Science of the Total Environment 566–567 (2015) 1194–1204

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Heavy metal mediated innate immune responses of the Indian green frog, *Euphlyctis hexadactylus* (Anura: Ranidae): Cellular profiles and associated Th1 skewed cytokine response

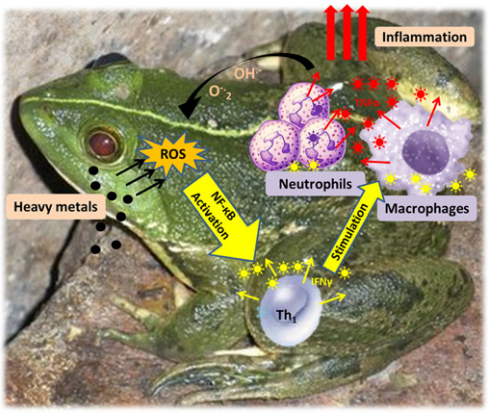
Uthpala A. Jayawardena, Wanigasekara D. Ratnasooriya, Deepthi D. Wickramasinghe, Preethi V. Udagama *

Department of Zoology, University of Colombo, Colombo 03, Sri Lanka

HIGHLIGHTS

- Heavy metal pollution in wetland ecosystem is a serious ecotoxicological issue of global dimension
- Immune cells and cytokine profile alternations were detected in heavy metal exposed *Euphylictis hexadactylus* under field and laboratory conditions
- Th1 immune response showed significant positive correlation with neutrophil counts explaining the role of neutrophil function under heavy metal burden.
- Heavy metal mediated inflammatory response in the liver elevated TNFα, IL6 and IL10 production, positively correlated with melanomacrophage aggregates
- Th1 skewed immune response may be attributed to oxidative stress mediated NFκB activation

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 26 March 2016
Received in revised form 23 May 2016
Accepted 24 May 2016
Available online 19 June 2016

Editor: D. Barcelo

Keywords:
Eco-immunotoxicity
Heavy metals
Innate immunity
Neutrophils
Th1/Th2 cytokine polarisation

ABSTRACT

Immune cell and cytokine profiles in relation to metal exposure though much studied in mammals has not been adequately investigated in amphibians, due mainly to lack of suitable reagents for cytokine profiling in non-model species. However, interspecies cross reactivity of cytokines permitted us to assay levels of IFNγ, TNFα, IL6 and IL10in a common anuran, the Indian green frog (*Euphlyctis hexadactylus*), exposed to heavy metals (Cd, Cr, Cu, Zn and Pb, at ~5 ppm each) under field and laboratory settings in Sri Lanka. Enumeration of immune cells in blood and melanomacrophages in the liver, assay of serum and hepatic cytokines, and Th1/Th2 cytokine polarisation were investigated.

Immune cell counts indicated overall immunosuppression with decreasing total WBC and splenocyte counts while neutrophil/lymphocyte ratio increased with metal exposure, indicating metal mediated stress. Serum IL6 levels of metal exposed frogs reported the highest (~9360 pg/mL) of all cytokines tested. Significantly elevated IFNγ production ($P < 0.05$) was evident in heavy metal exposed frogs. Th1/Th2 cytokine ratio in both serum and liver tissue homogenates was Th1 skewed due to significantly higher production of pro-inflammatory cytokines, IFNγ in serum and TNFα in the liver ($P < 0.01$).Metal mediated aggregations of melanomacrophages in the liver were positively and significantly ($P < 0.05$) correlated with the hepatic expression of TNFα, IL6 and IL10

* Correspondence author.
E-mail addresses: dappvr@yahoo.com, preethi@zoology.cmb.ac.lk (P.V. Udagama).

http://dx.doi.org/10.1016/j.scitotenv.2016.05.171
0048-9697/

activity. Overall, Th1 skewed response may well be due to oxidative stress mediated nuclear factor κ-light chain enhancer of activated B cells (NFκB) which enhances the transcription of pro-inflammatory cytokines. Xenobiotic stress has recently imposed an unprecedented level of threat to wildlife, particularly to sensitive species such as amphibians. Therefore, understanding the interactions between physiological stress and related immune responses is fundamental to conserve these environmental sentinels in the face of emerging eco-challenges.

