Title: Acute exposure to environmentally relevant Pb levels induces oxidative stress and neurobehavioral alterations in larval zebrafish (*Danio rerio*)

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Abstract

The ubiquitous contamination of environmental lead (Pb) remains a worldwide threat. Improper Pb mine waste disposal from an abandoned lead-zinc mine has recently unearthed a widespread Pb poisoning in children in Kabwe Zambia. Although the adverse effects of Pb on human health have begun to receive attention, the ecotoxicological effects on aquatic vertebrates still need further investigation. In addition, there is paucity in the knowledge on the behavioural and molecular subcellular responses in larval zebrafish exposed to Pb within the range of environmental relevant concentration (average 3 µg/L with maximum of 94 µg/L) on aquatic organisms such as zebrafish. The adverse effects of environmentally relevant levels of Pb on larval zebrafish was evaluated by measuring swimming behaviour under alternating dark and light conditions. Larval zebrafish acutely exposed to environmentally relevant Pb exhibited neuro-behavioural alteration including enhanced hyperactivity under light conditions evidenced by increased distanced covered and speed compared to the control. The alteration of entire behavioral profiles was further associated with the disturbed expression patterns of mRNA level of key genes associated with antioxidant (HO-1, Ucp-2 and CoxI), proapoptotic gene (TP53), and antiapoptotic gene (Bcl-2). To our knowledge, this is the first report on the effects of environmentally relevant Pb levels from Kabwe, Zambia and their adverse neurobehavioural effects and subcellular molecular oxidative responses in larval zebrafish acutely exposed within a 30 minutes period. The current results would be beneficial in our understanding of the effects of low Pb levels acutely discharged into an aquatic environment and the life of aquatic organisms.

Keywords: Lead; Zebrafish larvae; Behavioral toxicology; Hormesis; Hyperactivity

1. Introduction

Lead (Pb) has been reported as the most abundant toxic metal element in the environment and is generally found in trace amounts in soils, plants, and water (Cheng and Hu, 2010; Wani et al., 2015). Although Pb is ubiquitous in aquatic environments, high levels of Pb could be attributed to anthropogenic activities including the manufacture of batteries, paint, cement, as well as mining and smelting (Kim and Kang, 2017). Because of its valuable use historically, lead was extensively mined, and very large piles of leaded soil waste tailings are still found in some parts of the world. One of the most well-known cases is a now closed lead-zinc mine that operated for over 90 years in Kabwe (Zambia) without any Pb waste management system (Ikenaka et al., 2010; Nakayama et al., 2011). Dispersion of the contaminated soils in the form of dust particles, by strong winds and occasional flooding, has led to the pollution of the city and eventual contamination of children; leaving the majority of them with blood Pb levels above the level of concern which is 5µg/dL by the Center for Disease Control and Prevention (Bose-O'Reilly et al., 2017; Yabe et al., 2020, 2015). Samples from domestic animals like cattle, goats, free range chickens and wild rodents within the vicinity of the closed mine had substantial Pb accumulation in their tissues (Nakata et al., 2016; Nakayama et al., 2011; Yabe et al., 2013, 2011). On the other hand, Pb levels in natural water bodies and ground water drawn from boreholes in the area were found to have the highest concentration of 94 µg/L from a given site with the average concentration of 3 µg/L, well below the Pb permissible values of 50 µg/L by the Zambia Bureau of Standards (Nachiyunde et al., 2013).

Lead is known to be deleterious to almost all living organisms even in smaller amounts (Pokras and Kneeland, 2008). In humans, especially children, deficits in cognitive and academic skills have been reported in blood Pb concentrations lower than 5 µg /dL (Lanphear et al., 2000). In both young animals and humans, the nervous system, especially the brain, has been widely reported as the most vulnerable to Pb toxicosis due to its rapid growth that may incorporate Pb and the immature blood brain barrier (Flora et al., 2012). Furthermore, associations between subclinical Pb toxicosis and altered behaviour such as delinquent, antisocial and aggressive behaviours in humans have been reported in several studies (Nevin, 2000; Olympio et al., 2010; Sciarillo et al., 1992). Zebrafish are increasingly being used as a model organism due to their close homology (71%) with the human genome (Howe et al., 2014) . In zebrafish, Pb exposure to various levels and at different stages of the zebrafish development has been known to induce an array of neurobehavioral derangements such as memory deficit, altered coloured preferences, altered responses to environmental stimuli such as locomotor activity patterns under light and dark illumination and sensorimotor responses among others (Chen et al., 2012; Dou and Zhang, 2011; Fraysse et al., 2006; Lefauve and Connaughton, 2017; Xu et al., 2016; Zhao et al., 2019). This neurotoxicity is suggested to arise due to direct damage to the nervous tissue through oxidative stress or alteration on the neurotransmitters and or their receptors (Lee and Freeman, 2014a; Tu et al., 2018; Weber et al., 1997). For example, Pb^{2+} arouses Ca^{2+} and calmodulin to stimulate and modulate the release of neurotransmitters in neurons (Zhong et al., 2017). In addition, Pb exposure disturbs the balance of pro-oxidants and antioxidants, causing oxidative stress and Pb poisoning (Kim and Kang, 2017). In vivo studies have suggested that Pb exposure might induce increases in antioxidant responses in fish through the production of reactive

oxygen species (ROS) (Kim and Kang, 2017; Maiti et al., 2010). Lead exposure in fish also has toxic effects on membrane structure and function owing to its high affinity to red blood cells, which increases susceptibility to oxidative stresses (Gurer and Ercal, 2000).

While environmental Pb exposure at low levels have been linked to wildlife mortality by hindering the complex mental processes and social behaviours required in general (Pokras and Kneeland, 2008) and in zebrafish a subset of low Pb level studies affecting developmental and behaviours in embryos and adult fish in particular (Chen et al., 2012; Lee and Freeman, 2014a, 2014b; Lefauve and Connaughton, 2017; Li et al., 2019a, 2019b; Tu et al., 2018; Weber et al., 1997; Zhao et al., 2019), no acute studies have examined the effect of very-low Pb exposure on the larval zebrafish neurobehavioural and oxidative stress responses. Since there is a paucity of information on the adverse effects of the reported Pb levels from water samples obtained from Kabwe, Zambia on aquatic life, this study was undertaken to fill the gap in our knowledge about the effects of low environmental Pb levels on behavioural and oxidative responses in larval zebrafish. To this end, the following questions were asked: Do acute exposure to levels of Pb, as they occur in Kabwe, causes locomotor pattern activity change under dark/light illumination? If so, are the resulting neurobehavioural changes accompanied by oxidative stress responses?

2. Materials and Methods

2.1 Fish husbandry and larviculture

Adult zebrafish from a wild-type laboratory strain specifically kept as a breeding stock were used. Fish were maintained at 26–28 ºC on a 14-hour light and 10-hour dark cycle in a ZebTec (Tecniplast, Italy) flow-through, reconstituted water system in the

National Aquatic Bioassay Facility (NABF) at North -West University, South Africa. All experimental procedures were conducted in accordance and adherence to guidelines approved by the North–West University AnimCare Ethics Committee (Ethics number NWU-00269-16-A5). Zebrafish were bred in a 60 L iSpawn breeding tank with a 1/8" nylon mesh false bottom to protect fertilized eggs from being consumed by the adults. Eggs were collected ≤ 2 hours post fertilization (hpf), counted, and placed into Pb-free, glass culture dishes containing an embryo development medium (E3 medium: each litre contains 0.875 g NaCl, 0.038 g KCl, 0.120 g $MgSO_4$, 0.021 g KH_2PO_4, and 0.006 g Na_2HPO_4). The unfertilized embryos were removed under a Zeiss stemi microscope and the viable embryos were kept in an incubator at 28 ºC in Pb-free embryo media. After 24 hpf again the embryos were examined under the stemi microscope to remove any coagulated embryos. Half of the embryo medium (E3 medium) was replaced daily with freshly oxygenated medium until the test started at 120 hours post fertilization (hpf). Larvae were not fed throughout the test.

2.2 Lead (Pb) stock solution and exposure protocols

Lead acetate trihydrate (PbAc; Purity 99.5%) was purchased from Fluka chemika (Sigma-Aldrich), Buchs, Switzerland. A Pb stock solution of 10 mg/L was prepared from PbAc in ultrapure water and stored at 4 °C. The exposure doses of Pb were prepared from the stock after appropriately diluting with Pb-free embryo media to 3 µg/L, 91 µg/L and 250 µg/L Pb concentrations, respectively. The 3 µg/L was based on the average Pb levels in water and 91 µg/L was estimated based on the maximum Pb sampled nearest to the point source (Nachiyunde et al., 2013) and 250 µg/L was the included as highest level of exposure approximately three times the maximum Pb level in Kabwe water bodies.

2.3 Acute Pb exposure, recording and analysis of locomotor behaviour in 120 hpf old larval zebrafish

Larval zebrafish, 120 hpf, reared in Pb-free embryo media were exposed to Pb at three concentrations (3 µg/L, 91 µg/L and 250 µg/L) and a control group (0 µg/L). Our choice of the age of the zebrafish larvae (120 hpf) was based on standard protocol that recommends that all behaviour studies are done between day 5-7 days post fertilization when fish are free swimming but feeding on the yolk sac (Strähle et al., 2012). The experimental design consisted of a negative control (n = 12) and exposure concentrations; 3 µg/L (n = 12), 91 µg/L (n = 12) and 250 µg/L (n =12). One larva per well was transferred to the 12 well testing plate using a plastic pipette with each well containing 3 mL of the respective exposure solution. The acute exposure was replicated four times in order to achieve twelve replicates (n=3 per group per run). The 12-well plate was placed in a Noldus DanioVision chamber (Wageningen, Netherlands) and recorded at 25 frames per second. Experiments were performed at 28 °C in embryo media at a 10-minute light: dark cycle intervals for a total time of 30 minutes. We excluded the first 2 minutes as habituation, and selected the 5^{th} minute under the first dark phase and 15 min time point in the light phase as the statistical behaviour reference point as previously described by Li et al., (2019a). In addition, zones (center and outer) within each well were setup by digitally dissecting zones per plate using Noldus EthoVision XT15 software. The exposure and recording were repeated four times while changing the position of each treatment to minimize the effect of positioning of the larvae in the DanioVision chamber. Thereafter, the larval zebrafish were sacrificed in cold ice water as approved by the North West University ethics committee and immediately preserved in 1.5 mL Eppendorf tubes

with cold RNAlater® (Ambion, South Africa). To obtain adequate pooled samples (5-10 larvae) for RNA extraction based on our pre-experimental trials, additional exposures were carried out across the groups (n=24) using same conditions and concentrations of test compound. The samples were stored at -80 °C prior to RNA extraction.

2.4 RNA extraction and real-time PCR analysis

An RNA isolation protocol was used where 5-10 pooled larvae samples were homogenized in TRI Reagent® with a zirconia bead using a tissue lyser; chloroform was added, and samples were vortexed and then centrifuged at 13,000 g for twenty minutes at 4 °C. The supernatant was then mixed with 350 µL of 70% ethanol and then placed in the FastGene® RNA binding column. Afterwards, the standard FastGene® RNA Basic kits protocol was followed for the rest of the steps. The RNA was eluted from the membrane using RNase free ultrapure MilliQ water. The RNA quality was assessed by spectrophotometry (OD 260:280 ratio) using a NanoDrop 1000A Spectrophotometer (Delaware, USA). The first strand cDNA synthesis kit ReverTra Ace-α (Toyobo) was used for cDNA synthesis according to manufacturer instructions.

