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Heavy metal mediated immunomodulation of the Indian green frog, *Euphlyctis hexadactylus* (Anura:Ranidae) in urban wetlands

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ABSTRACT

Impacts of heavy metal toxicity on the immune system of the Indian green frog, *Euphlyctis hexadactylus,* in Bellanwila Attidiya, an urban wetland polluted with high levels of heavy metals, compared to the reference site in Bolgoda, in Sri Lanka was investigated. Significantly higher accumulation of selected heavy metals, copper (Cu), zinc (Zn), lead (Pb), and cadmium (Cd) were detected by AAS in frog liver and gastrocnemius muscle, in the polluted site than in the reference site. Non-functional immunotoxicity tests; total WBC, splenocyte and bone marrow cell counts, spleen weight/body weight ratio, neutrophil/lymphocyte ratio and basal immunoglobulin levels, and phagocytic capacity of peritoneal macrophages (immune functional test) were carried out using standard methodology. Test parameters recorded significantly lower values for frogs of the polluted site compared with their reference site counterparts, indicative of lowered immune response of frogs in the former site. *In vitro* phagocytic assay based on NBT dye reduction, measured the stimulation index (SI) of *E. hexadactylus* blood leukocytes, splenocytes and peritoneal macrophages, where SIs of frogs in the polluted site were significantly lower. Also, *in vitro* exposure of frog phagocytes to Cu, Zn, Pb and Cd at 10^{-2}–10^{-10} M, showed immunomodulation *i.e.* low concentrations stimulated phagocytosis while increased concentrations showed a trend towards immunosuppression. IC_{50} values indicated Cd > Zn > Cu > Pb as the decreasing order of the potential of phagocytosis inhibition. In conclusion, this study clearly demonstrated immunomodulation of *E. hexadactylus,* stimulated by heavy metals. *In-vitro* studies evidently suggested the use of phagocytosis as a biomarker in Ecoimmunotoxicology to detect aquatic heavy metal pollution.

1. Introduction

Contamination of aquatic environments, particularly wetlands, due to anthropogenic activity is increasingly evident globally. Urban wetlands are strongly influenced by long term discharge of untreated domestic and industrial wastewaters, storm water run-off, accidental spills and direct solid waste dumping due to rapid urbanization accompanied by weak urban planning (Mackintosh and Davis, 2014). In Sri Lanka, urbanization has caused a steady degradation of urban wetlands during the past few decades (CEA, 1994; Hettiarachchi et al., 2011), threatening the sustainability of ecosystem services provided. Heavy metals disposed of through industrial and domestic wastes and effluents are amongst the most ubiquitous pollutants, posing severe threats to biota in the wetland ecosystems (Rai, 2009).

Owing to the intimate association with the wetland ecosystem through diverse life-stages and other vulnerable traits, amphibians appear to be particularly sensitive to xenobiotics, including heavy metals (Hopkins et al., 2007; Gürkan et al., 2014; Franco-de-S and Val, 2014; Zhelev et al., 2013a, 2014a). The exceptionally sensitive nature of amphibians qualifies them as sentinel species which warn mankind of serious biodiversity issues. Recent decline and malformations reported in amphibians from different geographical regions of the world were associated with aquatic pollution

Abbreviations: Cu, copper; Zn, zinc; Pb, lead; Cd, cadmium; Cr, chromium; Hg, mercury; Fe, iron; Mn, manganese; Ni, nickel; GFAAS, graphite furnace atomic absorption spectrophotometry; WBC, white blood cells; RBC, red blood cells; ppm, parts per million; Ig, immunoglobulin; DO, dissolved oxygen; BOD, biological oxygen demand; SVL, snout-vent length; HL, head length; HuL, humerus length; RL, radius length; FL, femur length; F, foot length; HW, head width; FTL, first toe length; FAAS, furnace atomic absorption spectrophotometry; ASTM, American society for testing and materials; EDTA, ethylenediaminetetraacetic acid; OD, optical density; PM, peritoneal macrophages; NBT, nitroblue tetrazolium; RPMI, Roswell Park Memorial Institute; CRPMI, complete RPMI; PBS, phosphate buffered saline; DMSO, dimethyl sulfoxide; IC50, inhibitory concentration; ROS, reactive oxygen species; LPO, lipid peroxidation; DNA, deoxyribonucleic acid; ATP, adenosine triphosphate; SI, stimulation index

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(Johnson and Chase, 2004; Taylor et al., 2005; Rohr et al., 2008). This issue supports multiple stressors hypothesis where xenobiotic induced susceptibility to diseases and other agents evidently elucidate the mass decline of amphibians reported in contaminated habitats (e.g. Hayes et al., 2010; Blaustein et al., 2011). Drastic reduction of amphibians due to disease outbreaks in contaminated habitats is suggestive of xenobiotic driven immunosuppression that increased their disease susceptibility (Carey et al., 1999). Hence, the extent to which xenobiotics contribute to immunosuppression warrants in depth study.

Heavy metal mediated immunosuppression in mammals can vary with the metal variety reporting mercury (Hg) > copper (Cu) > manganese (Mn) > cobalt (Co) > cadmium (Cd) > chromium (Cr) > zinc (Zn) > lead (Pb) > nickel (Ni) as the toxicity lowering order (Lawrance, 1984). Ionization state of metal may also play a role in toxicity (Levis et al., 1978; Von Burg and Liu, 1993). Immunosuppressive behavior of these metals is associated with decreased humoral and cellular responses, resulting in decreased lymphoid organ cellularity and weight (Bernier et al., 1995), decreased phagocytic activity and lymphocyte proliferation (Omara et al., 1997), and cytokine production (Bernier et al., 1995; Omara et al., 1997). Epidemiologic data obtained from several studies of occupational Pb exposure, reported alterations in humoral immunity parameters such as IgG, IgM and IgA levels compared with unexposed individuals (Kimber et al., 1986; Ewers et al., 1982). Moreover, decreased splenic and bone marrow macrophages, and reduced macrophage activity were reported in several instances in laboratory mice treated with Pb and Cd (Koller, 1980; Burchiel et al., 2000). Data obtained from *in vitro* studies claimed that Cd at non-toxic levels is a potential immunomodulator (Bernier et al., 1995), eliciting quantitative and functional alterations of different phagocytic cells. Therefore, it is apparent that the immune system is specifically sensitive to toxic effects of chemicals of environmental concern (Luster and Rosenthal, 1993) including heavy metal pollution. Similarly, immunotoxicity resulted when animals were exposed to environmental relevant levels of heavy metals (Omara et al., 1997; Savabieasfahani et al., 1998). As a corollary to this, we witness the emergence of immuno markers as indicators of environmental health, associated with heavy metal pollution.

