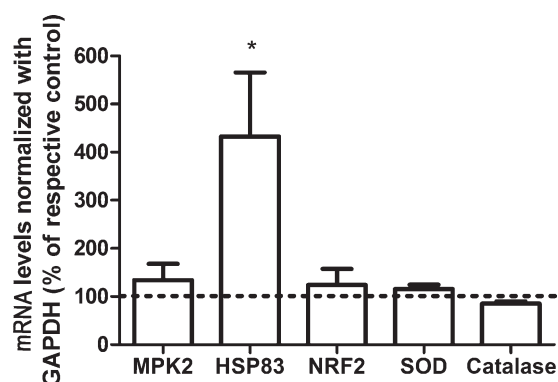


Fig. 5. Quantitative real time PCR (qRT-PCR) analysis of MPK-2, Hsp83, NRF-2, SOD, and catalase mRNA in flies exposed to Hg(II). Flies were exposed for 48 h in 100 μ M HgCl₂ solutions diluted in 2 M sucrose. We used qRT-PCR to quantify levels of mRNA, relative to respective controls, after exposure. The data were normalized against GAPDH transcript levels and each bar represents the mean \pm SEM of four independent experiments expressed as percent of its respective control (dotted line). * p < 0.05.

documenting a variety of effects including respiratory disturbances in shrimp larvae, embryotoxicity and teratogenicity in fishes, birds, and mammals (reviewed in Zahir et al., 2005). Recently, Allatia et al. (2011) demonstrated that flies were susceptible to organic mercury exposure, finding potent γ -secretase inhibition in the fruit fly *D. melanogaster* and disrupted embryonic neural development. Previous studies have shown that exposure of fruit flies to iron (Fe), manganese (Mn), and copper (Cu) caused locomotor deficits, associated with neuron degeneration in longer periods of treatments of 72 h and from the concentration of 500 μ M (Bonilla-Ramirez et al., 2011).

Here, we show that in the fly model acute Hg(II) exposure also has deleterious effects on survivability, locomotor performance, and a series of biochemical and physiological characteristics. There are relatively few previous studies of the absorption and accumulation of metal by flies, but it has been shown that *D. melanogaster* accumulate Pb when this metal is present in the diet, increasing Pb level content in the whole body (Hirsch et al., 2003). Although accumulation was found throughout the body, the midgut is considered the primary region for absorption of nutrients in *Drosophila* presenting specialized sites for absorption and accumulation of essential metals (Wang et al., 2009). We found that 48-h exposure to 100 μ M HgCl₂ resulted in an 80 \times increase in Hg(II) concentration compared to the control group. The same concentration and exposure time also caused locomotor deficits and inhibited acetylcholinesterase protein activity by 30%. In previous *in vitro* studies with purified *D. melanogaster* acetylcholinesterase, HgCl₂ caused some inhibition in this enzyme but only at mM range (Frasco et al., 2007). In contrast, in this study, *in vivo* exposure to Hg (II) caused inhibition of Ache at μ M range, emphasizing the use of Ache as a biomarker to survey heavy metals polluted areas.

The transcription factor NF-E2-related factor 2 (Nrf-2) regulates the expression of several cytoprotective enzymes, resulting in protection against toxic insults from exposure to electrophilic and oxidative chemicals, including mercury (Osburn et al., 2008; Rand et al., 2009). Nrf2-regulated antioxidant enzymes include glutathione reductase, peroxiredoxin, thioredoxin and thioredoxin reductase, catalase, superoxide dismutase, and glutathione peroxidase. Nrf2 is also implicated in controlling the inducible expression of a number of enzymes responsible for the synthesis of GSH including GSH-S-transferases (Osburn et al., 2008). In this study, we find that mercury exposure did not alter gene expression of transcription factor NRF-2, SOD, or catalase, in the flies, but did cause significant reductions in the SOD and GST enzyme, suggesting regulation may be through post-translational regulation of enzyme activity not gene expression.

We do find significant evidence of regulation of at least one gene; mercury exposure induced a 450% increase in expression of HSP83 gene, the *Drosophila* HSP90 homolog. The HSP83 protein physically associates with Raf protein

(Van der Straten and Rommel, 1997) contributing positively to Raf activation. The activation of receptor tyrosine kinase (RTK) in response to external stimulus lead to Ras activation. This kinase recruits the protein Raf to the plasma membrane stimulating its kinase activity and inducing MAPKs phosphorylation and translocation to the nucleus, leading to phosphorylation of transcription factors and consequent gene expression (Van der Straten and Rommel, 1997). In agreement with this, the present study demonstrates a significant stimulation in phosphorylation of MAPKs ERK and JNK, without altering p38^{MAPK} phosphorylation.

MAPK proteins participate of cell signaling pathways, mediating several cell responses including cell differentiation, death, and survival. ERK protein regulates cell growth and cell differentiation, and ERK phosphorylation may be induced by oxidative stress (Ho et al., 2007; Posser et al., 2009). Activation of JNK and p38^{MAPK}, in contrast, primarily regulates apoptosis, inflammation, and the differentiation process (Matsuoka and Igisu, 2002). Furthermore, MAPKs have been shown to regulate the meiotic cell cycle in both marine invertebrates and vertebrates (Sackton et al., 2007). Previous research has also demonstrated that metals, including Hg(II), are able to modulate the phosphorylation of MAPKs in vertebrates and this phosphorylation has been correlated with toxic effects of metals observed in *in vivo* and *in vitro* models of metal toxicity (Posser et al., 2007; Rigon et al., 2008; Hasse et al., 2011).

Mercury chloride has been shown to increase JNK phosphorylation in LLC-PK1 cells (Matsuoka and Igisu, 2002) and JNK, in combination with p38^{MAPK}, has been associated with Hg induced apoptotic cell death in human kidney cells (Hao et al., 2009). It has also been recently demonstrated that JNK has a role in regulation of the cytoskeleton and cell shape remodeling associated with stretch in *Drosophila* cells (Pereira et al., 2011). Treatment with organic mercury has also been demonstrated to induce cell death in vertebrate neurons, and this induction could be avoided by blocking ERK and p38^{MAPK} phosphorylation with antioxidant treatment, implicating both kinases in the cytotoxic effects incited by mercury presence in the medium (Lu et al., 2011). Our study extends this work to also suggest a possible role in toxic response to mercury exposure in invertebrates for MAPK. Further studies are necessary to clarify the signaling cascades modulated by Hg(II) in *Drosophila* and how important is this modulation for Hg(II) toxicity.

Our results demonstrating that Hg(II) exposure induced inhibition of antioxidant enzyme activity, overproduction of reactive oxygen species (ROS), and lipid peroxidation in flies are particularly notable. Various neurodegenerative conditions resulting from metal-induced neurotoxicity appear to be the result of oxidative stress (i.e. an excess of ROS), triggering a MAPK-mediated cell death process (Lu et al., 2011). The results presented here extend the known molecular targets of mercury-based cytotoxicity.

Overall, this study shows that contamination with Hg(II) affects biochemical and behavioral parameters in a well characterized model of insect showing that organisms other than vertebrates are potential targets for heavy metal contamination. Moreover, this study reinforces the use of *D. melanogaster* as a model for toxicological analysis, including for Hg(II) exposure.

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