1. Introduction

Eco-immunotoxicology that investigates effects of environmental toxicants on wildlife immunity is an emerging area of research globally. Functional and non-functional biomarkers of immunotoxicity were researched extensively in this quest. Most immunologic markers however, are capable of detecting only a segment of the immune response, rather than perceiving the overall response. Cytokines as messengers which trigger a wide variety of immune responses, provide comprehensive countenance of the health status including stress, disease resistance and the cost of inflammatory response (Zimmerman et al., 2014). Despite its wide immunotoxicological relevance, only a fraction of the cytokine responses to xenobiotic stress in non-model systems, such as amphibians were studied. This is due mainly to knowledge gaps that exist in the understanding of immune functions in non-mammalian vertebrates and more so due to unavailability of pertinent reagents for non-model systems.

The adaptive immune response is believed to have first evolved in the jawed vertebrates and their activity is well established in mammalian systems (Kaiser et al., 2004). However, T and B cell subpopulations do exist in lower vertebrates, fishes, amphibians and birds. However, among vertebrate immune systems amphibian granulocytes remain relatively obscure. Based on Wright (2001) amphibians possess eosinophilic granulocytes, basophilic granulocytes, and neutrophilic granulocytes described as eosinophils, basophils, and neutrophils, respectively (Wright, 2001). Neutrophils and monocytes are phagocytic and associated basically with the innate immune responses while lymphocytes are associated with the adaptive immunity. Eosinophils are cytotoxic in function and stimulate other WBCs to release histamine during parasitic invasions (Edwards, 1994). In amphibians neutrophil/lymphocyte ratio is considered a predictor of inflammation manifesting as a general response towards stressors (ForbesMR and Shutler, 2006; DavisAK and Maerz, 2008).

Cytokines, particularly, pro-inflammatory ones may be common to all vertebrates or at least have functional alternatives in lower animals (Kaiser et al., 2004). Interferons (IFNs) and interleukins (ILs) are major groups of cytokines present in the vertebrate immune system. IFNs induce antiviral function in cells to defend against viral infections of vertebrates (Samuel, 2001). Unlike type I IFN, IFNγ is produced exclusively by cells of the immune system, particularly by CD_4^+ T helper 1cells (Th1) and CD_8^+ cytotoxic T cells in response to major histocompatibility complex (MHC) presented antigens (Biron and Sen, 2001). IFNγ plays a major role in the adaptive, cell mediated immune response. Being the hallmark of Th1 response, IFNγ has been studied extensively in mammals and other vertebrate species including amphibians (Savan et al., 2009). Since the IFNγ molecule has a wide range of immunomodulatory properties due to its ability to up regulate the expression of several transcription factors and cytokines, studying IFNγ activity is imperative in eco-immunological investigations. Tumour necrosis factors (TNFs) are cytokines involved in inflammation, apoptosis, and cell proliferation of the immune system (Goetz et al., 2004). TNFα is produced by macrophages, lymphocytes and NK cells in response to antigens or chemical stimuli, including toxins, parasites or pathogens, and other cytokines (Kushibiki, 2011). Interleukins are crucial components in both adaptive and innate immune responses in the mammalian immune system. Records of the activity of ILs in lower vertebrates such as fishes and amphibians are scarce, and anti-inflammatory cytokine, IL10 (Th2 type) has not been reported from fishes and lower vertebrates (Kaiser et al., 2004). IL6 induces the development of Th17 cells from naïve T cells together with TGFβ while it inhibits TGFβ induced Treg differentiation (Kimura and Kishimoto, 2010). The balance between pro-inflammatory/anti-inflammatory cytokines correspond to that of Th1/Th2activity. Th1 responses are dominated by innate and cell mediated responses whereas Th2 cytokines drive the antibody mediated humoral response. Cytokines produced by Th1 cells down regulate Th2 responses and vice versa, resulting in the polarisation of the immune response (Elenkov et al., 2005).

Since cytokine genes are conserved across vertebrate evolution, these show fairly high interspecies cross reactivity (Zimmerman et al., 2014) allowing eco-immunologists to study cytokine activity even in non-model species such as amphibians. Savan and colleagues (Savan et al., 2009) revealed structural and functional conservation of the IFNγ gene in lower vertebrates suggesting the presence of an innate, NK cell activity and Th1 response in lower vertebrates. Recent data strongly suggest that teleost fishes and lower vertebrates possess one IFNγ gene that has the same exon/intron structure as the IFNγ of higher vertebrates (Robertsen, 2006). Fish and mammalian TNFα shows high structural and genomic conservation, where "TNF family signature" in TNFα gene shares 70 amino acids in the amino terminus (Goetz et al., 2004).Therefore, cross reactivity has been observed in fish and mammalian TNFα where human recombinant TNFα was reported to enhance neutrophil and lymphocyte activity in the rainbow trout (Hardie et al., 1994). IL6 is reported to share a high percentage of similarity in its structure across vertebrate evolution (Zimmerman et al., 2014) where human recombinant IL6 reportedly induced acute phase proteins in fish liver tissue (Jørgensen et al., 2000). Moreover, a cDNA sequence study reported that chicken IL6 shared 35% amino acid identity with mammalian IL6 (Van Snick, 1990).