Consequently, phagocytosis was used as a biomarkerto detect environmental pollution by different pollutants including heavy metals (Zelikoff et al., 2000; Witeska et al., 2010; Fournier et al., 2000). Use of biomarkers specifically as a measure of immunotoxicity has received considerable attention, especially in chronic and low levels of exposure to xenobiotics; These may be more sensitive than other biomarkers often used for acute exposure (Brousseau et al., 1997). For instance, sub lethal doses of Pb exposure resulted in no clinical signs of toxicity, but did result in the suppression of cell mediated immune function (Faith et al., 1979). They observed a significantly decreased thymic weight, responsiveness of lymphocytes to mitogen stimulation and in delayed hypersensitivity responsiveness. Since, immunosuppression may exist without expressing any clinical symptoms in the victim, immunotoxicological studies may be more sensitive and provide greater information regarding contaminant exposure and toxicity.

A majority of immunotoxicological studies are devoted to impacts on mammalian models, including occupational exposure studies with humans. Conversely, limited information is currently available on the effects of heavy metals on the immune system of adult and larval amphibians. In few instances, decreased phagocytic and lytic activity of leukocytes (Rosenberg et al., 2003) and elevated levels of immunoglobulins (Chiesa et al., 2006) were reported due to sub lethal doses of Pb in *Bufo arenarum*. Similarly, *Pelophylax ridibundus* (Pallas, 1771) associated with low concentrations of Fe, Mn, Cu and Pb reported low lymphocytes, basophils, eosinophils and segmented nuclei neutrophils, while the number of monocytes increased (Zhelev et al., 2013b, 2014b). Parallel to changes found in WBC counts, splenocyte counts were also altered significantly in frogs inhabiting polluted wetlands (Zhelev et al., 2014c). Sublethal doses of Cd were reported to increase agglutination titers of hemoagglutination assay in adult *Rana pipiens* and larvae of *Rana catesbeiana* (Zettergren et al., 1988, 2001).

As a country with enormous amphibian diversity that harbor more than 119 species of which more than85% are endemic, and 19 species extinct up to date (Bambaradeniya, 2006; IUCN List IR, 2012), it is pertinent to study impacts of heavy metal pollution in wetland associated amphibian species in Sri Lanka. Bellanwila-Attidiya Sanctuary (BAS) is located in the Southeastern outskirts of the Colombo city, in the Western province of Sri Lanka. This is primarily a freshwater marsh ecosystem surrounded by a rapidly developing urban environment. Besides its value as a protected area rich in wildlife, it acts as a flood retention area which provides protection to inhabitants. However, this wetland is in a degraded status due to anthropogenic activity (CEA, 1994). The major water ways were polluted with litter and untreated industrial waste, and sewage was discharged into the canal from the tributary channels (CEA, 1994).The disturbances and threats were so great that some amphibian species were reported to experience rapid decline in the 1997–2000 period, that warranted the need to urge for mitigatory measures (CEA and IUCN, 2006).

We undertook the present field study in the polluted Bellanwila Attidiya Sanctuaryto examine the immunotoxic effectsof selected heavy metals on a common anuran species, the Indian green frog, *Euphlyctis hexadactylus* (Lesson, 1834). *In vitro* testing of heavy metals was used to validate some of the field assessments.

2. Materials and methods

Approval for collection of wildlife specimens from protected areas, and ethical approval for conducting animal research was obtained from the Department of Wildlife Conservation (W/L/3/2/1/6) and the Ethical Research Committee, Faculty of Medicine, University of Colombo (ERC/12/176), Sri Lanka, respectively.

2.1. Field sampling and heavy metal analysis

The Bellanwila Attidiya wetland (E. 79.55′–79.58′, N. 6.40′–6.48′), was the selected polluted site reported to be highly contaminated with domestic sewage, industrial effluents and agricultural runoff, while Bolgoda South Lake (E. 79.90′–79.89′, N. 6.76′–6.78′) with negligible human disturbances and pollution was selected as the reference site. Water quality parameters such as pH, temperature, DO (Dissolved Oxygen) and BOD (Biological Oxygen Demand) were measured at each site during sampling events carried out biweekly for a period of four months. Water samples were collected in to thick plastic bottles and preserved with conc. HNO_3 acid 3% (v/v) for heavy metal analysis (UNEP/WHO, 1996).

The test animal, adult Indian green frogs ($N=10$ per site) were randomly captured from water ways using bait. Some measurements were recorded in situ. Body weights were recorded using a digital weighing balance (A 40409/A-M 6005, Philip Harris, China). Standard external morphometric parameters, such as snout-vent length (SVL), head length (HL), humerus length (HuL), radius length (RL), femur length (FL), foot length (F), head width (HW) and first toe length (FTL) were measured using an electronic digital caliper (Grade 03, Control Company, USA). Animals were transported to the laboratoryfor further analysis in aerated plastic bags half filled with water and housed in aerated glass tanks half

filled with dechlorinated tap water (pH=6.0–8.5, hardness=250–350 ppm, 27–30 °C). Frogs were acclimatized for two days prior to further analysis, providing them with metal free chopped meat (~10% of their body weight) once daily (CCAC, 1984).

The preserved water samples, and digested frog gastrocnemius muscle and liver tissue were analyzed by graphite furnace atomic absorption spectrophotometry (GFAAS;Perkin-Elmer 1100B FAAS) for selected heavy metals Cu, Zn, Pb, Cd (ASTM, 2003). These metals were selected after a pilot study which indicated a significant difference of these metals in the polluted site compared with the reference site.

2.2. Immunotoxicologic assays: non-functional tests

Adult frogs were anesthetized with diethyl-ether and were dissected under aseptic conditions. The perisac of the heart was removed and blood was collected by heart puncture into EDTA containing tubes (Turgeon, 2005). Using anticoagulated blood diluted in Turck's solution, total WBC counts were obtained using Neubauer haemocytometer (Arserim and Mermer, 2008). Stained thin blood smears (Arserim and Mermer, 2008) observed under oil immersion microscopy (100 ×) to obtain differential WBC counts to calculate, neutrophil/ymphocyte ratio.

Serum was separated from blood dispensed in to anticoagulant free tubes (Hudson and Hay, 1989) and stored at −20 °C until further use. Partially purified immunoglobulins (Ig) obtained from Ammonium Sulfate precipitation were dialyzed and the optical density (OD) was measured at 260 and 280 nm using UV/VIS/NIR spectrophotometer (V 560, Jasco cooperation, Japan) (Hudson and Hay, 1989). The OD values thus obtained were converted into Ig concentrations where the concentration was given by $OD_{280} \times [OD_{280}/OD_{260}]$ratio (Garcia-Romo et al., 2011).