The real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis (StepOnePlus Real-Time PCR System, Applied Biosystems, USA) was performed using a 10 µL PCR reaction mixture containing 5 µL of Fast SYBR Green Master Mix (Applied Biosystems, USA), 0.4 µL of 5 µM forward and reverse primers (Thermo Fisher Scientific, Life Technologies Japan Ltd., Japan), 20 ng of cDNA of each pooled zebrafish larvae samples, and 2.2 µL of distilled water. The qRT-PCR condition for all target genes was 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s, and 60 °C, 62 °C or 65 °C for 30 s depending on the annealing temperature of a given primer set as

shown in **Table 1**. Most of the primers used were obtained from published works (Jin et al., 2010; Shi and Zhou, 2010; Stancová et al., 2015) and some were generated using the NCBI and Primer3 tools. The gene expressions of various oxidative stress related genes were quantified with the relative absolute method using tubulin 1a (Tuba1) as a house keeping reference gene (**Table 1)**. The choice of Tuba I as a housekeeping gene was based on its stability and constant expressions in both control and Pb exposed groups after preliminary validation when compared with beta actin, which is in agreement with (Mccurley and Callard, 2008) in zebrafish at various developmental stages following chemical treatment. The primer efficiency range was from 96.5% to 100.4%

Table 1: Primers used for real-time qPCR with annealing temperatures used and PCR product lengths

Target gene	Primer Sequence (5'-3')	Annealing Temperature (◦C)	Product length (bp)	Reference
Tubulin alpha I (Tuba I)	**F:** TTTGTGCACTGGTACGTGGG **R:** CCACACTCTCAGCTCCAACCTC	60	112	a
Glutathione-S-transferase (GST)	**F:** ACAACCTGTTCGATCTCCTGCTGA **R:** GTTGCCGTTGATGGGCAGTTTCTT	60	161	(Jin et al., 2010)
Superoxidase dismutase (SOD2)	**F:** CGCATGTTCCCAGACATCTA **R:** GAGCGGAAGATTGAGGATTG	60	100	(Stancová et al., 2015)
Catalase (CAT)	**F:** AGTGCTCCTGACGTCCAGCCA **R:** TGAAGAACGTGCGCACCTGGG	65	115	(Jin et al., 2010)
Glutathione Peroxidase (GPX 4b)	**F:** GGACGATCCAAGCGTGGTGGA **R:** CAGCCGTCACACGTCTGGGC	60	148	(Stancová et al., 2015)
Uncoupling protein 2 (Ucp-2)	**F:** TGGCTCAACCCACTGATGTA **R:** CAATGGTCCGATATGCGTC	62	102	(Jin et al., 2010)
Heme Oxygenase 1 (HO-1)	**F:** GGAAGAGCTGGACAGAAACG **R:** CGAAGAAGTGCTCCAAGTCC	62	107	(Shi and Zhou, 2010)
B-cell lymphoma 2 (Bcl2)	**F:** AGGAAAATGGAGGTTGGGATG **R**: TGTTAGGTATGAAAACGGGTGGA	62	83	(Jin et al., 2010)
Tumour protein p53 (TP53)	**F:** CCCAGGTGGTGGCTCTTGCT **R:** GAGTGGATGGCTGAGGCTGTTCT	62	113	a
Nuclear factor erythroid 2 (Nrf2)	**F:** GACAAAATCGGCGACAAAAT **R:** TTAGGCCATGTCCACACGTA	65	165	(Shi and Zhou, 2010)
Cytochrome c oxidase subunit I (COXI)	**F:** GGATTTGGAAACTGACTTGTG **R:** AAGAAGAAATGAGGGTGGAAG	60	105	(Jin et al., 2010)

Note: a: Primers designed by the authors using NCBI and Primer3 tools. F and R represents forward primer and reverse primer sequences, respectively.

2.5 Measurement of Pb in exposure test solutions

The Pb in exposure media were measured for verification purposes. Freshly constituted Pb exposure dose solutions using 99.5% lead acetate trihydrate and the actual Pb exposure solutions that remained in wells after sacrificing the zebrafish larvae during the study were collected and analyzed. After acidification, concentration of Pb was measured using the inductively coupled plasma mass spectrometry (ICP-MS, 7700 series; Agilent Technologies, Tokyo, Japan). The instrument detection limit for Pb was 0.001 µg/L.

2.6 Data Analysis

The larval locomotor behaviour data and gene expression data were analysed using GraphPad Prism software (Prism 7 for Windows; Version 5.02, California USA). The data were reported as mean and SEM (standard error of the mean). Data were tested for criteria of normality using the Kolmogorov–Smirnov test and homogeneity of variance using Levene's test. If data were normally distributed an analysis of variance (one-way ANOVA) was performed and the differences among test groups were assessed with the Tukey's test. For non-parametric data the Kruskal–Wallis test and Dunn's Multiple Comparison test or Mann-Whitney U test was used. In our experiment the difference between groups was assessed to be significant at $P < 0.05$ (*) and $P < 0.01$ (**).

3. Results

Concentration of Pb in exposure test solutions

The nominal Pb dose represents the theoretical doses upon which the exposure solutions were prepared (0, 3, 91 and 250 µg/L) using pure grade 99.5% lead acetate trihydrate. The actual Pb concentrations detected by ICP-MS (100 – 106.7% recovery) in the exposure solutions were within an 8% range (0, 3.2, 93 and 252.6 µg/L, respectively).

3.1.1 Effect of acute Pb exposure on locomotor behaviour and molecular subcellular responses in larval zebrafish (120 hpf old *Danio rerio* larvae)

Under locomotor behaviour, we investigated the mean distance covered, the mean speed, cumulative mobility, cumulative immobility, mobile frequency, immobile frequency, durations (time) spent in the center/outer of the plate (**Fig. 1**), dark/light illumination related locomotor behaviour with emphasis on the distance covered and speed as shown in **Fig. 2**. and **Fig. 3**.

3.1.2 Pb effects on the mean distance covered and speed of larval zebrafish

The mean total distance travelled across the exposed group was significantly higher (Tukey test, $p < 0.01$) than that of the control group. The mean distance for the control, 3 µg/L, 91 µg/L and 250 µg/L were 51,891 ± 3054 mm,77,857 ± 5108 mm, 88,623 ± 4737 mm and 85,287 ± 3545 mm, respectively as shown in **Fig. 1A**. However, there was no significant difference in the mean distance covered between the Pb exposed groups.

Regarding the mean swimming speed, we observed similar trend with the pattern of the mean distance covered. The exposed groups had significantly higher speed implying faster speed compared with that of the control (Tukey test, $p < 0.01$). The mean speed for the control, 3 µg/L, 91 µg/L and 250 µg/L were 28.8 ± 1.7 mm/s, 43.3 ± 2.8 mm/s, 49.3 ± 2.6 mm/s and 47.4 ± 2 mm/s, respectively (**Fig. 1B**).

3.1.3 Pb effects on the cumulative mobility time

Significant differences in cumulative mobility (Dunn's multiple comparison test, $p < 0.001$) were observed in the control (133.6 ± 15.4 s) versus 91 µg/L (275.9 ± 11.9 s) and 250 µg/L (273.4 ± 15.3 s) as shown in **Fig. 1C**. Among the exposed groups compared with that of the control we observed a reduction in the time spent immobile by the larval zebrafish during the behavioural assessment period. The control spent statistically significant less time being immobile with mean of 1652 ± 17.9 s while the larval zebrafish in the 3 µg/L, 91 µg/L and 250 µg/L spent 1531 ± 21.8 s, 1466 ± 17.4 s and 1478 ±16.3 s respectively, as shown in **Fig. 1D**.

3.1.4 Pb effects on the mean mobile and immobile frequency

The total number of times the larval zebrafish spent mobile were significantly higher in the exposed groups compared to the control. The average mean frequencies were 1348 ± 158.1 s; 2115 ± 157.3 s; 2638 ± 11.9 s and 2580 ± 107.8 s for the control, 3 µg/L, 91 µg/L and 250 µg/L, respectively (**Fig. 1E).** Regarding the immobility frequency, the control group recorded a significantly lower frequency of 1247 ± 139 s when compared

to the exposed groups namely; 3 µg/L which recorded 1770 ± 118.2 s; 91 µg/L with 2184 ± 82.8 s and 250 µg/L with 2210 ± 90 s. Among the exposed groups, there was an increased pattern in the immobility frequency with an increased in Pb exposure dose. A significant difference was seen between 3 µg/L and 250 µg/L immobility frequencies (**Fig. 1F)**.

3.1.5 Pb effects on the time spent by larval zebrafish in the centre/outer zones

At the lowest level of exposure (3 µg/L - mean time was 164.6 ± 22.5 s) no difference in the centre zone preferences to that of the control group (155.7 ± 29 s) were recorded. However, a significant reduction in the time spent in the center zones was observed at 91 µg/L (86.4 ± 19.3 s) compared to the control group **(Fig. 1G)**. The exposure groups spent more time in the outer zones of the well although not statistically significant when compared to the control group. Comparing the behaviour among the exposed groups only showed a significant ($p < 0.05$) outer zones preference between 3µg/L (90.9 ± 1.6 s) and 91 µg/L (95.2 ± 1.1 s) with the of the larval zebrafish spending more time in the outer zone at 91 µg/L (**Fig. 1H**).

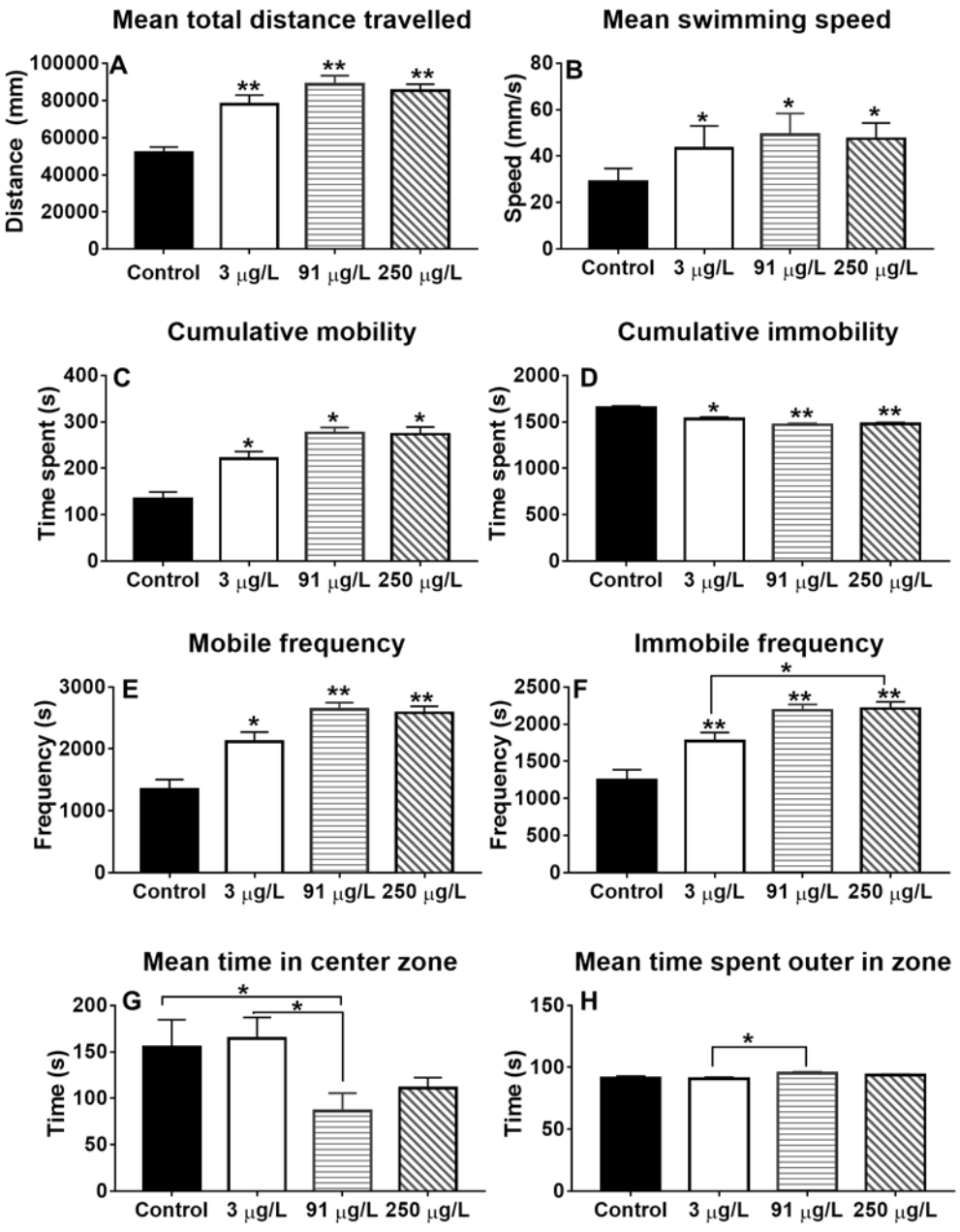

Fig. 1. Locomotor behaviour of larval zebrafish (120 hpf) exposed to 3 µg/L ,91 µg/L and 250 µg/L concentrations of Pb for 30 minutes during the test. For each exposure treatment n =12. Significance was regarded at $p<0.05$* and at $p<0.01$**.

3.2 Pb effects on the distance covered and speed of larval zebrafish in the dark/light phases

In general, the exposed groups covered longer distances in both the dark and light phase compared to that of the control group (**Fig. 2**). Mean distance recorded at 5 minutes during the first dark phase was significantly higher in 91 µg/L (2642 ± 193.1 mm) than that of the control (1655 ± 163.7 mm). Under the light phase, the control group (1199 ± 126.2 mm) had covered significantly lower ($p < 0.01$) distance at 15 minutes time bin compared to the 3 µg/L (2773 ± 177.6 mm), 91 µg/L (3156 ± 305.4 mm) and 250 µg/L (3289 ± 177.6 mm) as shown in **Fig. 2 (A, B** and **C)**, respectively. During the second dark phase, the distance covered by the control group (2182 ± 318.5 mm) was lower than the exposed groups and the difference was significant in the 91 µg/L group (3585 ± 242.7 mm) and 250 µg/L group (3396 ± 328.6 mm) at 25 minutes time bin (**Fig. 2 B and C),** respectively**.**

For swimming speed, the trend was similar as observed with the distance covered. At 5 minutes time bin, the speed of the 91 µg/L group (44 ± 3.2 mm/s) was significantly faster ($p < 0.05$) than that of the control group (27.8 ± 2.7 mm/s) as shown in **Fig. 3B**. At the 15 minutes time bin of the light phase, the larval zebrafish exposed to Pb were significantly faster than the control group ($p < 0.01$). The mean speeds at 15 minutes time bin were 20 ± 2.1 mm/s, 46.2 ± 5.8 mm/s, 52.6 ± 5.1 mm/s and 54.8 ± 3 mm/s for the control, 3 µg/L, 91 µg/L and 250 µg/L, respectively (**Fig. 3 B** and **C).**

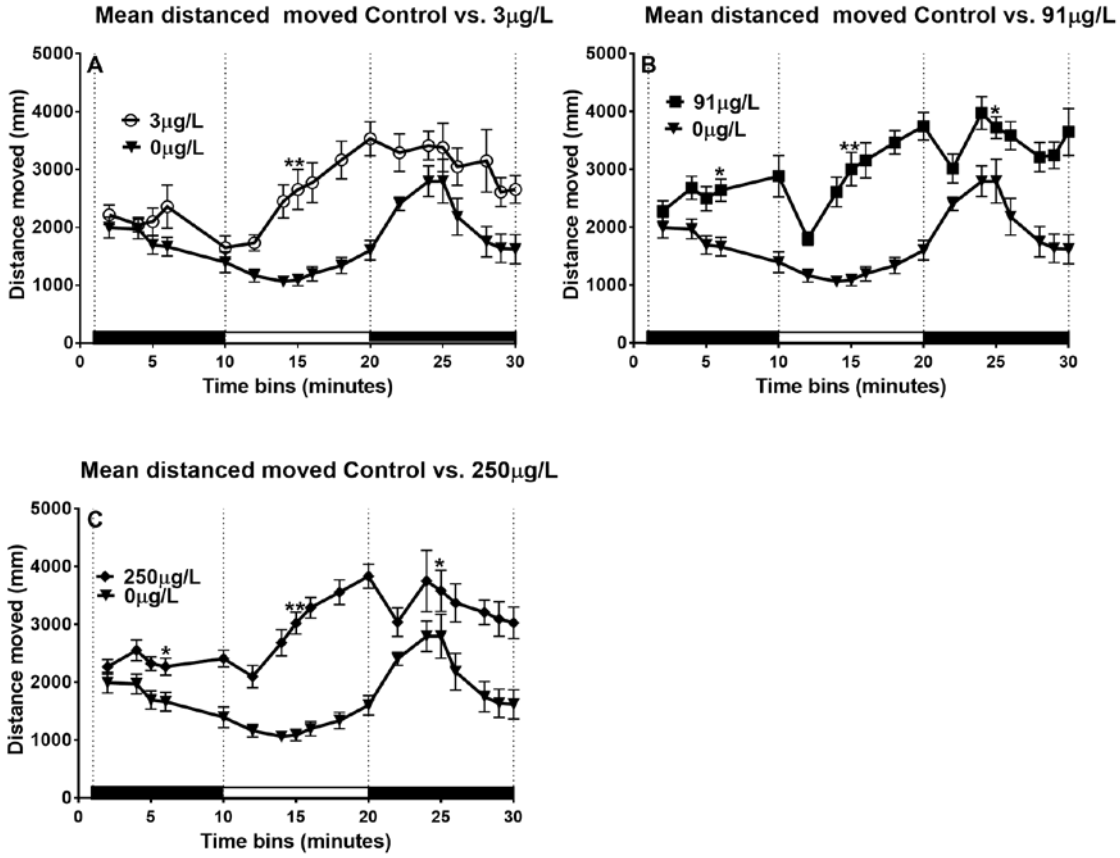

Fig. 2. Locomotor behaviour of larval Zebrafish (120 hpf) distance moved for exposed to 3 µg/L(A), 91 µg/L(B) and 250 µg/L (C) Pb concentrations and control for 30 minutes under dark/light transition illumination (5,15 and 25 minutes) during the test. The dark and white bar represents dark and light phases, respectively. For each exposure treatment n=12 Significance was regarded at $p<0.05$* and at $p<0.01$**.

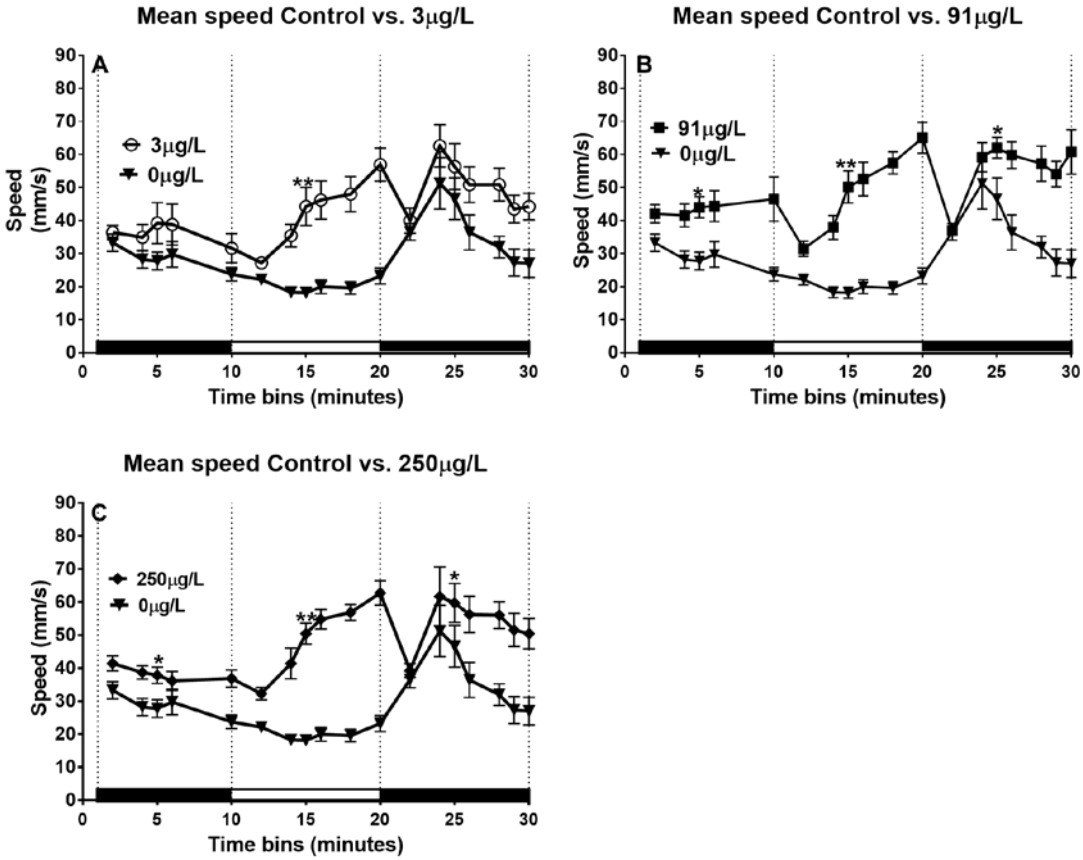

Fig. 3. Locomotor behaviour of larval Zebrafish (120 hpf) speed for exposed to 3 µg/L(A), 91 µg/L (B) and 250 µg/L (C) Pb concentrations and control for 30 minutes under dark/light transition illumination (5,15 and 25 minutes) during the test. The dark and white bar represents dark and light phases, respectively. For each exposure treatment n = 12. Significance was regarded at $p<0.05$* and at $p<0.01$**.

3.3 Pb effects on the subcellular oxidative stress responses in larval zebrafish

The messenger ribonucleic acid (mRNA) level of SOD2 in the larval zebrafish samples showed a slight increased level of expression in the exposed groups with 1.6-fold change at 3 µg/L, 1.3-fold change at 91 µg/L and 1.7-fold change at 250 µg/L levels of exposure though not statistically significant (**Fig. 4A**). On the other hand, acute effect of Pb was not accompanied by statistically significant GST mRNA across the exposed groups though a slight increase in the mRNA levels was seen at 250 µg/L with 1.5-fold change (**Fig. 4B**). Similarly, the mRNA for GPX expression levels showed a slight

increase at 3 µg/L of 1.2-fold change and at 250 µg/L of 1.5-fold change though not statistically significant. At 91 µg/L, we observed a slight decrease in the mRNA levels of GPX of 0.8-fold change which was not statistically significant (**Fig. 4C**). Additionally, we observed that the mRNA levels of HO-1 were upregulated in exposed groups when compared to the control with 2.8-fold change, 2.1-fold change and 1.8-fold change for the 3 µg/L Pb, 91 µg/L Pb and 250 µg/L Pb, respectively. The upregulation was statistically significant in the 3 µg/L exposed group compared to the control (**Fig. 3D)**.

The mRNA levels of Ucp-2 were significantly upregulated ($p < 0.05$) in exposed groups when compared to the control with 3.9-fold change, 3.5-fold change and 3.4-fold change for the 3 µg/L, 91 µg/L and 250 µg/L, respectively (F**ig. 4E**). The expression of the mRNA levels for the Bcl-2 gene were significantly upregulated following the Pb exposure across the exposed groups. The fold changes were 4.6-fold change, 6.3-fold change and 5.5-fold change at 3 µg/L Pb, 91 µg/L Pb and 250 µg/L Pb, respectively (**Fig. 4F).** In addition, the expression of CoxI which is involved in the mitochondrial respiratory chain and ATP synthesis was significantly upregulated in 3 µg/L Pb and 91 µg/L Pb groups when compared to the control with 3.9-fold change and 3.2-fold change, respectively (**Fig. 4G)**. The expression of the mRNA levels for the TP53 gene were slightly upregulated following the Pb exposure across the exposed groups. However, only the mRNA levels in 250 µg/L Pb group with a 2.5-fold (**Fig. 4H**) change were statistically significant when compared to the control ($p < 0.05$).

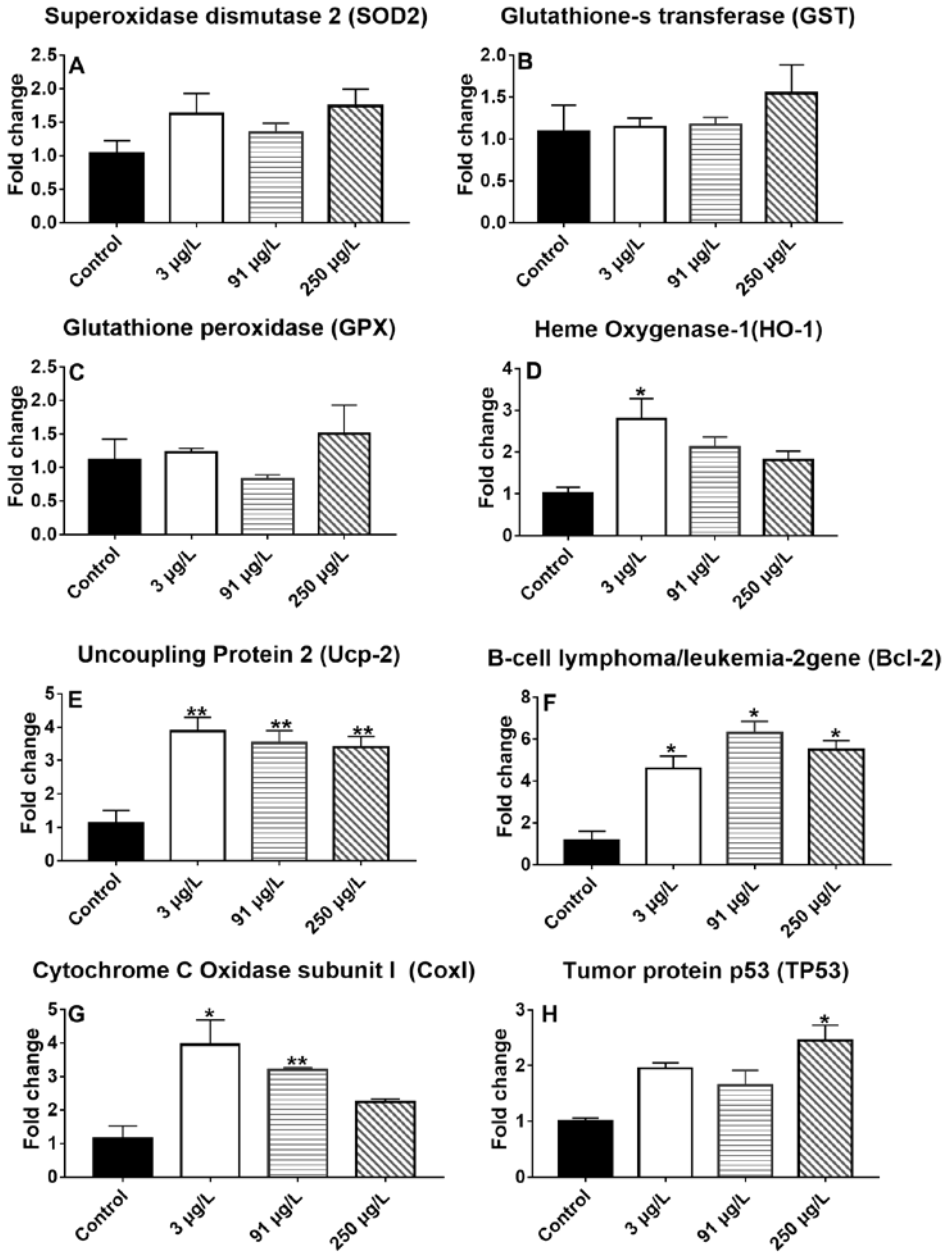

Fig. 4. Expression of SOD2, GST, GPX, HO-1, Ucp-2, TP53, COX1 and Bcl-2 (A, B, C, D,E,F,H and G) in the pooled samples from the larval zebrafish after the acute Pb 30 minutes dark/light illuminations (120 hpf old larval zebrafish) groups at 3 µg/L Pb, 91 µg/L Pb and 250 µg/L Pb concentrations and control. Values normalized against Tubulin alpha-1A (used as house-keeping gene) and represent the mean mRNA expression value ± SEM (n = 3 pooled samples) relative to those of the controls. The asterisk represents a statistically significant difference when compared with the controls *at $p < 0.05$ and **at $p < 0.01$ levels.

4. Discussion

In this study, we investigated the effects of environmentally relevant levels of Pb (as reported in Kabwe town and Zambia (Nachiyunde et al., 2013) on ensuing subcellular oxidative stress responses of the primary antioxidant, pro-apoptotic and anti-apoptotic genes and locomotor behaviour of larval zebrafish. Acute Pb exposure to larval zebrafish for 30 minutes induced hyperactivity characterised by significant increases in distance covered, swimming speed and mobile frequency as well as increased distance covered and speed under light illumination. We observed that the hyperactivity was significantly increased in exposed groups under light conditions when the zebrafish are reported to assume relaxed state (Lee and Freeman, 2014b) indicating Pb's potentiating neurobehavioural effects. Our results were similar to the activity of zebrafish larval that were exposed as embryos to low Pb of 25 µg/L (Chen et al., 2012) and contrary to findings reported in other studies where they observed hypoactivity (Dou and Zhang, 2011; Lefauve and Connaughton, 2017; Li et al., 2019a). The difference between some of the foregoing studies and the present study could have been related to the dose of Pb, the duration of exposure and the developmental stages of the zebrafish. For instance Li et al., (2019a) reported a concentration dependent reduction in locomotion of adult zebrafish exposed Pb over a 14 day period to 1 µg/L , 10 µg/L and 100 µg/L Pb, respectively. Dose and duration effects in Pb exposure in fish have been reported in the Mirror carp exposed to 50 µg/L of Pb over a 30 day period with hyperactivity reported in the first week and last week of exposure (Rehman, 2003). Other neuroactive chemicals such an cholinesterase inhibitor, paraoxon showed hyperactivity in a lower exposure range and hypoactive with a 100-fold increase in exposure concentration (Yozzo et al., 2013). This study confirms the responses that were obtained using the mammalian models,

humans and rodents, exposed to chronic low Pb concentrations where hyperactivity was been reported as the neurobehavioural end point (Ma et al., 1999); thereby showing the application value of the zebrafish model to assess Pb exposure responses. While our experimental design was not aimed at explaining the mechanism behind this behaviour, our results demonstrated alterations in free locomotor activity manifested by hyperactivity, a recognized effect arising from Pb metal neurotoxicity (Atchison et al., 1987). In Mirror carp that exhibited Pb induced hyperactivity, a positive correlation between enhanced lipid peroxidation, a marker of oxidative damage in the brain and increased behavioural activity was reported (Rehman, 2003) . The abnormal behaviour of increased locomotor activity in larval zebrafish during daylight phases has ecological potential ecological implications in the form of increased predation. South et al. (2019) observed similar reduced anti-predator behaviour (i.e. increased locomotion) in mosquito larvae following exposure to low levels of the insecticide, dichlorodiphenyltrichloroethane (DDT). The authors indicated that the pollutant not only mediates the behaviour of the prey but also the interactions of the natural predator. This would inevitably result in potential alterations of the strength of trophic interactions (South et al., 2019).

In case of the mRNA responses to acute exposure of environmentally relevant Pb in larval zebrafish, we observed a non-significant induction of the primary antioxidant enzymes apart from HO-1 that was significantly upregulated at 3 µg/L. Functionally, HO-1 catabolize free heme, that is, iron (Fe) protoporphyrin (IX), into equimolar amounts of Fe^{2+}, carbon monoxide (CO), and biliverdin (Gozzelino et al., 2010). In our study, the upregulation of HO-1 at 3 µg/L Pb and its accompanying lack of significant upregulation at high levels (91 and 250 µg/L Pb) may have been a protective response against acute

Pb exposure or a hormetic response (Calabrese et al., 2012). Heme oxygenase (HO-1) gene has been classified among the vitagene family exhibiting hormetic responses (Calabrese et al., 2004). According to the hormetic principles, low doses of drugs, toxicants, and natural substances may elicit a positive response in terms of adaptation to or protection from the stressor, whereas at higher concentration the toxic effect prevails (Calabrese et al., 2008). The hormetic dose – response can occur through different mechanisms: as a direct stimulatory response; after an initial disruption in homeostasis followed by the modest overcompensation response; or as a response to an "adapting" or "preconditioning" dose that is followed by a more challenging dose (Piantadosi, 2008). Furthermore, we observed significant upregulation of the Ucp-2 gene and Bcl-2 across the exposed group. The upregulation of CoxI and the proapoptotic TP53 genes seem to suggest an acute upregulation of ROS that led to an enhanced mobilization of the Ucp-2 and Bcl2 genes to quail the ROS production as an initial protective mechanism (Craig et al., 2007). Ucp-2 functions, by an incompletely defined mechanism, to reduce the production of reactive oxygen species during mitochondrial electron transport (Giardina et al., 2008), while Bcl-2 works to counter the effect of the TP53 elevated gene expression and has been said to be an indicator of a conspicuous increase in ROS (Kowaltowski and Fiskum, 2005). Similarly, the significant upregulation of CoxI expression in the acutely Pb exposed larval zebrafish points to the acute requirement for cellular responses to the generated ROS following exposure (Bourens et al., 2013).

While Pb has been reported as a neurotoxic element that causes behavioural dysfunction in fishes within days of exposure to sublethal concentrations (Weber et al., 1997), our study has demonstrated that effects in zebrafish larvae manifest within a very short period following exposure to environmentally relevant Pb levels. Taken together,

our results showed that the lowest average Pb level of 3 µg/L Pb as found in Kabwe, Zambia may have deleterious effects to the same degree as higher exposure concentrations (91 µg/L and 250 µg/L) on aquatic life triggering a surge in ROS generation and hyperactive swimming behaviour as observed in the larval zebrafish.

The following limitations of using toxicogenomics in this study were identified. There is a long-standing debate as to what fold change in gene expression can be considered to be biologically significant (Mccarthy and Smyth, 2009). In this study we regarded a significant difference from the control to reflect those genes that are differentially expressed. It is however acknowledged that there is a threshold for minimum fold gene change below, which differential expression is unlikely to be of any biological interest for a particular gene. Therefore, this ad hoc approach probably provides an over estimation of the effects likely to occur following exposure to low levels of Pb. It is further acknowledged that the use of gene expression profiling as an indicator of sub-cellular changes needs to be verified through assessment of molecular and biochemical to reveal and confirm precise mechanisms of action (Fielden and Zacharewski, 2001). Due to the small sample volume we were not able to verify increased antioxidant responses through analyses of enzymatic (e.g. SOD and catalase) and non-enzymatic (lipid peroxidase and protein carbonyl) compounds. However, based on the studies (Ahmadifar et al., 2019; Parolini et al., 2017; and Safari et al., 2017) where similar fold changes were accompanied by significant biochemical changes, seem to support that the gene expression (i.e. upregulation of the anti-oxidant genes) results in increase of the enzymes thereby combatting ROS formation. It is then this premise that we use to postulate that the gene expression maybe a good indicator (biomarker) of antioxidant responses against ROS formation.

5. Conclusion

Environmentally relevant concentrations of Pb as they occur in Kabwe could be detrimental to aquatic life especially in larval fish. Acute exposure to the environmentally relevant Pb levels attenuated larval zebrafish behaviour by inducing hyperactivity under dark/light illumination. This locomotor activity pattern alteration could be linked to altered neurobehavior via neurotoxicity mediated by oxidative stress or direct Pb neuro-intoxication due to lack of fully formed brain blood barrier. This has potential ecological ramifications through alterations in predator-prey interactions. However, the degree to which these observed effects following an acute exposure period to low Pb levels will persist during prolonged exposure needs to be investigated further.

6. Ethical statement

All experimental procedures were approved by the AnimCare animal research ethics committee (NHREC reg. number AREC-130913-015) of the North-West University. All animals were maintained, and procedures performed in accordance with the code of ethics in research, training and testing of drugs in South Africa and complied with national legislation (ethics approval number: NWU-00269-16-A5).

7. Declaration of Competing Interest

The authors declare that they have no conflict of interest relating to the work presented in this manuscript.

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