Among ubiquitous environmental pollutants, heavy metals known for their toxicity are of prime concern. Although, immunotoxicology of heavy metals has been widely studied, scant information is available on the immunomodulation of heavy metals with respect to cytokine profiles, particularly in amphibians. Mammalian systems, mostly rat models, have been studied widely in association with occupational exposure of humans, showing inconsistent results depending upon the concentration and the type of metal, differences in the path of cell activation, age and sex of the target individual. In vitro exposure of $CdCl_2$ (5–100 μM) to blood leukocytes elevated TNFα at low concentrations but had an insignificant effect at higher concentrations. IFNγ decreased significantly at higher concentrations whereas IL6 increased at low concentrations (Funkhouser et al., 1994). IFNγ activity was unaffected in mice injected with $CdCl_2$ (6.25 mg/kg, (Daniels et al., 1987)). Occupational exposure to chromium (Cr) down regulated IL6 production (64% of the control, (Snyder et al., 1991)) and human macrophages treated with Cr showed increased production of TNFα (Wang et al., 1996). Available data concerning the effects of metals on cytokine responses are thus fragmentary and contradictory. In this respect, not only the systemic cytokine profile, but also that of hepatic cytokines may help predict the overall response of heavy metal mediated toxicity, as the liver is the major organ of heavy metal accumulation and detoxification.

The liver is associated with the production and the elimination of cytokines. Further, all cell types present in the liver produce cytokines

(Simpson et al., 1997) and hepatic cytokines are associated with diseases such as cirrhosis and fibrosis (Hsieh et al., 2011). During tissue injury, the activated *Kupffer* cells (macrophages) and T cells in the liver secrete cytokines, particularly TNFα which is the central in the augmentation of liver injury mainly by inducing hepatocyte apoptosis to activate hepatic stellate cells through membrane protein synthesis (Knittel et al., 1997).As a counter-regulatory effect, hepatic macrophages also secrete IL4, IL6, and IL10 which may diminish hepatic injury by either direct or indirect mechanisms and are associated with hepatocellular damage described in cirrhosis and other inflammatory liver tissue injuries (Nagano et al., 1999). IL6 is an efficient stimulator for the production of acute phase proteins in the liver (Demas, 2004). Therefore, hepatic cytokine profiling is a diagnostic tool for a wide range of disorders (Aggarwal and Puri, 1995). Aggregates of *Kupffer* cells (macrophages) in some animals form melanomacrophage centres and are reported to increase in size or percentage under environmental or physiological stress and therefore were suggested as a biomarkers for xenobiotic mediated toxicity studies (Agius and Roberts, 2003).

Amphibians represent a key evolutionary landmark as a turning point of aquatic life forms to terrestrial ones, and are frequently proposed as sentinels of environmental degradation (Burlibaşa and Gavrilă, 2011; Mann et al., 2009; Roy, 2002; Van der Schalie et al., 1999). Amphibian immune system, particularly in *Xenopus* is remarkably conserved with respect to the mammalian system, especially *T*-cell development of the thymus and peripheral T-cell function (Robert and Ohta, 2009). Accordingly, *Xenopus* is a versatile model to study ontogeny and phylogeny of humoral and cell-mediated immunity against tumours and pathogens. However, amphibian cytokine profiles and cytokine genes have not been studied adequately. Cytokine activity, particularly leukocyte derived IL1, IL12 and transforming growth factor (TGF), macrophage migration inhibitory factor (MIF) activity were evaluated (Watkins et al., 1987; Haynes and Cohen, 1993).Savan et al. *(Savan et al., 2009)* identified the IFNγ gene in *Xenopus tropicalis*; with X-ray crystallography analysis; They reported strict conservation of the poly-cationic *C*-terminus tail of the IFNγ structure compared with the mammalian IFNγ structure, suggesting its function as an IFNγ specific binding site. However, analysis of immune responses in wildlife including amphibians has been hindered due to lack of specific reagents for these non-model species (Zimmerman et al., 2014).Therefore, the current study assayed selected cytokine levels in frogs using reagents available to assess rat cytokines.

Heavy metals, Cd, Cr, Cu, Zn and Pb (~5 ppm of each) present in the waters of a polluted urban wetland, Bellanwila-Attidiya Sanctuary (BAS), in Sri Lanka were reported to impair the immune system of a common anuran amphibian species, the Indian green frog, *Euphlyctis hexadactylus*, by reducing WBC, platelet, splenocyte, bone marrow counts and phagocytic activity of macrophages (Priyadarshani et al., 2015). Twenty eight days of laboratory exposure to the same heavy metal mixture (5 ppm) of each metal validated the field study suggesting heavy metal mediated immunosuppression in *E. hexadactylus* (unpublished data). The present study entailed immune cell profiling and liver melanomacrophage enumeration conjointly with assay of serum and hepatic levels of IFNγ and TNFα (Th1 type cytokines), IL10 (Th2 type cytokine), and IL6 (regulator of Treg/Th17 balance), and also investigated the Th1/Th2 cytokine polarisation in both field and laboratory exposed adult frogs to selected heavy metals using commercially available rat cytokine sandwich ELISA kits.

2. Materials and methods

Approval for the collection of adult *E. hexadactylus* from the study sites was obtained from the Department of Wildlife Conservation (WL/3/2/10/13), Sri Lanka. Ethical approval for conducting animal research was granted by the Ethics Review Committee, Faculty of Medicine, University of Colombo (ERC/12/176). Therefore, all experiments conducted were in compliance with the ethical guidelines provided by these two authorities.

2.1. Study sites and sample collection

Bellanwila-Attidiya sanctuary (BAS), (6° 48′-52′ N and 79° 52′-56′ E) was selected as the polluted site, while Labugama reservoir and the catchment area (7° 1′-60′ N and 79° 52′-0′ E) served as the reference site, with both sites situated in the western province of Sri Lanka. This sanctuary is heavily degraded due to uncontrolled anthropogenic activity such as, garbage dumping and input of untreated industrial sewage. A pilot study revealed contamination in waterways of the sanctuary with metals, Zn (2.71 ppm), Pb (0.95 ppm), Cu (0.04 ppm)and Cd (0.019 ppm) (Priyadarshani et al., 2015).Water quality parameters(pH, temperature, DO[Dissolved Oxygen] and BOD[Biological Oxygen Demand]) were measured at each site during sampling carried out bimonthly from Feb. 2013–Aug. 2014. Water samples were collected bimonthly during March 2013 to May 2015 into acid washed plastic bottles and were preserved with 3% (v/v) conc. HNO_3 acid for heavy metal analysis (ASTM, 2003).

The test animals, adult Indian green frogs, *E. hexadactylus*, ($n = 15$ per site) were randomly captured using bait from waterways of the two study sites. Frogs with average 100 g body weight and ~100 mm body length were selected for the study. The collected frogs were transported to the laboratory in aerated plastic bags half filled with water and transferred in to aerated glass tanks, containing metal free dechlorinated tap water in order to acclimatise frogs for two days prior to further analysis, providing them with chopped meat (~10% of their body weight) once daily (ASTM, 2003).

2.2. Laboratory exposure

Another set of adult *E. hexadactylus*, ($n = 15$) was collected from the reference site to conduct laboratory exposure. They were acclimatized in the same conditions as above for two days prior to metal exposure. Frogs were exposed to a mixture of heavy metals (Cu, Cd, Cr, Zn and Pb [5 ppm each]) prepared in dechlorinated tap water, for a period of 28 days. The exposure medium was renewed every second day and the frogs were fed with chopped meat (10% of bodyweight; (OECD, 2008)) once daily, throughout the exposure period.

2.3. Tissue sampling and processing

Field collected and laboratory exposed frogs were sacrificed using MS222 (0.2% solution). Body weight was recorded using a digital weighing balance up to 0.01 g accuracy (LP 202A, Ningbo Hinotek Tech, Zhejiang, China) and standard external morphometric parameters, such as snout-vent length (SVL) and body width (W) were measured using an electronic digital calliper (Grade 03, Control Company, Friendswood, USA). Blood was drawn by heart puncture to obtain total and differential WBC counts, and to prepare serum according to standard procedure (Hudson et al., 1989). Spleen and bone marrow were excised and the cellularity of both organs was enumerated (Hudson et al., 1989).Frog gastrocnemius muscle from the left leg and the liver were preserved at −20 °C for heavy metal analysis. Parts of liver were also preserved in 10% buffered formalin for histological enumeration of melanomacrophages. The frozen liver tissues were also used for the preparation of liver tissue homogenates.

2.4. Measuring heavy metal concentrations

Water samples were filtered to remove any solid particles and analysed using a graphite furnace atomic absorption spectrophotometer (AAS, AA-6650, Shimadzu, Japan). Frog liver and gastrocnemius muscle tissues were burnt to ash (450 °C) in a muffle furnace (JSMF-45 J, research Inc., Korea) and acid digested (1HNO_3: 1H_2SO_4, (ASTM,

2003)). The digested samples were filtered to remove any solid particles and analysed under Graphite AAS.

2.5. Preparation of Liver tissue homogenates

Ice cold liver tissue was placed in a glass homogenizer filled with ice cold STKM (250 mM sucrose, 50 mM TrisHCl, 25 mM KCl, 5 mM $MgCl_2$) buffer (16–20 mg/mL).Tissue was homogenized and the extract was cold centrifuged at 2000 g for 20 min. During all preparative procedures tissue samples were maintained near 0 °C. Supernatant was separated immediately and stored at −80 °C for cytokine analysis, the following day.

2.6. Enumeration of liver melanomacrophages

Liver tissue, fixed in 10% buffered formalin were processed after 48 h. At the outset, tissue samples were dehydrated through an ascending series of ethanol; thereafter were cleared in Histoclear, clearing agent (H-2779, Sigma Aldrich, Taufkirchen, Germany) and embedded in paraffin wax. Tissue sections of 5–8 μm thickness were prepared by using a rotary microtome (Yamato, Kohki, Japan) and stained with standard double staining procedure using Hematoxylin and Eosin. Cytological scoring of melanomacrophage aggregates/ hepatocytes ratio and other morphometrics were obtained by the Infinity analyze software (version 6.3, Lumenera Corporation, Canada).

2.7. Sandwich-ELISA for cytokine analysis

Concentrations of cytokines in frog serum and liver tissue homogenates were measured by sandwich enzyme linked immunosorbent assay (ELISA) following the manufacturer's instructions using kits designed for IFNγ, TNFα, IL6 and IL10 (BD Opt EIA™, BD Bioscience, USA).Briefly, ELISA plates were coated with the relevant capture antibody (anti-rat IFNγ, TNFα, IL6 and IL10). Subsequent to blocking the plates with assay diluent (10% foetal bovine serum in phosphate buffered saline), serum or liver homogenate was added and incubated. After washing, plates were interacted with the detection antibody (e.g. Biotinylated anti rat IFNγ for IFNγ analysis) followed by addition of enzyme conjugate (Avidin-Horseradish peroxidase; HRPO) and the chromogen, O-phenylaminedichloride (OPD). Optical density was measured at 490 nm, using an ELISA plate reader (Bio-Rad, Model 680, USA). The concentration of each cytokine was calculated from a standard curve constructed with cytokine standards provided in the kit.

2.8. Statistical analyses

Statistically unbiased sample was obtained with the equation, $ME = t \times S/\sqrt{n}$, where ME = marginal error, t = t score, s = standard deviation and n = sample size. Thus, N = 15 in each test group represented a statistically unswaying sample for the analyses conducted. Data were expressed as means ± standard error of mean (SEM) and consequently, bars drawn in the graphs represent SEM. Body morphometrics of the adult frogs between study sites were analysed with simple t test. Mean comparisons of physicochemical parameters, heavy metal accumulation and immunotoxicity data among treatment groups were conducted with one way ANOVA, followed by Tukey's HSD. The analyses were conducted with SPSS 20.0 (IBM, USA).

3. Results

As the metabolic activity varies with the morphometrics and affects their physiology and the fitness (Hudson et al., 1989), use of specimens with similar morphometric parameters was essential for comparison purposes. There was no significant difference ($P > 0.05$) in the morphometrics such as body weight and the snout vent length of the adult frogs used in the three experimental groups, viz a viz. field exposed, laboratory exposed and reference groups. Since, the body weight was considered as the selection criterion during capture of animals, their age was assumed to be comparable. The body weight and the snout vent length on average were 120.7 ± 26.8 g and 107.3 ± 8.4 mm, respectively with no significant differences between test groups ($P > 0.05$, t-test).Health status of the frogs, revealed by their external appearance, general activity such as escaping behaviour, alertness, competition for feeding and the type of external and internal parasite association was common to all. Therefore, immune status of the test populations was considered congruent at the onset of the study.

Water quality parameters of the two study sites such as temperature, pH, BOD and DO did not vary significantly in the two study sites ($P > 0.05$, ANOVA).Water samples collected from the polluted site at BAS were contaminated with, Cu (18.39 ppm), Cr (9.51 ppm), Pb (7.24 ppm), Zn (4.68 ppm), Cd (3.75 ppm) in significantly higher concentrations ($P < 0.05$, ANOVA) compared with the reference site[Cu (0.06 ppm), Cr (0.72 ppm), Pb(0.93 ppm), Zn (0.36 ppm), Cd (0.06 ppm)]. Accumulation of Cu, Cr and Pb in liver and gastrocnemius muscle tissue ($n = 15$ *each*) of *E. hexadactylus*, collected from the polluted site and the laboratory study were significantly higher (~1500 mg/kg, $P < 0.05$; Fig. 1), than those of the reference site.

3.1. Immune cell profiling

White blood cells (WBCs) of *E. hexadactylus* comprise of granulocytes (neutrophils, eosinophils and basophils) and agranulocytes (lymphocytes and monocytes) (Fig. 2). Total WBC and splenocyte counts were significantly lower in metal exposed frogs than those of the reference site ($P < 0.05$, ANOVA). Bone marrow cell counts did not significantly differ among the study groups ($P > 0.05$, Fig. 3a). Neutrophil/lymphocyte ratio of the metal exposed frogs showed significant elevation ($P < 0.05$) than those of the reference site (Fig. 3b). Similarly, the number of neutrophils per 1000 RBCs was also elevated significantly with metal exposure ($P < 0.05$; Fig. 3b). Conversely, the number of eosinophils per 1000 RBCs decreased with metal exposure.

3.2. Serum cytokine concentrations

The serum levels of selected cytokines of adult *E. hexadactylus* recorded in different test groups are presented in Fig. 4a. IFNγ level was significantly increased in both field and laboratory metal exposure ($F_{2,44} = 5.78$, $P = 0.01$, ANOVA). Unlike IFNγ, serum TNFα was slightly down regulated with metal exposure ($F_{2,44} = 1.43$, $P = 0.184$) compared to the reference levels. However, serum IL10 levels reported marked decline in field ($F_{2,44} = 1.58$, $P = 0.146$) and laboratory exposed frogs ($P = 0.006$) than in their reference counterpart. Among all the cytokines tested, IL6 reported the highest level in frog serum, with a reference of 11,540 ± 156 $pgmL^{-1}$.Serum IL6 of metal exposed frogs was slightly down regulated in field ($F_{2,44} = 0.89$, $P = 0.413$) and laboratory exposed frogs ($P = 0.194$).

Estimating the ratio of IFNγ/IL10 and TNFα/IL10 presented the Th1/Th2 cytokine balance (Fig. 4b). The ratio of IFNγ/IL10 showed significant elevation with metal exposure in the field ($F_{2,44} = 2.79$, $P = 0.031$) and laboratory exposed frogs ($P = 0.011$). Similarly, TNFα/IL10 was significantly increased in the field exposed frogs ($F_{2,44} = 2.55$, $P = 0.029$) with further elevation in laboratory exposed frogs ($P = 0.032$).

3.3. Correlation between neutrophil counts and serum cytokine levels

Correlation of neutrophil counts per 1000 RBCs with serum cytokine levels was plotted by pooling the data of all the adult *E. hexadactylus* used in the study (Fig. 5). The neutrophil counts showed significant positive correlation with IFNγ level ($r = 0.549$, $P = 0.045$, Fig. 5a) and significant negative correlation with the IL10 ($r = -0.685$, $P = 0.014$, Fig. 5d) level in serum. Correlation of neutrophil counts with serum

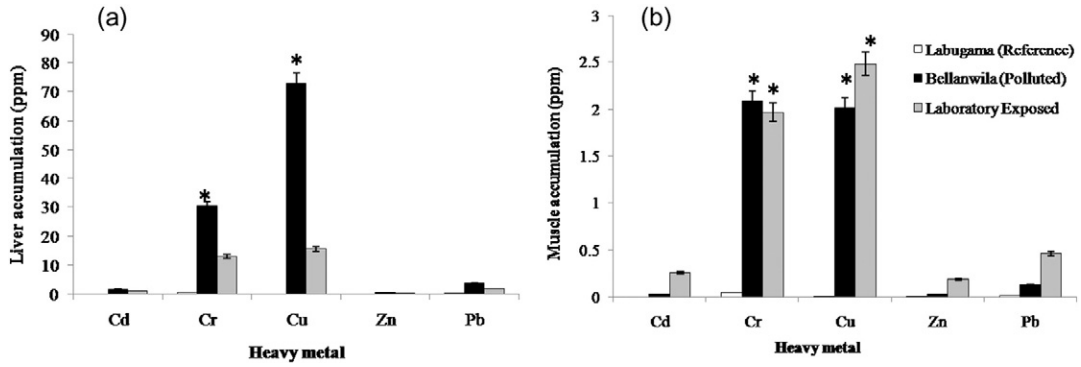


Fig. 1. Bioaccumulation, calculated as the amount of metal ions per tissue wet weight (μg/g or ppm), of the heavy metals in (a) frog liver and (b) gastrocnemius tissue of *E. hexadactylus*. *Significant accumulation ($P < 0.05$) compared to the reference value.

TNFα was near positive but with no significance ($P > 0.05$, Fig. 5b). IL6 levels and neutrophil counts showed no correlation (Fig. 5c).

3.4. Hepatic expression of cytokines

Hepatic expression of cytokines deviated from general body circulating levels, specified by the serum cytokine levels (Fig. 6a). There was no significant change of hepatic IFNγ levels in metal exposed frogs, compared to reference frogs where slight elevations only were reported in the former ($F_{2,44} = 1.24$, $P = 0.271$). However, hepatic TNFα levels showed marked elevation in the field ($F_{2,44} = 2.90$, $P = 0.063$) and laboratory exposed ($P = 0.027$) frogs compared with the reference level. In contrast, hepatic IL10 production was significantly down regulated in metal exposed frogs under field ($F_{2,44} = 5.0$, $P = 0.004$) and laboratory exposure ($P = 0.003$). IL6 production in the liver showed exponential elevation in laboratory exposed frogs (11,299 ± 767 pg mL^{-1}; $F_{2,44} = 4.08$, $P = 0.01$) compared with reference and field exposed frogs ($P = 0.472$).

Results similar to serum levels were obtained for hepatic cytokines when ratios of IFNγ/IL10 and TNFα/IL10 were compared, which reiterated a Th1 polarised immune response (Fig. 6b). The IFNγ/IL10 was elevated significantly in the metal exposed *E. hexadactylus* in field ($F_{2,44} = 1.34$, $P = 0.252$) and laboratory exposure ($P = 0.05$). Accordingly, hepatic TNFα/IL10 was significantly increased in field exposed frogs($F_{2,44} = 2.95$, $P = 0.048$) with further elevation under laboratory exposure ($P = 0.025$).

E. hexadactylus liver possessed distinct aggregations of *Kupffer* cells into melanomacrophage centres (MMCs) which increased significantly ($P < 0.05$) in number with metal exposure (Fig. 7a & b). % MMCs revealed significant positive correlation with TNF, IL6 and IL10 levels ($P < 0.05$) (Fig. 7c–f).

3.5. Correlation between serum levels and hepatic expression of cytokines

Hepatic and serum levels of cytokines were correlated by pooling data of all the adult *E. hexadactylus* used in the study (Fig. 8). Serum IFNγ levels positively and significantly correlated with hepatic IFNγ

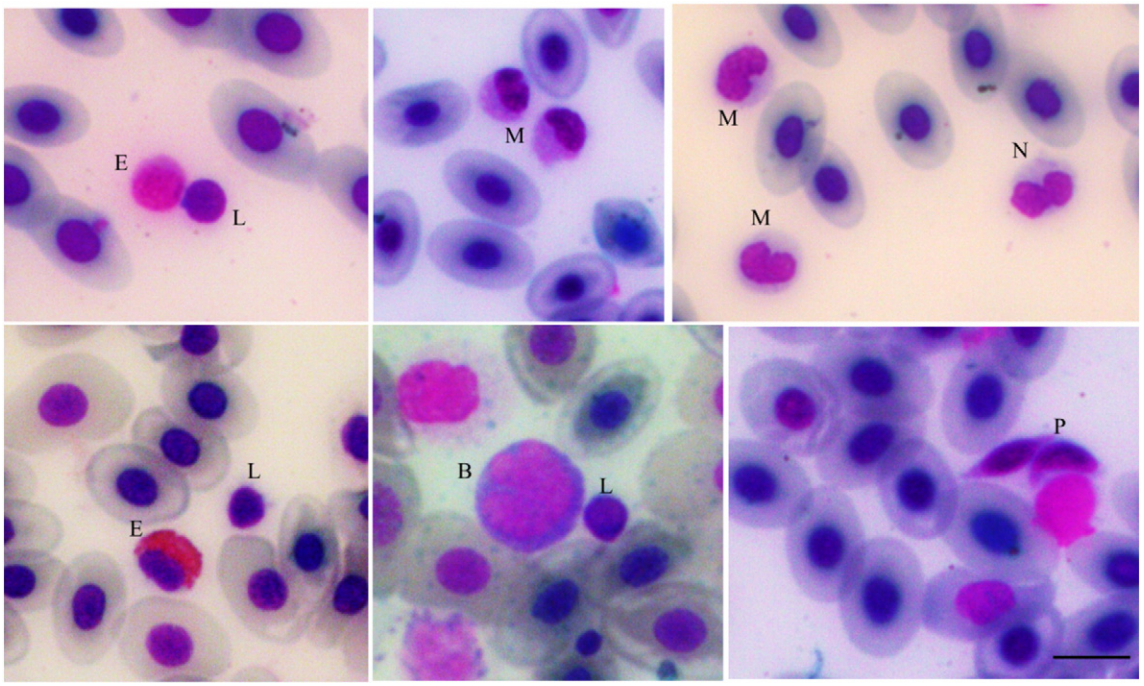


Fig. 2. *Euphlyctis hexadactylus* blood smears stained with Wright's stain, showing white blood cells and platelets. Agranulocytes; (L) lymphocytes with orbicular body and bluish violet nucleus, (M) monocytes with orbicular body and dumbbell shaped nuclei, (N) neutrophil with bilobed nucleus stained in violet red, (E) eosinophils with dark purple nucleus and deep orange eosinophilic granules, (B) basophils with orbicular body, violet red nucleus and bluish violet granules in the cytoplasm, (P) platelets/thrombocytes spindle-shaped body and oval nuclei stained in dark purple. Scale bar represents 20 μm.

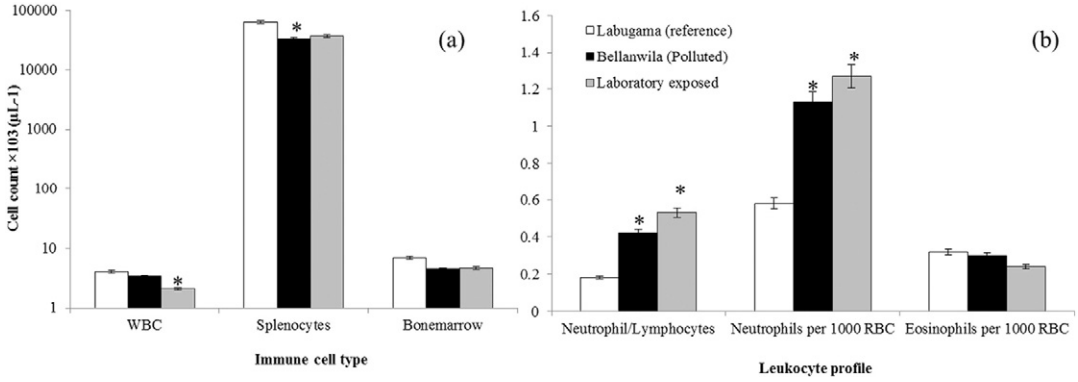


Fig. 3. Immune cell profiles of adult *E. hexadactylus* in different exposure groups. (a) WBC, splenocyte and bone marrow cell counts. (b) Neutrophil/ lymphocyte ratio, and neutrophil and eosinophil counts derived by differential WBC counts. *Significant alteration compared with reference value (Mann Whitney *U* test, CI at 95%).

levels($r = 0.427$, $P = 0.048$). However, serum TNFα and IL6 showed no correlation with the corresponding hepatic cytokine levels ($P > 0.05$), while serum IL10 was negatively and significantly correlated with the hepatic IL10 level ($r = -0.588$, $P = 0.044$).

4. Discussion

The effects of xenobiotics on various aspects of the immune system of amphibians for e.g. leukocyte profiles remain essentially obscure and are limited to a few studies (Rosenberg et al., 2002). Among the reported investigations, a decrease in total WBC and splenocyte counts was not commonly encountered as we observed in the metal exposed *E. hexadactylus* in the current and in a previous study (Priyadarshani et al., 2015). Nevertheless, laboratory exposure to high concentrations of atrazine and nitrate resulted in lowering WBC counts in *Ambystoma tigrinum* (Forson, 2006). Conversely, adult *Bufo maculatus*, exposed to cadmium (0.25–2 ppm concentration) resulted in an elevation of total WBC counts which was interpreted as immunostimulation in response to the tissue damage caused by cadmium toxicity (Ezemonye and Enuneku, 2011). