

**Original article:**

***DROSOPHILA MELANOGASTER* – AN EMBRYONIC MODEL FOR  
STUDYING BEHAVIORAL AND BIOCHEMICAL EFFECTS OF  
MANGANESE EXPOSURE**

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**ABSTRACT**

Embryonic animals are especially susceptible to metal exposure. Manganese (Mn) is an essential element, but in excess it can induce toxicity. In this study we used *Drosophila melanogaster* as an embryonic model to investigate biochemical and behavioral alterations due to Mn exposure. Flies were treated with standard medium supplemented with MnCl<sub>2</sub> at 0.1 mM, 0.5 mM or 1 mM from the egg to the adult stage. At 0.5 mM and 1 mM Mn, newly eclosed flies showed significantly enhanced locomotor activity when assessed by negative geotaxis behavior. In addition, a significant increase in Mn levels ( $p < 0.0001$ ) was observed, while Ca, Fe, Cu, Zn and S levels were significantly decreased. A significant drop in cell viability occurred in flies exposed to 1 mM Mn. There was also an induction of reactive oxygen species at 0.5 mM and 1 mM Mn ( $p < 0.05$ ). At 1 mM, Mn increased Catalase ( $p < 0.005$ ), Superoxide Dismutase ( $p < 0.005$ ) and Hsp83 ( $p < 0.0001$ ) mRNA expression, without altering Catalase or Superoxide Dismutase activity; the activity of Thioredoxin reductase and Glutathione-S-transferase enzymes was increased. Mn treatment did not alter ERK or JNK1/2 phosphorylation, but at 1 mM caused an inhibition of p38<sup>MAPK</sup> phosphorylation. Together these data suggest mechanisms of adaptation in the fly response to Mn exposure in embryonic life.

**Keywords:** Manganese, *Drosophila melanogaster*, MAPK, oxidative stress, Thioredoxin reductase

## INTRODUCTION

Manganese (Mn) is an essential element for living organisms. It is the twelfth most abundant element in earth's crust and is present in rocks, water, soil and food, normally associated with other elements (Santamaria, 2008; Farina et al., 2013). Environmental and occupational exposure to Mn may occur by contact with fungicides, such as Maneb, Manconzeb, methylcyclopentadienyl manganese tricarbonyl (MTT)-an anti-knock agent in gasoline, Mn-ore mining, Mn alloy production and dry alkaline battery manufacture (Mergler and Baldwin, 1997; Mergler, 1999).

Dietary ingestion is the main source of Mn for humans and Mn absorption takes place mostly in the gastrointestinal tract where it is homeostatically controlled in the intestinal wall (Au et al., 2008). The brain is especially susceptible to metal intoxication during embryonic development, when it is known that Mn is able to cross the placenta and to be excreted in the maternal milk (Betharia and Maher, 2012). Mn absorption is increased during the neonatal period, when biliary excretion is poorly developed, leading to elevated concentrations of Mn in the brain and other tissues (Aschner and Aschner, 2005). In children, Mn exposure is associated with alterations in psychomotor and cognitive development; furthermore a positive correlation exists between Mn exposure and hyperactivity (Menezes-Filho et al., 2011; Roels et al., 2012; Torres-Agustín et al., 2013).

Exposure to high levels of Mn can lead to pathological conditions, including neurodegeneration (Mergler et al., 1994). The mechanisms mediating Mn toxicity are complex and not completely understood. Some of them include:

- (1) Mn accumulation in astrocytes leading to disruption of their ability to promote neuronal differentiation and decreasing glutamate uptake by astrocytes (Erikson and Aschner, 2003; Giordano et al., 2009);
- (2) Mn induced- loss of dopaminergic neurons (Stanwood et al., 2009);

- (3) Inhibition of respiratory chain complexes and induction of reactive oxygen species (ROS) (Zhang et al., 2004; Sriram et al., 2010).

The use of alternative models in toxicological studies has been growing over the years. The fruit fly *Drosophila melanogaster* has served as a unique and powerful model for studies on human genetics and diseases. Although humans and flies are only distantly related, almost 75 % of disease related genes in humans have functional orthologs in the fly (Deepa et al., 2009; Pandey and Nichols, 2011). Moreover, the fast and external developmental cycle of this organism enable the study of toxicological effects of compounds during the developmental period. All these advantages make flies an appropriate model for studies related with metal toxicity (Bonilla-Ramirez et al., 2011; Paula et al., 2012) and human neurodegeneration (Hirth, 2010).

As the embryonic development period is particularly sensitive to Mn exposure, in this paper we aimed to investigate the behavior and biochemical alterations caused by Mn exposure during the embryonic development of *Drosophila melanogaster*, focusing on adaptations in the antioxidant systems and MAPK signaling pathways. The levels of Mn and major essential elements were also determined.

## MATERIALS AND METHODS

### Reagents

Anti-phospho-p38<sup>MAPK</sup>, anti-phospho JNK, anti-phospho ERK, anti ERK and  $\beta$ -actin antibodies were purchased from Cell Signaling Technology (Danvers, MA, United States). EDTA (CAS 60-00-4), glycine (CAS 56-40-6), tris(hydroxymethyl) aminomethane (CAS 77-86-1) and ammonium persulfate (CAS 7727-54-0) were purchased from Serva (Heidelberg, Germany). L-Glutathione reduced (CAS 70-18-8), 1-chloro-2,4-dinitrobenzene (CAS 97-00-7), sodium orthovanadate (CAS 13721-39-6), manganese (II) chloride tetrahydrate (CAS 13446-34-9),  $\beta$ -mercaptoethanol (CAS 60-24-2), methanol

(CAS 67-56-1), tween 20 (CAS 9005-64-5), potassium phosphate dibasic (CAS 7758-11-4), potassium phosphate monobasic (CAS 7778-77-0), potassium bicarbonate (CAS 298-14-6) and anti-rabbit immunoglobulin antibody, *N,N,N',N'*-Tetramethylethylenediamine (CAS 110-18-9), quercetin (CAS 117-39-5), protease inhibitor cocktail for use with mammalian cell and tissue extracts, 5,5'-dithiobis(2-nitrobenzoic acid) (CAS 69-78-3), 2',7'-dichlorofluorescein diacetate (CAS 2044-85-1), glycerol (CAS 56-81-5), resazurin sodium salt (CAS 62758-13-8), triton x-100 (CAS 9002-93-1), sodium chloride (CAS 7647-14-5), albumin from bovine serum (CAS 9048-46-8), HEPES (CAS 7365-45-9),  $\beta$ -nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt were obtained from Sigma Aldrich (St. Louis, MO, United States). Bis-acrylamide, hybond nitrocellulose, acrylamide (CAS 79-06-1), sodium dodecyl sulfate (CAS 151-21-3), boric acid (CAS 10043-35-3) were purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). All other reagents were commercial products of the highest purity grade available.

### **Animals**

*Drosophila melanogaster* (Harwich strain) was obtained from the National Species Stock Center, Bowling Green, OH, USA. The flies were maintained at 25 °C on 12 h light/dark cycle in glass bottles containing 10 mL of standard medium (mixture of 39 % coarse and 32 % medium corn flour, 10 % wheat germ, 14 % sugar, 2 % milk powder, 1 % salt, 1 % soybean flour, 1 % rye flour, a pinch of methyl paraben (99-76-3) and lyophilized yeast. All experiments were performed with the same strain, and both genders were used at random.

### **Animal treatment**

Adults flies were placed in 10 mL of standard medium supplemented with 3 mL of a fresh solution (0.1 mM, 0.5 mM or 1 mM) of manganese chloride (MnCl<sub>2</sub>). In the control group the standard medium were

supplemented with 3 mL of ultrapure water. After ten days laying eggs the adult flies were removed. When eggs were newly eclosed, 1 to 3 day old flies were used for all analyses. The MnCl<sub>2</sub> concentrations were chosen based on previous studies (Bonilla-Ramirez et al., 2011).

### **Locomotor assay**

Locomotor activity was determined using the negative geotaxis assay as described by Bland et al. (2009) with minor modifications. Briefly, for each assay, individual flies (1-3 days old) were immobilized on ice and placed separately in a glass tube; this method of immobilization does not affect fly neurology (Deepa et al., 2009). After 15 minutes the flies were gently tapped to the bottom of the tube and the time required to climb up 8 cm of the tube wall was recorded. Each fly was tested 4 times at 1 minute intervals. For each experiment, the climbing mean was calculated.

### **Metal content**

Two hundred flies per group were washed three times in ultrapure water and then dried on a filter paper in the incubator at 37 °C for 90 minutes. Flies were digested in closed vessels according to the procedure described previously by Bizzi et al. (2010). Flies (~ 70 mg) were transferred to quartz vessels with 6 mL of nitric acid 3 mol L<sup>-1</sup>. After closing and capping the rotor, the vessels were pressurized with 7.5 bar of oxygen using the valve originally designed for pressure release after conventional acid sample digestion. Then, the rotor was placed inside a microwave oven (Multiwave 3000 Microwave Sample Preparation System, Anton Paar, Graz, Austria). The system was equipped with eight high-pressure quartz vessels (volume of 80 mL, maximum operational temperature and pressure of 280 °C and 80 bar, respectively). Pressure was monitored in each vessel during all the runs. Microwave heating program was as follows:

- (1) 1000 W, with a ramp of 5 min;
- (2) 1000 W for 10 min; and

(3) 0 W for 20 min (cooling step).

After digestion, the pressure in each vessel was carefully released. The resulting solutions were transferred to polypropylene vials and diluted to 25 mL with water. Determination of calcium (Ca), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), phosphorus (P), sulfur (S), and zinc (Zn) was performed using an inductively coupled plasma optical emission spectrometer (Optima 4300 DV, PerkinElmer, Shelton, USA) with axial view configuration. A concentric nebulizer and cyclonic spray chamber were used. Argon 99.996 % (White Martins, São Paulo, Brazil) was used for plasma generation, nebulization and as auxiliary gas. The instrumental parameters were carried out in accordance with previous work (Pereira et al., 2013). Two readings were averaged to give one value per biological replicate and expressed as a mean ( $\pm$ ) standard deviation of the mean (SD). Metals levels were expressed relative to the weight of flies used for analysis ( $\mu\text{g}$  metal/g of dried weight tissue).

### **Cellular viability**

Cellular viability was measured by two different methods. Firstly, cellular viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay as described by Sudati et al. (2013) with minor modifications. The analysis was performed on the whole body of female flies. The flies were incubated in MTT for 60 min (37 °C), after MTT was removed the sample was incubated in DMSO for 30 min (37 °C). The absorbance from formazan dissolution by addition of DMSO was monitored in an EnsPire<sup>R</sup> multimode plate reader (PerkinElmer, USA) at 540 nm.

The second method used was the resazurin reduction assay. The method uses the indicator resazurin to measure the metabolic capacity of cells. Viable cells reduce resazurin into resorufin, a fluorescent compound (Franco et al., 2009). Groups of 40 flies were mechanically homogenized in 1 mL 20 mM

Tris buffer (pH 7.0) and centrifuged at 1,000 RPM for 10 min at 4 °C. The supernatant was incubated in Elisa plates with 20 mM Tris buffer (pH 7.0) and resazurin for two hours. The fluorescence was recorded using EnsPire<sup>R</sup> multimode plate reader (Perkin Elmer, USA) at  $\text{ex}579\text{nm}$  and  $\text{em}584\text{nm}$ .

### **DCF-DA oxidation assay**

Groups of 20 flies were mechanically homogenized in 1 mL 20 mM Tris buffer (pH 7.0), and centrifuged at 1,000 RPM for 10 min, 4 °C. The supernatant was used to quantify 2',7'-dichlorofluorescein diacetate (DCF-DA) oxidation as a general index of oxidative stress as described by Perez-Severiano et al. (2004). The fluorescence emission of DCF resulting from DCF-DA oxidation was monitored at regular intervals ( $\text{ex}488\text{nm}$  and  $\text{em}530\text{nm}$ ) in an EnsPire<sup>R</sup> multimode plate reader (PerkinElmer, USA).

### **Determination of gene expression by real-time quantitative PCR (qPCR)**

Real-time quantitative PCR (qRT-PCR) was performed according to the method described by Paula et al. (2012). The primers utilized are shown in Table 1. All samples were analyzed as technical and biological triplicates with a negative control. Threshold and 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  $2^{-\Delta\Delta\text{CT}}$  method (Livak and Schmittgen, 2001). The GPDH gene was used as an endogenous reference showing no alterations in response to the treatment. For each well, analyzed in quadruplicate, a  $\Delta\text{C}_\text{T}$  value was obtained by subtracting the GPDH  $\text{C}_\text{T}$  value from the  $\text{C}_\text{T}$  value of the interest gene (sequences of tested genes are represented in Table 1). The  $\Delta\text{C}_\text{T}$  mean value obtained from the control group of each gene was used to calculate the  $\Delta\Delta\text{CT}$  of the respective gene ( $2^{-\Delta\Delta\text{CT}}$ ).

**Table 1:** Genes tested by quantitative real-time RT-PCR analysis and used forward and reverse primers

Genes	Primer sequences
GPDH	LEFT 5' ATGGAGATGATTTCGCTTCGT 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'

**Enzyme assays**

For enzyme activity measurements, groups of 40 flies were mechanically homogenized in 1 mL 20 mM HEPES buffer (pH 7.0), and centrifuged at 14.000 RPM for 30 min at 4 °C (Franco et al., 2009). The supernatant was used for determination of Glutathione S-Transferase (GST), Catalase (CAT), Superoxide Dismutase (SOD) and Thioredoxin Reductase (TrxR).

The GST 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 340nm. 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. CAT activity was assayed following the clearance of H<sub>2</sub>O<sub>2</sub> at 240 nm in a reaction media containing 50 mM phosphate buffer (pH 7.0), 0.5 mM EDTA, 10 mM H<sub>2</sub>O<sub>2</sub>, 0.012 % TRITON x100 as described by Aebi (1984). SOD activity assay was performed as described by 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 μM quercetin. TrxR activity was assayed as described by Holmgren and Björnstedt (1995). The test is based on the reduction of oxidized thioredoxin (Trx-S<sub>2</sub>) to reduced thi-

oredoxin [Trx-(SH)<sub>2</sub>], using NADPH at 412nm in a reaction media containing 0.1M phosphate buffer (pH 7.0), 10 mM EDTA, 5 mM DTNB, 0.2mg/mL BSA, 0.2 mM NADPH. All enzyme activities were performed at room temperature (25 ± 1 °C) using a Thermo Scientific Evolution 60s UV-Vis spectrophotometer. The enzyme activities were expressed in milliunits per milligram of total protein content, which was quantified following Bradford (1976).

**Western blotting**

Quantification of the phosphorylation of mitogen-activated protein kinases (MAPKs) was performed by Western blotting as described by Posser et al. (2009) with minor modifications. Groups of 40 flies were mechanically homogenized at 4 °C in 200 μL of buffer (pH 7.0) containing 50 mM Tris, 1 mM EDTA, 20 mM Na<sub>3</sub>VO<sub>4</sub>, 100 mM sodium fluoride and protease inhibitor cocktail. The homogenate were centrifuged at 4000 RPM for 10 min at 4 °C and the supernatants collected. After protein determination following Bradford (1976), 4 % SDS solution, β-mercaptoethanol and glycerol was added to samples to a final concentration of 100, 8 and 25 %, respectively and the samples frozen for further analysis. Proteins were separated using SDS-PAGE with 10 % gels, and then electrotransferred to nitrocellulose membranes (Paula et al., 2012). 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 at 4 °C with specific primary antibodies (anti-phospho-p38<sup>MAPK</sup>, anti-phospho JNK, anti-phospho ERK, anti ERK and anti  $\beta$ -actin). Following incubation, membranes were washed in tris-buffered saline with Tween and incubated for 1 h at 25 °C with anti rabbit-IgG secondary specific antibodies. Antibody binding was visualized using the ECL Western Blotting substrate Kit (Promega). Band staining density was quantified using the Scion Image software (Scion Image for Windows) and expressed as the percentage (%) of the control group (mean  $\pm$  standard deviation of the mean). The values were normalized using total proteins (total ERK and  $\beta$ -actin).

### Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. Pearson's correlation test was applied for detection of significant statistical differences among the metals. Differences were considered statistically significant when  $p < 0.05$ . GraphPad Prism 5 Software was used for artwork creation.

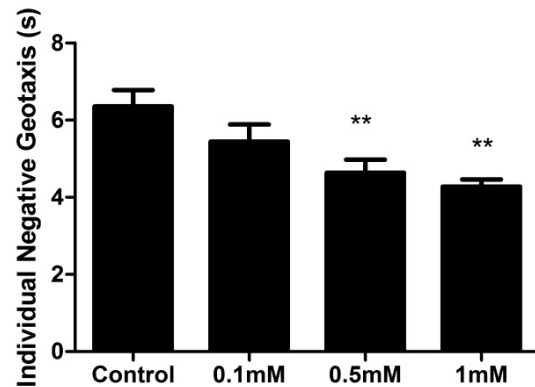
## RESULTS

### Exposure to Mn causes hyperactive behaviors and alters metal levels in *Drosophila melanogaster*

The evaluation of climbing behavior performance by negative geotaxis showed that flies exposed to 0.5 mM and 1 mM of Mn

reached the limit of columns significantly ( $p < 0.005$ ) faster than controls (Figure 1).

Levels of Mn and other essential metals were measured in *D. melanogaster* exposed to Mn. At concentrations of 0.5 and 1 mM, the levels of Mn in flies increased almost two and three fold respectively when compared with the control group (Table 2), whereas Ca, Cu, Na and Zn levels decreased significantly in flies treated with Mn. Statistically, a significant negative relationship between Mn uptake and levels of Ca ( $r = -0,7966$ ), Fe ( $r = -0,6635$ ), Cu ( $r = -0,8028$ ), S ( $r = -0,6018$ ) and Zn ( $r = -0,9802$ ) occurred in response to Mn treatment (Table 3).



**Figure 1:** Effects of exposure to Mn during the embryonic development on locomotor performance in *Drosophila melanogaster*. Results are expressed as mean ( $\pm$ ) standard deviation (SD) and represent the time spent to climb up to 8cm in a glass tube. (n = 20-24). \*\* indicates a significant effect of Mn in comparison to control group ( $p < 0.005$ ).

**Table 2:** Metal levels in flies Mn-exposed during the embryonic development

	Ca	Cu	Fe	K	Mg	Mn	Na	P	S	Zn
<b>Control</b>	494 $\pm 21$	15,3 $\pm 0,5$	290 $\pm 3$	8505 $\pm 83$	1001 $\pm 1$	35 $\pm 0,2$	2188 $\pm 3$	9505 $\pm 14$	5049 $\pm 44$	286 $\pm 1$
<b>0.1 mM</b>	471 $\pm 13$	16,3 $\pm 0,5$	303 $\pm 7$	6945 $\pm 58$	956 $\pm 3$	37 $\pm 0,8$	1674 $\pm 5^{***}$	9112 $\pm 28$	5071 $\pm 43$	289 $\pm 8$
<b>0.5 mM</b>	445 $\pm 45$	14 $\pm 0,4$	266 $\pm 17$	8190 $\pm 657$	947 $\pm 80$	63 $\pm 3,2^{***}$	1813 $\pm 136^{***}$	8990 $\pm 638$	4784 $\pm 347$	261 $\pm 19$
<b>1 mM</b>	405 $\pm 4^*$	13 $\pm 0,3^*$	269 $\pm 5$	8508 $\pm 126$	931 $\pm 4$	101 $\pm 1^{***}$	1881 $\pm 10^{**}$	9197 $\pm 100$	4748 $\pm 82$	239 $\pm 4^{**}$

Effect of exposure to Mn during embryonic development on metal levels of *Drosophila melanogaster*. Value were showed in  $\mu\text{g}\cdot\text{g}^{-1}$ . Results were expressed as a mean ( $\pm$ ) standard deviation (n = 2). \*, \*\* and \*\*\* indicates a significant difference in comparison to control group ( $p < 0.05$ ,  $p < 0.005$  and  $p < 0.0001$ , respectively).

**Table 3:** The relationship between Mn concentration and metal levels

Variables	Correlation coefficient	P-value
Ca	-0.7960**	0.0020
Fe	-0.6635*	0.0187
Cu	-0.8028**	0.0017
K	0.4581	0.1342
Mg	-0.4457	0.1465
Na	-0.1368	0,6715
P	-0.1453	0.6522
S	-0.6018*	0.0384
Zn	-0.8902***	0.0001

Using Pearson correlation coefficient test we evaluated the relationship between Mn concentration and other metal concentrations (n = 12)

\*, \*\* and \*\*\* indicate a significant negative correlation between Mn levels and Ca, Fe, Cu, S and Zn ( $p < 0.05$ ,  $p < 0.005$  and  $p < 0.0001$ , respectively).

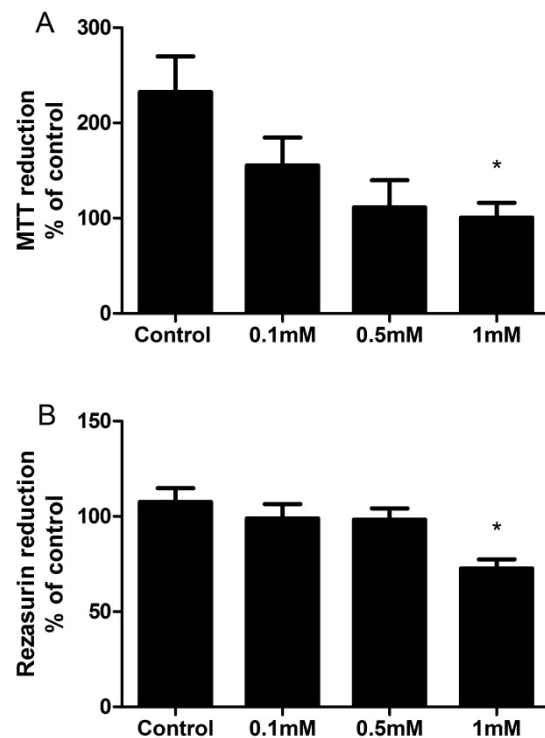
### ***Mn exposure during embryonic development decreased cell viability and increased ROS production in flies***

Cell viability was evaluated through two different tests, MTT and Resazurin. Both procedures showed a significant drop in cell viability at higher concentrations of Mn, confirming its toxicity at the cellular level (Figure 2A and 2B). Many factors have been implicated in Mn-induced neurotoxicity, among them the oxidative stress caused by dopamine oxidation, or its ability in interfering with cellular respiration (Aschner and Aschner, 2005). In this study, exposure to Mn leads to an increase in DCF-DA oxidation, a general index of oxidative stress from 0.5 mM (Figure 3).

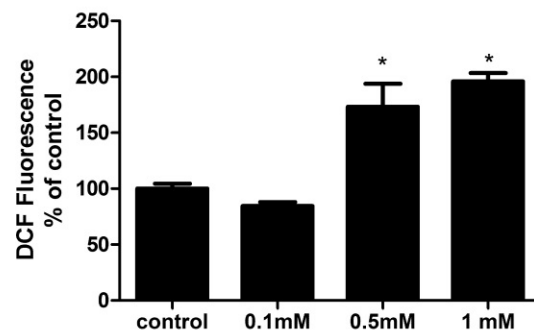
### ***Mn increased CAT and SOD mRNA expression, without altering their enzymatic activity***

Expression of mRNA for Cat, Sod and Hsp83 (an homolog of mammalian HSP90) (Bandura et al., 2013) was analyzed by qRT-PCR using specific primers (Table 1) in flies treated with 1 mM Mn.

CAT and SOD mRNA expression was 250 % higher in treated flies whereas Hsp83 expression was 1000 % higher (Figure 4).



**Figure 2:** Effect of Mn exposure on cell viability in homogenate of flies treated with metal over embryonic development performed by MTT (A) and Resazurin (B) cell viability assays. For MTT, the average of absorbance in control group was 0.017 (n = 3-4); by Resazurin, absorbance in control group was 229.03 (n = 4-5). Results were expressed as the percentage (%) of the control group (mean  $\pm$  standard deviation) \* indicates a significant effect of Mn in comparison to control group ( $p < 0.05$ ).



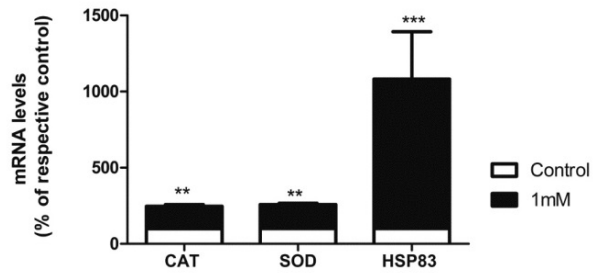
**Figure 3:** Analysis of ROS production in response to Mn-exposure during embryonic development in flies. The data showed the DCF-DA intensity of fluorescence in total flies homogenate, expressed as percentage of the control group (mean  $\pm$  standard deviation) (n = 3-10). \* indicates a significant effect of Mn in comparison to control group ( $p < 0.05$ ).



The levels of the antioxidant enzymes activity TrxR, GST, SOD and CAT were determined. TrxR and GST activity were increased at concentrations of 0.5 mM and 1 mM (Figure 5), while CAT and SOD activity showed no significant differences.

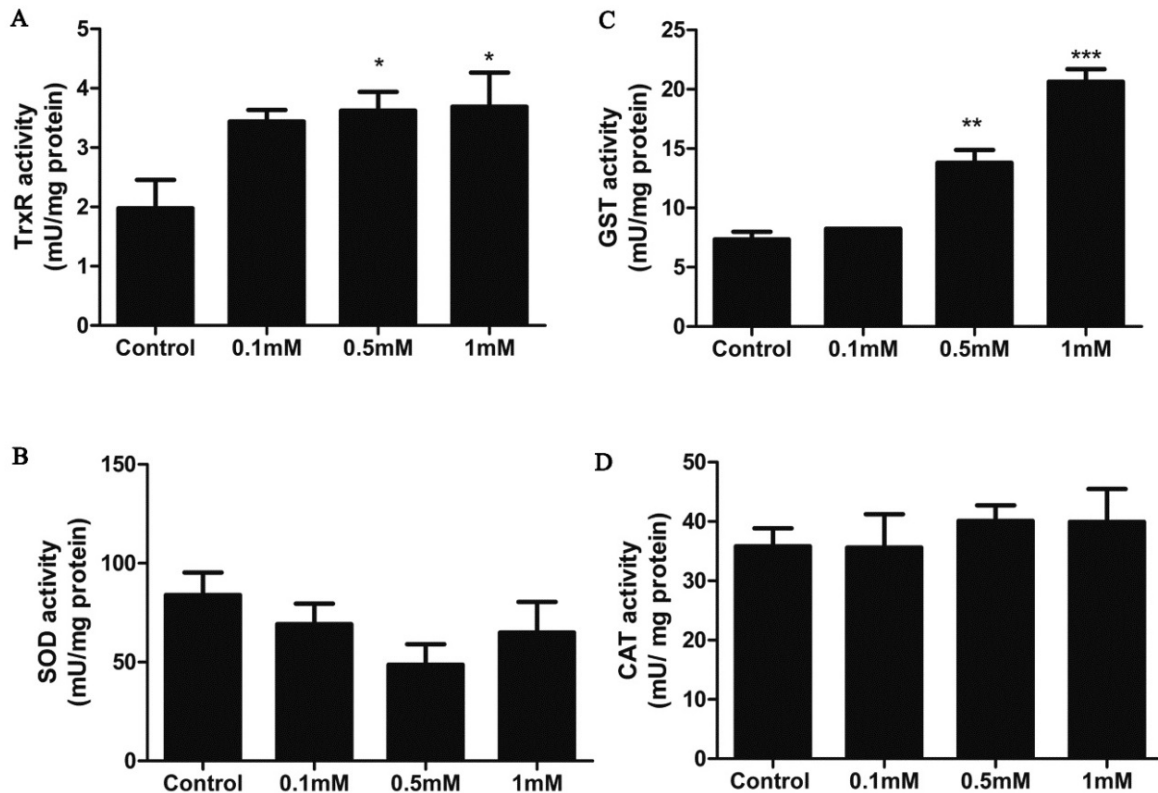
### Mn exposure inhibited p38<sup>MAPK</sup> phosphorylation

MAPKs phosphorylation levels were investigated in flies exposed to Mn. There was a 40 % inhibition of p38<sup>MAPK</sup> phosphorylation in flies exposed to Mn at 1 mM, while the phosphorylation level of extracellular signal-regulated kinases (ERK) was unaltered. C-Jun-N-terminal Kinases 2 (JNK2) phosphorylation was not statistically different from controls (Figure 6).



**Figure 4:** Quantitative real time PCR (qRT-PCR) analysis of CAT, SOD and HSP83 mRNA in flies Mn-exposed at 1 mM. We used qRT-PCR to quantify levels of mRNA, relative to respective controls, after exposure. The data were normalized against GPDH transcript levels and each bar represents the mean  $\pm$  standard deviation expressed as percent of its respective control (n = 3-4)

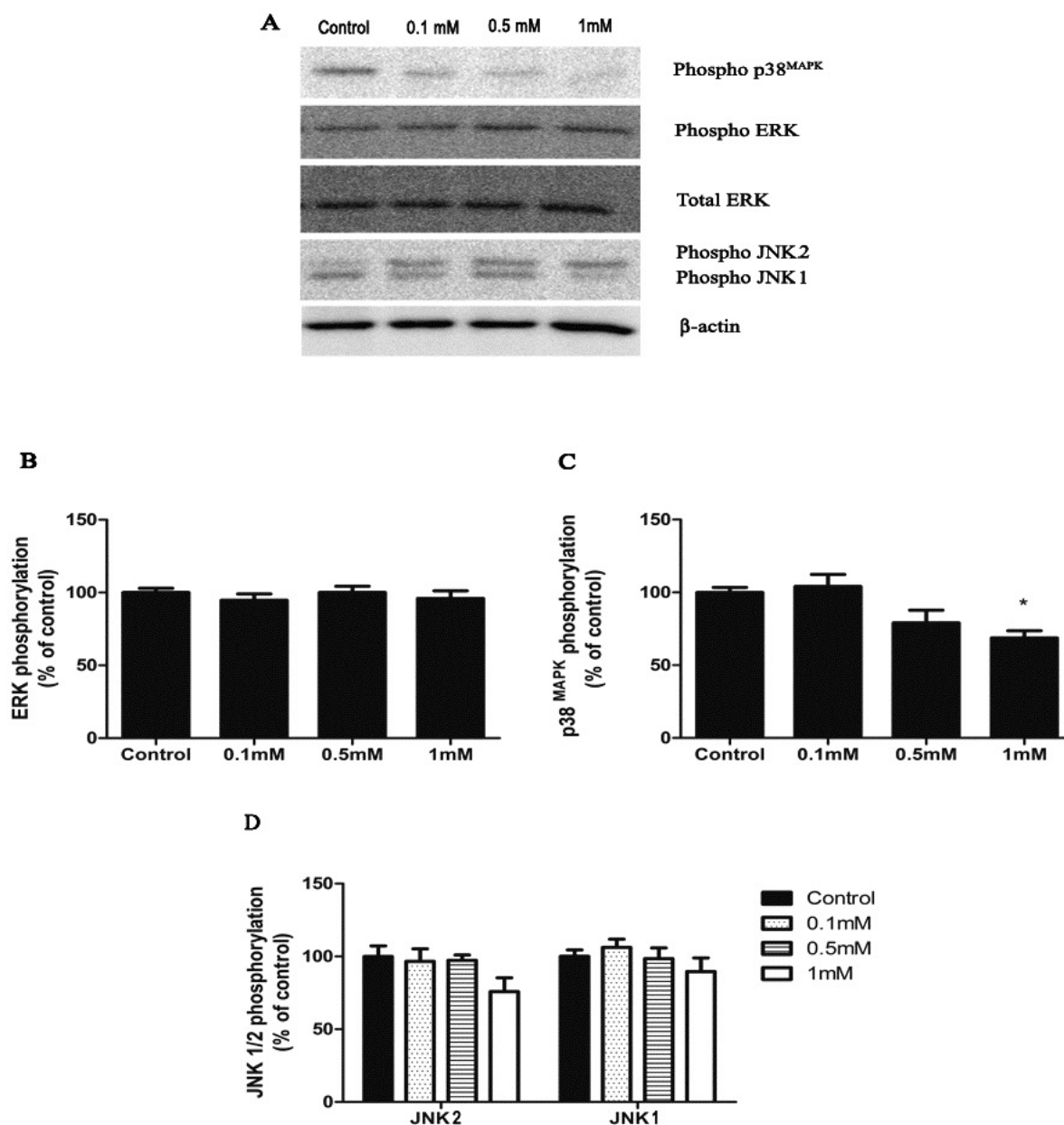
\*\* and \*\*\* indicate a significant effect of Mn in comparison to control group ( $p < 0.005$  and  $p < 0.0001$ , respectively).



**Figure 5:** Effects observed on enzymatic activities in flies Mn-exposed during embryonic development. TrxR activity (n = 5-7) (A), GST activity (n = 3-4) (B), SOD activity (n = 6-8) (C) and CAT activity (n = 5-6) (D). The data shows the enzymatic activities in flies homogenate expressed as mean (mU/mg protein)  $\pm$  standard deviation.

\* and \*\*\* indicate a significant effect of Mn in comparison to control group ( $p < 0.05$  and  $p < 0.0001$ , respectively).





**Figure 6:** Modulation of MAPKs phosphorylation in response to Mn-exposed flies during the embryonic development. The upper panel is a western blot showing phosphorylated forms of p38<sup>MAPK</sup>, ERK and JNK 1/2 and total forms of ERK and β-actin (A). The graphs are showing the quantification (percentage of control) of immunoreactive bands, each bar represents the mean ± standard deviation. ERK phosphorylation was normalized with total ERK expression (n=5) (B). p38<sup>MAPK</sup> phosphorylation was normalized with β-actin expression (n=5) (C). JNK 1/2 phosphorylation was normalized with β-actin expression (n = 3-5) (D).

\* Indicates a significant effect of Mn in comparison to control group (p < 0.05).

## DISCUSSION

In this study, we determined the biochemical and behavioral alterations in *Drosophila melanogaster* in response to Mn exposure during embryonic development. Mn

is an essential element required in key biological processes; however, high levels of Mn are associated with neurological and neuropsychiatric disorders (Mergler, 1999). The risk of Mn overexposure comes from both occupational and environmental sources

(Mergler and Baldwin, 1997). Mn intoxication, a syndrome known as Manganism, is characterized by an extrapyramidal dysfunction and neuropsychiatric symptomatology and is associated with prolonged occupational exposure to high concentrations of this metal. Classical symptoms include irritability, intellectual deficits, compulsive behaviors, tremors and cock-like walk (Mergler, 1999; Roth, 2006).

In rodents, Krishna et al. (2014) showed that adult mice exposed to Mn through the drinking water presented neurobehavioral deficits and glial activation related with Mn deposition in brain. Moreover, others studies demonstrated that Mn toxicity in rats is accompanied by increased cholesterol biosynthesis and impairments in neuronal function of the hippocampus, which is involved in learning and memory (Öner and Sentürk, 1995; Sentürk and Öner, 1996). It has been shown that Mn supplementation during the neonatal period of rats resulted in increased Mn concentrations in tissues leading to adverse effects on motor development and behavior (Tran et al., 2002).

Mn uptake is increased during the neonatal period as biliary excretion, which has been suggested as a pathway for Mn elimination from the body, is poorly developed at this stage (Aschner and Aschner, 2005). Exposure to Mn during the embryonic and early postnatal periods may result in increased levels of Mn in the brain and other tissues including bone, liver, pancreas and kidney (Aschner and Aschner, 2005; Roels et al., 2012). Higher levels of Mn retention *in utero* may affect children's psychomotor development (Takser et al., 2003). Possible adverse effects of Mn exposure on children's health include cognitive deficits and hyperactive behaviors (Menezes-Filho et al., 2009; Torres-Agustín et al., 2013). Children exposed to high levels of Mn during the fetal period were more impulsive, inattentive, aggressive, defiant, disobedient, destructive and hyperactive (Ericson et al., 2007). It is recognized that factors such as the source and the duration of exposure, as well as nutritional status,

can interfere in the intensity and incidence of neurological symptoms associated with Mn exposure in humans. Chronic consumption of drinking-water containing Mn at levels ranging from 81 to 2300 µg/l was associated with progressively higher prevalence of neurological symptoms (Kondakis et al., 1989). The concentrations used in this study were from 0.1 mM of Mn in food (corresponding to 19 mg/L in the medium). Despite the use of relatively elevated concentrations, body levels of Mn were not altered at 0.1 mM. In previous studies, adult flies were acutely exposed to Mn (0.5-20 mM) diluted in sucrose, as the only source of food and liquid, which lead to significant locomotor deficits (Bonilla-Ramirez et al., 2011). Our study is the first where Mn was provided as a cereal based diet over all the embryonic period. Thus, more studies are necessary to understand the rate of Mn uptake from diet in flies and how it may affect neurological behaviour.

In our study, flies exposed to Mn at 0.5 mM and 1 mM showed increased locomotor speed in the locomotor behavior test (assayed as negative geotaxis behavior), pointing to a hyperactive-like behavior in *Drosophila melanogaster*. Furthermore, Mn levels were substantially increased in treated flies, while Ca, Cu, Zn, Fe and S levels were all decreased. This relationship may be in part associated with a competition of the metals for the same mechanism of transport into the flies cells. Facilitated diffusion, active transport, divalent metal transport 1 (DMT1), ZIP8 and transferrin (Tf)-dependent transport mechanisms are all involved in cellular Mn transportation (Aschner et al., 2007). Among these metal transport systems, DMT1 has a very broad substrate specificity and is likely to be the major transmembrane protein responsible for the uptake of a variety of divalent cations, including  $Mn^{2+}$ ,  $Cd^{2+}$ ,  $Zn^{2+}$ ,  $Co^{2+}$ ,  $Ni^{2+}$ ,  $Cu^{2+}$  and  $Pb^{2+}$  (Gunshin et al., 1997). In the flies, many proteins involved in the metabolism of biometals such as ferritin, transferrin, iron regulatory proteins, divalent metal transporter are ex-

pressed (Bonilla-Ramirez et al., 2011). In this context, Mn uptake is less frequently studied in comparison with other metals and the mechanisms related to Mn transport are considerably more complex, occurring in most part in the divalent (II) and oxide forms (Tebo et al., 2004). Mn has the capacity to interact and /or compete with Ca (Dittman and Buchwalter, 2010). In a study performed in the aquatic insect *Hydropsyche sparna*, Mn exposure decreased cadmium (Cd) and Zn accumulation. Furthermore, increased Ca concentrations significantly reduced Mn accumulation in the insect (Poteat et al., 2012). Dittman and Buchwalter (2010) suggested that Mn is also absorbed by Ca transporters in aquatic insects, where increasing ambient Ca concentrations decrease Mn accumulation.

There was also a negative correlation between Mn and Fe levels. Iron deficiency has been suggested as a possible contributing cause of attention deficit and hyperactivity disorder (ADHD) in children (Konofal et al., 2008). Concomitantly, children with ADHD showed elevated serum Mn concentrations (Konofal et al., 2008). Recent studies have suggested that Mn accumulates in dopaminergic neurons via the presynaptic dopamine transporter (DAT) and an altered functioning of the dopaminergic system has been well established in the etiology of ADHD (Farias et al., 2010).

Our results showed decreased cell viability using two different methodologies and increased ROS generation in flies exposed to Mn during development. Previous work by our group in PC12 cells, demonstrated that Mn leads to increased production of hydrogen peroxide ( $H_2O_2$ ) (Posser et al., 2009).  $H_2O_2$  is a highly permeable and reactive molecule being able to react with metals such as Fe, thus generating hydroxyl radicals (Jiménez Del Río M and Vélez-Pardo, 2004; Barbosa et al., 2010) resulting in a propagation of oxidative damage in cells.

Tissues can respond to oxidative stress by modulating antioxidant defenses (Halliwell and Gutteridge, 2007). We measured

the gene expression of Hsp83, CAT and SOD in *Drosophila melanogaster*. Earlier studies showed that cellular stress may induce heat shock proteins in parallel with ROS production (Kim et al., 2004; Paula et al., 2012). Our results showed a significant increase in Hsp83 mRNA levels in Mn treated flies. Previously, our group demonstrated that exposure of flies to heavy metals such as mercury causes increases in the expression of Hsp83 (Paula et al., 2012). CAT and SOD mRNA levels were significantly increased by Mn, but the enzymatic activity of these proteins was unchanged. The antioxidant enzyme SOD converts superoxide radicals ( $O_2^{\cdot -}$ ) to  $H_2O_2$  and CAT catalyzes the conversion of  $H_2O_2$  to oxygen ( $O_2$ ) and water (Barbosa et al., 2010), thus neutralizing these reactive species. Considering that both SOD and CAT are crucial in the cell defense against oxidative stress (Halliwell and Gutteridge, 2007), it might be expected that a posttranscriptional regulation mechanisms could maintain adequate levels of these proteins, however, further studies are necessary to elucidate this. Our results also showed that the activity of TrxR and GST was enhanced in Mn exposed flies. These two enzymes play an important role in protection against oxidative stress (Mustacich and Powis, 2000). GST is a complex group of phase II detoxifying enzymes that participate in the metabolism of electrophilic substances, including carcinogenic, mutagenic and toxic compounds (Hayes et al., 2005). TrxR is a dimeric FAD-containing enzyme that catalyzes the NADPH-dependent reduction of the active-site disulfide in Trx-S<sub>2</sub> to give a dithiol in Trx-(SH)<sub>2</sub> (Zhao et al., 2002). Thioredoxin consists in one of the major redox-regulating proteins displaying a number of biological activities, including cytoprotection against ROS, protein repairing and protein disulfide reduction and modulation of signaling pathways (Yan et al., 2012). Our data suggest that the increase in the levels of Mn and TrxR activity could represent a response to oxidation of thioredoxin in response to Mn-induced oxidative stress.

MAPKs regulate the activity of a range of transcription factors thereby controlling gene expression and cellular function. The three most-studied MAPKs are ERK1/2, JNK1/2 and p38<sup>MAPK</sup> (Ichijo, 1999). ASK1 (Apoptosis Signaling Kinase 1) is a member of mitogen activated protein kinase kinase family (MAPKK) and an upstream activator of MAPK signaling pathway (Yan et al., 2012). The redox state of thioredoxin regulates ASK1. Under normal conditions, ASK1 is bound to and inhibited by thioredoxin and when thioredoxin is oxidized, ASK1 can be activated and apoptotic signaling through the p38<sup>MAPK</sup>/JNK1/2 MAPKs initiated (Ichijo et al., 1997). Studies conducted by Yan et al. (2012) in a pancreatic carcinoma cell line, showed inhibition of TrxR by indolequinones resulting in a change of Thioredoxin-1 redox state to an oxidized form and activation of p38<sup>MAPK</sup> /JNK1/2. Similarly, Cd treatment activated ASK1 and its downstream MAPK in neuronal cells (Kim et al., 2005), and inhibits components of thioredoxin system (Chrestensen et al., 2000), while that Liedhegner et al. (2011) demonstrated that knockdown of ASK1 as well as chemical inhibition of p38<sup>MAPK</sup> and JNK played protective effects against L-DOPA induced apoptosis. We show that Mn induced TrxR activity while p38<sup>MAPK</sup> /JNK1/2 phosphorylation were inhibited. This suggests an involvement of thioredoxin system in the mechanism of Mn induced toxicity. Augmented TrxR activity may represent a cellular response to high levels of ROS induced by Mn exposure, thus preventing the oxidation of thioredoxin and its dissociation of ASK1. This could contribute to diminished activation of p38<sup>MAPK</sup> pathway upstream kinases resulting in lower levels of phosphorylation of this MAPK thus minimizing apoptotic cell death.

In summary, our study demonstrate for a first time that developmental exposure to Mn leads to hyperactive-like behavior and accumulation of this metal in *Drosophila melanogaster*. The observed raise in Mn

levels is negatively correlated with levels of other essential metals. This result fits with previous studies showing that Mn accumulation and Fe deficiency are associated with hyperactive behavior in children (Ericson et al., 2007; Konofal et al., 2008). The induction of stress responsive genes and antioxidant enzyme activity associated with inhibition of p38<sup>MAPK</sup> phosphorylation at higher concentrations of Mn may represent an adaptive response to oxidative stress generated by this metal, in an attempt to avoid exacerbated cellular damage.

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### CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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