Spleen weight was recorded using an electronic balance (±0.001 g, EB-3200H-A, Shimadzu cooperation, Japan), and the spleen weight/ body weight ratio was calculated. Total splenocyte and bone marrow cell counts of the left femur were made using a Neubauer haemocytometer (Hudson and Hay, 1989).

2.3. Immune functional assays

2.3.1. Phagocytic capacity

Peritoneal macrophages (PM) aspirated from the frog body cavity were treated with 2 drops of 1% Neutral Red in amphibian saline per mL of aspirate, mixed well and allowed to stand for 20 min (Hudson and Hay, 1989). The aspirate was centrifuged at 500 × *g* for 5 min, the pellet resuspended in PBS and a total of 200 phagocytic cells counted using Neubauer haemocytometer (B.S. 748, Weber, England). Those peritoneal macrophages that stained red pink were considered as functionally active cells. The phagocytic capacity was calculated as the ratio of the number of active cells/total number of phagocytic cells.

2.3.2. In vitro phagocytic assay based on nitroblue tetrazolium (NBT) dye reduction

The method described by Manosroi et al. (2006) for the *in vitro* phagocytic assay on NBT dye reduction was followed with modifications. Dried *Saccharomyces cerevisiae* (0.5 g) was heated to 100 °C for 1 h in 10 mL of 50 mM-phosphate buffer (PB), pH 7.4 (Jensen and Bainton, 1973). The suspension was centrifuged at l000 g for *5* min, the supernatant discarded and the pellet washed twice in 5 mL of PB. The heat-killed organisms were stored as a thick slurry at 4 °C for a week until required (Edwards and Doulah, 1982).

Stock solutions (0.1 M) of heavy metals were prepared by using water soluble salts of heavy metals (Fournier et al., 2000; ASTM, 2003). The metals tested were zinc sulfate, cadmium sulfate, copper sulfate and lead nitrate. The purity of the metal compounds was ≥99.0%. EFrog peritoneal macrophages, total WBC, and splenocytes were exposed to metal concentrations of 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}, and 10^{-10} M.

Frog peritoneal macrophages were harvested as described above (Hudson and Hay, 1989). Buffy coat was separated out for total WBC, where RBCs were removed using RBC lysis buffer (Mrowiec et al., 1995). Splenocytes were isolated under aseptic conditions using the method described by McIntosh et al. (1986) with slight modifications. Spleen was placed in complete RPMI 1640 medium with 10% FBS (CRPMI) and macerated through a 100 μm cell strainer using the medium, and centrifuged at 400 g at 4 °C for 3 min. RBCs were lyzed using RBC lysis buffer.

2.3.2.1. Heavy metal non treated assay. 20 μL of 10^6 cells/mL adherent macrophages (peritoneal macrophages, WBC, splenocytes) and 40 μL of CRPMI were added in to triplicate wells of a 96 well flat bottom culture plate (Linbro cat no. 6070013, US), and incubated for 18 h in a carbon dioxide incubator, humidified with 5% CO_2, at 37 °C. Plates were washed three times with RPMI medium to remove non adherent macrophages. Twenty microliter of yeast suspension (5×10^7 particles/mL) and 20 μL of NBT (sigma, Germany) solution in PBS (1.5 mg/mL) were added to treated wells, while 20 μL of NBT only was dispensed in to control wells. After incubation for 1 h, the adherent macrophages were rinsed vigorously with RPMI medium, and washed with 200 μL Methanol. After air drying, formazan granules that resulted from reduction of NBT were dissolved in 120 μL of 2 M KOH and 140 μLof DMSO. The absorbance was measured at 655 nm using a microplate reader (Model 680 micro plate reader, BioRad, US). Stimulation Index (SI) was calculated as the ratio of the optical density (OD) value of the treated cells/OD value of the control cells.

2.3.2.2. Heavy metal treated assay. Reference site frog cell types were assayed for heavy metal modulation by the *in vitro* phagocytic assay. All three phagocytic macrophage types (peritoneal macrophages, WBC, splenocytes) were assayed in the presence of known concentrations ($C_1=10^{-10}$ M, $C_2=10^{-8}$ M, $C_3=10^{-6}$, $C_4=10^{-4}$, $C_5=10^{-2}$ M) of selected heavy metals-Cu, Zn, Pb and Cd. The procedure was similar to that of the non treated assay, and was conducted by adding 20 μL of heavy metal solution (C_1–C_5) with 20 μL of 10^6 cells/mL adherent macrophages and 40 μL of CPRMI at the first step except for controls of each cell type. SI was calculated as the ratio of OD of wells treated with heavy metal/OD of wells without heavy metal treatment (control). The IC_{50} values, *i.e.* concentration of each metal which induced 50% suppression of phagocytosis, were calculated according to Alexander et al. (1999).

2.4. Reproductive biological parameters

The whole egg mass of the frog removed without fat bodies was placed in a graduated cylinder (±0.5 mL) and the volume of the mass recorded. The technique described in Kouba and Vance (2009) was followed to obtain the total egg count. According to the total volume, the whole egg mass was divided in to five equal parts, the average number of eggs per single equal portion counted and the total egg count was estimated by extrapolation of this value.

2.5. Statistical analyses

Data analysis was performed using SPSS 16 for windows. Data was presented as mean ± standard deviation and as median values. Water quality parameters (BOD, DO, pH and temperature) were compared using one-way ANOVA. Morphometric variables of frogs (N=10/site) from the two study sites were compared using

simple t test. Comparison of immunological parameters; total WBC, splenocyte and bone marrow cell counts, basal Ig levels, were carried out using Mann–Whitney *U* test. Differential WBC counts were compared using the Chi-squared test. Spleen weight/ body weight ratio and neutrophil/ lymphocyte ratio were analyzed using one sample *t* test. Level of significance was set at $P < 0.05$.

3. Results

3.1. Test populations from polluted and reference sites

No significant differences ($P > 0.05$; *t*-test) were evident between the body weights (reference 159.65 ± 9.61 g; polluted 153.71 ± 10.36 g), snout vent lengths (SVL, reference 105.73 ± 11.55 mm, polluted 113.44 ± 5.29 mm) and snout vent length/body weight ratios (reference 0.665 ± 0.097, polluted 0.741 ± 0.074) of adults frogs collected from the two study sites. Standard health conditions were detected in frogs collected from both study sites as both groups expressed similar activities, such as ordinary escaping behavior, alertness, etc. and were infected with common parasites such as gut nematodes. Hence, pathogen/ parasite related immune stimulation was considered equal. Water quality parameters (Mean ± SD) of the two study sites such as temperature (reference $30.25 \pm 0.64°$ C, polluted $30.45 \pm 0.53°$ C), pH (reference 6.723 ± 0.245, polluted 6.8975 ± 0.0971), BOD (reference 1.793 ± 0.235 mg/L, polluted 1.998 ± 0.217 mg/L) did not vary significantly in the two study sites with the exception of DO which was significantly higher in the polluted site (2.985 ± 0.1994 mg/L) compared to that of the reference site (3.695 ± 0.126 mg/L) ($P < 0.05$).

3.2. Heavy metal analysis

Selected heavy metal concentrations of water samples collected from the two study sites recorded higher concentrations of Zn (2.71 ppm) and Pb (0.955 ppm) ions in the waters of the polluted site while Cu (0.04 ppm) and Cd (0.019 ppm) were present in relatively low levels. Reference samples were detected with less than 1 ppb concentration for each heavy metal. Accumulation of metals in frog liver and musculature from the polluted site was significantly higher ($P < 0.05$) than that of the reference site (Fig. 1).

3.3. Non-functional immunotoxicologic assays

Total WBC (polluted site $3485 \times 10^4 \pm 698/\mu L$; reference site $6610 \times 10^4 \pm 1041/\mu L$) and splenocyte counts (polluted site $2.079 \times 10^5 \pm 0.39/\mu L$; reference site $2.518 \times 10^5 \pm 0.42/\mu L$) were significantly lower in frogs of the polluted site than those of the reference site ($P < 0.05$), while the bone marrow cell counts did not significantly differ (polluted site $6.99 \times 10^4 \pm 1.25/\mu L$; reference site $8.13 \times 10^4 \pm 0.83/\mu L$, $P > 0.05$, Fig. 2). Both the neutrophil/lymphocyte ratio (polluted site 1.7280 ± 0.122; reference site 1.3304 ± 0.252) and the spleen weight/ body weight ratio (polluted site 0.0020 ± 0.00; reference site 0.0028 ± 0.00) in the polluted site were significantly lower compared to that of the reference site ($P < 0.05$). This trend was also observed for total frog Ig levels, where on average 0.5397 ± 0.2053 mg/L of the polluted site was significantly lower than of the reference site (0.7740 ± 0.0898 mg/L) ($P < 0.05$).

3.4. Immune functional assays

The phagocytic capacity of frog peritoneal macrophages was significantly lower in the polluted site than that of the reference site ($P < 0.05$) (Fig. 3A). The Stimulation Index (SI) for each frog phagocytic cell type (peritoneal macrophages, WBC and splenocytes) of the polluted site was significantly lower than the corresponding values from the reference site ($P < 0.05$; pooled *t*-test) (Fig. 3B). In both sites, higher SIs of splenocytes and peritoneal macrophages were observed compared to that of blood leukocytes.

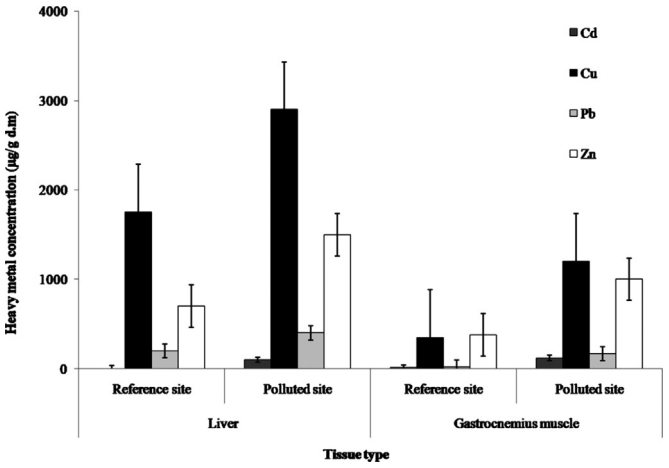

Fig. 1. Accumulation of selected heavy metals in frog gastrocnemius muscle and liver tissue of polluted and reference sites ($N = 10$/site). Accumulation of heavy metals in frog tissue in the polluted site was significantly higher compared to the reference site ($P < 0.05$). Heavy metal accumulation in the liver in both sites was significantly higher than that of the gastrocnemius muscle ($P < 0.05$). (Polluted site – BellanwilaAttidiya; Reference site – Bolgoda, error bars represent ± SEM).

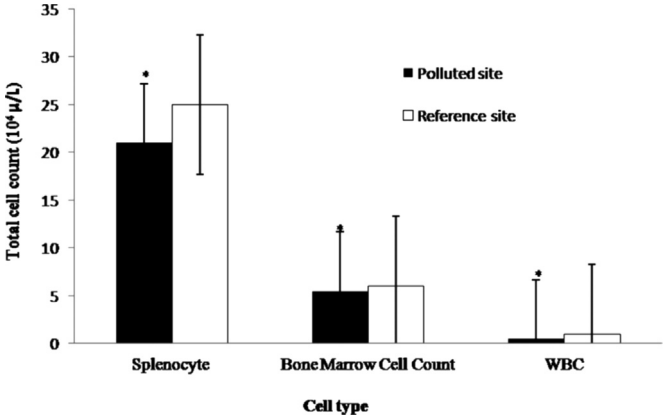

Fig. 2. Total splenocyte, WBC and bone marrow cell counts of frogs of the two study sites ($N = 10$/site). Total splenocyte and WBC counts were significantly lower in the polluted site compared to those of the reference site ($^*P < 0.05$). (Polluted site – BellanwilaAttidiya; Reference site – Bolgoda, error bars represent ± SEM).

3.5. Effects of heavy metals on in vitro phagocytic response

Effects of heavy metals on phagocytic activity of blood leukocytes, splenocytes and peritoneal macrophages are presented in Figs. 4, 5 and 6, respectively. All selected cell types showed immunomodulation following *in vitro* exposure to selected heavy metals, Cu, Zn, Pb and Cd at concentrations increasing in two fold dilutions, ranging from 10^{-2} to 10^{-10} M. At low concentrations all metals except Cu were observed to have the potential to stimulate phagocytosis, and as metal concentrations increased the trend was towards immunosuppression.SI of blood leukocytes significantly increased at lower concentrations (10^{-10}, 10^{-8} M) of Pb and Cd ($P < 0.05$), while this significantly ($p < 0.05$) decreased at higher concentrations for Cu showing immunosuppression (Fig. 4). Similarly, SI for splenocytes significantly increased at lower concentrations of Zn, Pb and Cd resulting in significantly higher ($p < 0.05$) immunostimulation. However, at the highest

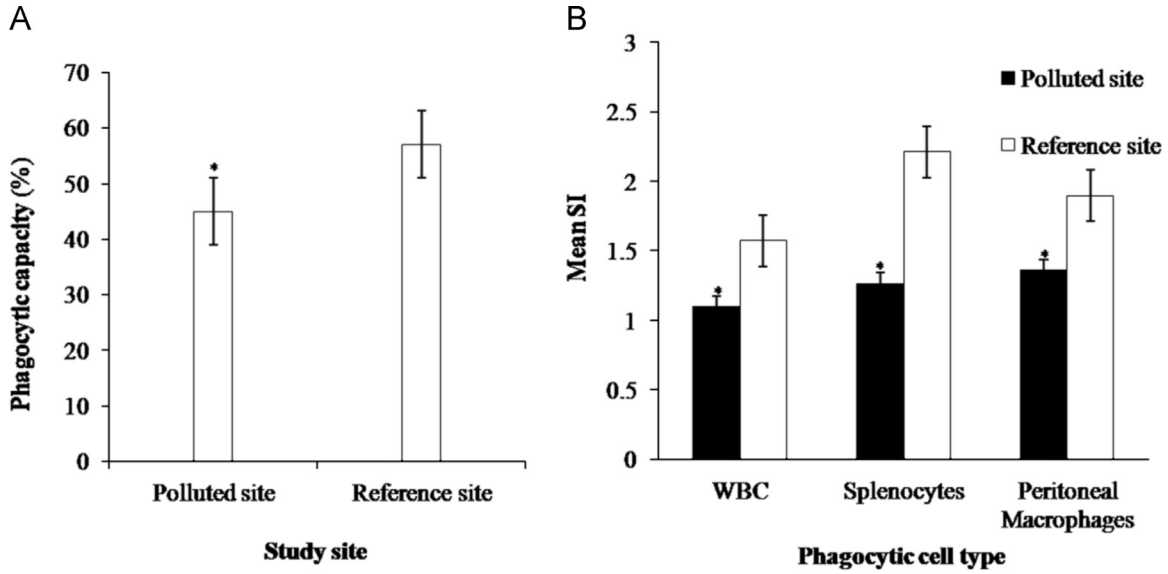

Fig. 3. Comparison of (A) phagocytic capacity and (B) stimulation index (SI) of the two study sites. Phagocytic capacity of frogs ($N=10$/site) assessed by the neutral red dye uptake assay, and the stimulation indices (SI) of frog phagocytic cell types calculated using NBT dye reduction assay, both recorded significantly lower values in the polluted site compared with those of the reference site ($P<0.05$). (Polluted site – BellanwilaAttidiya, Reference site – Bolgoda, error bars represent ± SEM).

concentration (10^{-2} M) of these metal ions, Pb and Cd but not Zn, recorded significantly low SI showing higher immunosuppression ($P<0.05$) (Fig. 5). However, peritoneal macrophages reported lower SI values showing clear immunosuppression for all the test concentrations of all metals tested (Fig. 6).

The concentration for each metal which induced 50% suppression of phagocytosis (IC_{50}) was calculated, for all different cell types used. Accordingly, Cd was the most potent inhibitor,

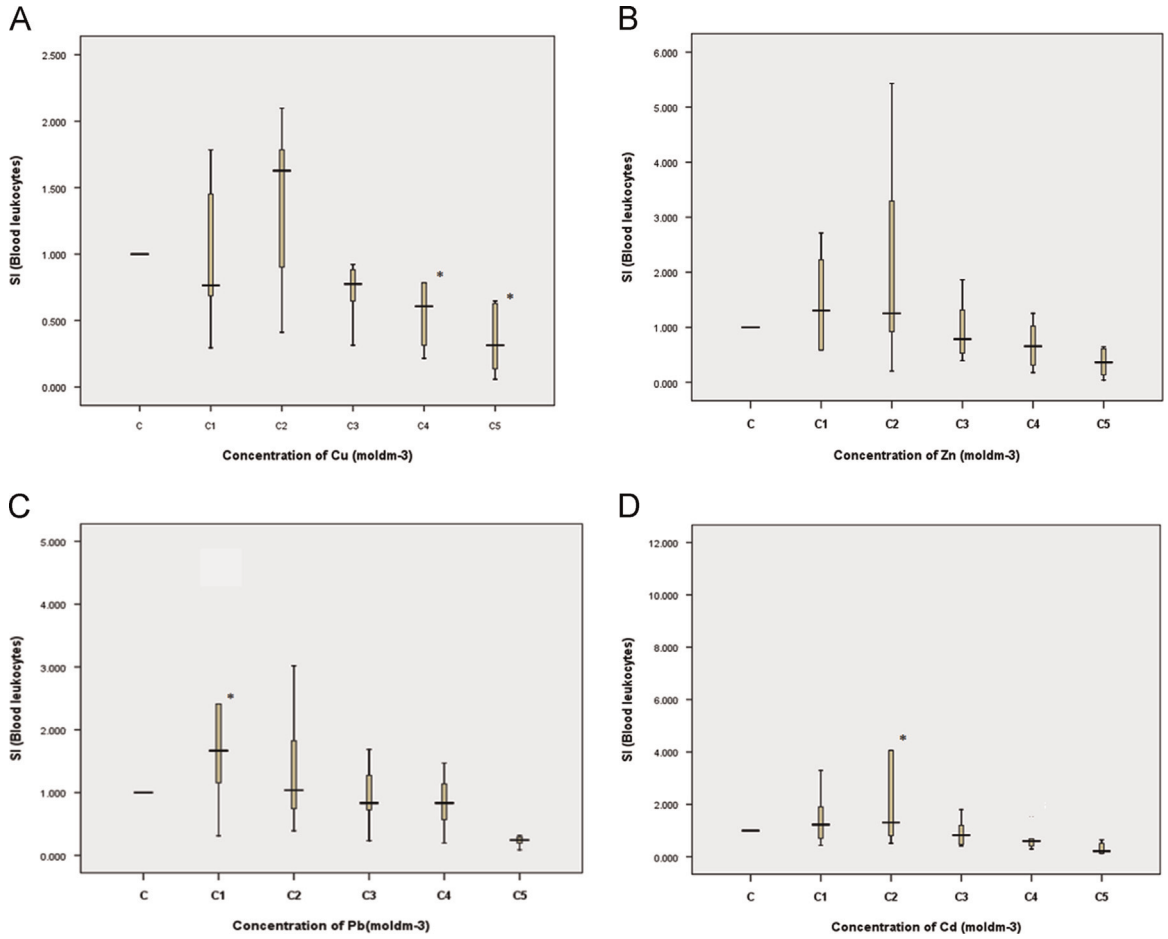

Fig. 4. Effects of (A) Cu, (B) Zn, (C) Pb and (D) Cd on *in vitro* phagocytic response of blood leukocytes. Frogs from the reference site ($N=10$) were tested under five concentrations (C_1–C_5) of each heavy metal. Significant differences in the SI of the metal exposed blood leucocytes were observed compared to the control ($^*P<0.05$). (C=no heavy metal treatment (control), $C_1=1\times10^{-10}$ M, $C_2=1\times10^{-8}$ M, $C_3=1\times10^{-6}$ M, $C_4=1\times10^{-4}$ M, $C_5=1\times10^{-2}$ M).

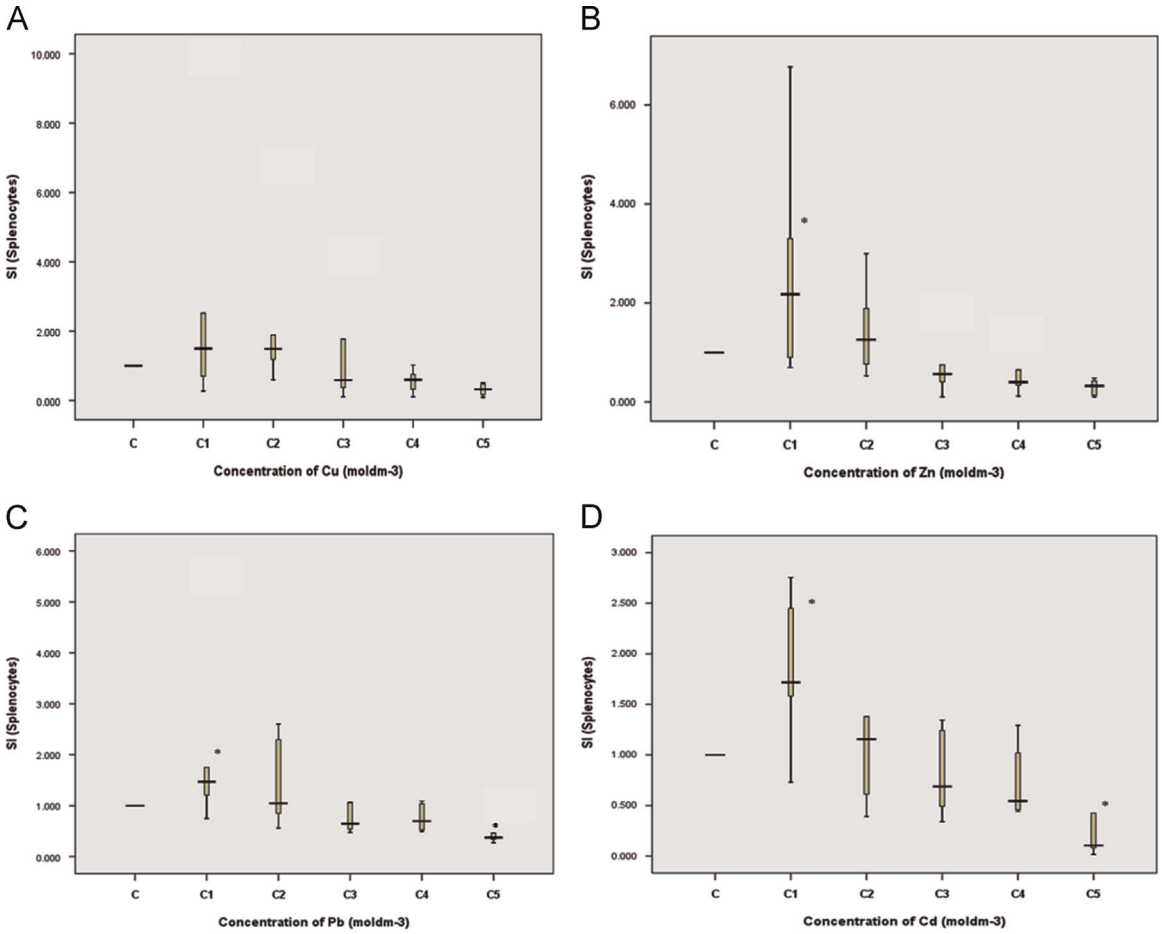

Fig. 5. Effects of (A) Cu, (B) Zn, (C) Pb and (D) Cd on *in vitro* phagocytic response of splenocytes. Frogs from the reference site ($N=10$) were tested under five concentrations (C_1–C_5) of each heavy metal. Significant differences in exposed splenocytes were observed compared to the control ($^*P<0.05$). (C=no heavy metal treatment (control), $C_1=1\times10^{-10}$ M, $C_2=1\times10^{-8}$ M, $C_3=1\times10^{-6}$ M, $C_4=1\times10^{-4}$ M, $C_5=1\times10^{-2}$ M).

(0.006×10^{-5} M for WBC and peritoneal macrophages, 0.05×10^{-5} for splenocytes) of phagocytosis followed by Zn (0.25×10^{-5} M for WBC, 0.2×10^{-5} M for peritoneal macrophages and 0.02×10^{-5} for splenocytes) and Cu (0.79×10^{-5} M for WBC, 0.79×10^{-5} M for peritoneal macrophages and 0.03×10^{-5} for splenocytes), while Pb (32×10^{-5} M for WBC, 130×10^{-5} M for peritoneal macrophages and 0.2×10^{-5} for splenocytes) was the least immunotoxic.

3.6. Reproductive measures

Total egg counts of *Euphlyctis hexadactylus* in the polluted site were significantly ($P<0.05$) higher than those of frogs in the reference site. On average, the egg count of mature female frogs in the polluted site (2986 ± 1713) was two folds higher than that of their counterparts in the reference site (1259 ± 625).

4. Discussion

Heavy metals being the most ubiquitous pollutants particularly influence sensitive species, such as aquatic amphibians by affecting their health, altering their behavior, physiology and anatomy which eventually pose adverse impacts on thier survival (Farombi et al., 2007). The outcome of our study indicated that heavy metals such as zinc, lead, copper and cadmium, present in the polluted Bellanwila Attidiya sanctuary that accumulate in frog liver and muscle tissue, may act as immunosuppressants. The immune system of *E. hexadactylus* was affected by altering the weight and cellularity of lymphoid organs, the quantity of the peripheral blood leukocytes and bone marrow, and finally modulating cellular functions such as phagocytosis. *In vitro* exposure of heavy metals to phagocytic cells, expressed similar immunosuppressive effects as those reported from the frogs naturally exposed to heavy metals in the polluted study site. This eco-immunotoxicological prototype study carried out in Sri Lanka further ramified the significance of immunotoxicologic tests such as phagocytosis, as potential biomarkers for evaluating heavy metal related health issues in wetland ecosystems.

Heavy metals in the polluted Bellanwila-Attidiya wetland waters were found to accumulate in liver and musculature of *E. hexadactylus* in significantly higher levels compared with counterpart frog tissue collected from the reference site. Even though, metals including Pb > Cu > Cd were found in low amounts (<1 ppm) in water compared with Zn (2.71 ppm), the tissue accumulation showed a reverse pattern where Cu was present in substantially higher levels ($1500–2800\ \mu g\ g^{-1}$) compared to Zn > Pb and Cd ($1–1500\ \mu g\ g^{-1}$). Hence, Cu seems to accumulate more prominently in tissues even with very low levels recorded in water (<6 ppb) of the reference site. The main route of exposure of *E. hexadactylus* to these metals may possibly be through wetland debris, consumed across the course of development from tadpole to frog. Nevertheless, their semi permeable skin and the buccal/gill respiration may provide easy internal access.

Heavy metals even at environmental levels elicit a number of immunosuppressive effects, which are numerous and diverse

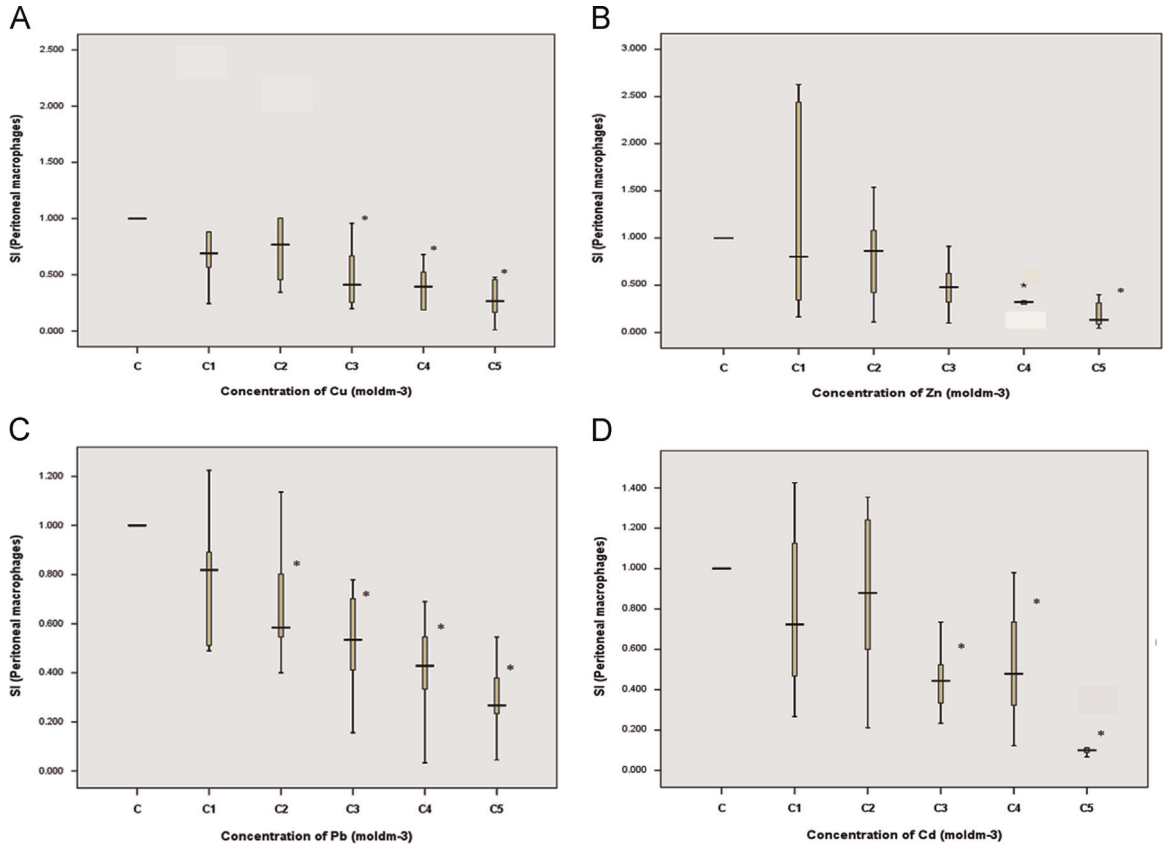

Fig. 6. Effects of (A) Cu, (B) Zn, (C) Pb and (D) Cd on *in vitro* phagocyttic response of peritoneal macrophages. Frogs from the reference site ($N=10$) were tested under five concentrations (C_1–C_5) of each heavy metal. Significant differences in the exposed peritoneal macrophages were observed compared to the control ($^*P<0.05$). (C=no heavy metal treatment (control), $C_1=1\times10^{-10}$ M, $C_2=1\times10^{-8}$ M, $C_3=1\times10^{-6}$ M, $C_4=1\times10^{-4}$ M, $C_5=1\times10^{-2}$ M).

among various animals, leading to enhanced vulnerability to disease and other stressors. Many *in vivo* and *in vitro* studies performed on animal models, such as fishes, birds, rats and mice (Gaworski and Sharma, 1978; Arunkumar et al., 2000; Holloway et al., 2003; Snoeijs et al., 2004) documented impacts of heavy metals on various aspects of humoral and cellular immunity. However, a majority of these studies are dedicated to mammalian responses in consequence to heavy metal administration. Conversely, scanty information is available on the immunosuppressive effects of lower vertebrates, including fishes and amphibians (Sparling et al., 2000). From this viewpoint the present study may provide a comprehensive account on amphibian immune responses against heavy metal mediated toxicity. According to Luster et al. (1988), systemic immunosuppression is indicated by decreased weight and histology of lymphoid organs, quantitative changes in cellularity of the lymphoid tissue, peripheral blood leukocytes and bone marrow, impairment of cell functions and finally the increased susceptibility to infectious agents or tumors. In the present study *E. hexadactylus* clearly showed most of the systemic immunosuppressive responses as a consequence of heavy metal exposure under both *in vivo* natural and *in vitro* empirical exposure. Among these measures of test animals in the polluted site, total blood leukocytes, splenocytes, neutrophils to lymphocytes ratio, total Ig levels, spleen weight to body weight ratio, phagocytic activity of peritoneal macrophages, splenocytes and leukocytes, showed significant suppression in comparison to counterparts of the reference site. Similar responses were reported in several other studies conducted with natural exposure or oral administration of heavy metal varieties including Zn, Pb, Cu and Cd; Relatively low, sub-toxic amounts of Zn, Pb, Cd and Hg tested *in vitro* reported a marked decline in phagocytic activity of mammalian splenocytes and peritoneal macrophages (Friberg et al., 1971). Conversely, Pb was reported to affect cellular aspects of the immune system following oral administration to rats while humoral parameters remained unchanged (Kimber et al., 1986). Moreover, decreased splenic and bone marrow macrophage levels were reported for mice treated with Pb (Burchiel et al., 2000). As lower vertebrates, fishes exposed to $CdCl_2$ (90–445 $\mu g\ L^{-1}$) were reported to have developed low WBC and lymphocyte counts (Murad and Hiouston, 1988). Hence, it is noteworthy that the reported immunosuppressive responses of *E. hexadactylus* following field exposure are comparable with the *in vitro* results and other heavy metal related immunotoxicity studies conducted elsewhere.

Often, immunotoxicity does not follow a dose–response relationship, as lower doses may enhance a specific immune function, middle range doses may have no effect while higher doses reflect immunosuppression (Gunnar and Quevedo, 2007). In our study, peritoneal macrophages and blood leukocytes showed a clear dose–response decline in phagocytosis when exposed to higher concentrations (10^{-3}–10^{-6} M) of Zn SO_4, $CdSO_4$, $CuSO_4$ and $PbNO_3$. However, at lower doses (10^{-10} M) immunostimulation was observed in phagocytic activity of frog blood leukocytes but not in other phagocytic cell types tested. Similar dose response relationships were observed by several other studies involving lead (Descotes et al., 1995), cadmium (Marth et al., 2000), chromium (Borella et al., 1990) and arsenic (McCabe et al., 1983) exposure. Conversely, contradictory results were reported by some other studies. For example, *Rana* tadpoles exposed to $CdCl_2$ at lower concentrations (0.1–0.2 ppm) reported no effect on lymphocyte numbers but reported higher B lymphocytes at higher (0.8 ppm) concentrations (Zettergren et al., 1991). However, a growing body of literature indicates that the exposure to sublethal

doses of xenobiotics elicit immunosupression in other animal species; Phagocytic cells collected from bovine leukocytes in response to 10^{-7} M of $CdCl_2$ and 10^{-4} M $PbCl_2$ (Brousseau et al., 2000) and earth worm coelomocytes in response to 10^{-6} M of $CdCl_2$ and $ZnCl_2$ exposure (Fugere et al., 1996). Most of these studies suggest that decrease in cell phagocytosis was not entirely a consequence of decline in cell viability but presumably due to some other causes.

Mechanisms by which metals express their toxicity in biological systems is diverse and involve several complex biochemical reactions including oxidative burst through the formation of reactive oxygen species (ROS). Hence, the mechanistic aspects of heavy metal cytotoxicity mediated damage to plasma membranes, may be attributed to direct binding to proteins and phospholipids, inhibition of Na^+, K^+ ion dependent ATPases, inhibition of transmembrane amino acid transport, enzyme inhibition, lipid peroxidation (LPO) and oxidative DNA damage, and depletion of antioxidant enzymes such as glutathione-group enzymes through the generation of ROS (Valavanidis and Thomie, 2010). Friberg et al. (1971) suggested that low/no immunosuppression at lower levels of heavy metals may be due to natural defense mechanisms such as metallothionine and glutathione family proteins. Related observations made by another study reported $CdCl_2$ and $HgCl_2$ induced thionine production in human peripheral leukocytes (Cherian and Goyer, 1978) occurred in a dose dependent manner and $CdCl_2 > HgCl_2 > PbCl_2$ induced metallothionine production in mouse splenocytes (Lynes et al., 1990). In contrast, disruption of calcium homeostasis by heavy metals could affect the phagocytic activity as the calcium flux is crucial for neutrophil chemotaxis and phagocytosis (Elferink and de Koster, 1994). Moreover, heavy metals affect the cytoskeleton that controls the ability of a phagocyte to alter its shape to interact with the foreign particles (Cima et al., 1998). Hence, higher affinity of heavy metals such as Cu and Cd to thiol group proteins such as calcium-ATPases, calcium channels may affect the membrane stability which in turn affects phagocytosis (Viarengo et al., 1994). Furthermore, copper is linked with the generation of ROS by the Fenton reaction, directly affecting cytoskeletal proteins (Halliwell and Gutteridge, 1984). As the highest accumulated metal species in the tissues of *E. hexadactylus* was copper, the other metal species, *i.e.* Cd, Pb, and Zn may act in one or more of these mechanistic ways to alter the immunosuppressive responses. However, exact mechanisms remain elusive warranting further mechanism-oriented studies.

Heavy metal induced immunotoxicity appeared to be associated with the metal binding protein, metallothionine, which plays a vital role in metal homeostasis and detoxification of heavy metals (Cherian and Goyer, 1978; Sone et al., 1988). Thionine production in human peripheral blood lymphocytes was known to be induced by metal chlorides such as $CdCl_2$ and $HgCl_2$ (Sone et al., 1988). Similarly, in mouse splenocytes $CdCl_2$was reported to be the prime agent for thionine induction followed by $HgCl_2$ and $PbCl_2$ (Lynes et al., 1990). Conversely, secretion of corticosterone and aldosterone due to metal mediated stress conditions, may further cause immune suppression by decreasing T-cell proliferation, antibody production, and by inducing lymphocyte apoptosis (Rollins-Smith and Blair, 1993; Ottaviani and Franceschi, 1996; Ducoroy et al., 1999). Hence, the overall effect of heavy metal exposure in the animal body may be a function of a corollary of all these factors. Even though, Cd ions induce thionine production to deal with oxidative stress it may generate ROS species with the presence of reducing agents in the body. The balance between these positive and negative factors may decide the overall effect of metal exposure. Likewise, cellular activities such as phagocytosis may be stimulated at lower concentrations of metals while inhibited at higher concentrations.

Besides the effect of stress hormones, resource partitioning or the energy trade-off between key physiological processes during high energy demands may provide an additional explanation to wildlife health in an eco-immunotoxicity context (Norris and Evans, 2000). Hence, for example, in a high-risk disease situation, devoting resources for reproduction may decrease at the cost of immune system functions whereas during reproduction, immune function may be compromised to allow the individual to maximize its reproductive effort. However, according to this explanation fast-paced environmental changes may pose immense pressure on the immune system of wildlife species, at the cost of population level damage. The alternative to withstand environmental stress conditions is to have a higher number of offspring to replace the immune impaired population that is more vulnerable to infections and disease. Therefore, as an evolutionary trade off, female frogs in the polluted Bellanwila site may have a higher rate of reproduction and oviposition than their counterparts from the reference site.

The results of the present study clearly demonstrated heavy metal mediated immunomodulation of the Indian green frog, *E. hexadactylus*, exposed to Cd, Zn, Cu, and Pb. This was confirmed by various functional and non-functional immunological tests. Long term exposure to heavy metals under field conditions, possibly elucidate better the overall schema of immunosuppression rather than, short-term empirical exposure. Thus, immunosuppressive parameters measured in this study may well be used as potential biomarkers of environmental quality in heavy metal contaminated wetland habitats, growingly evident in urban Sri Lanka.

Conflict of interest

The authors declare that they have no conflict of interest.

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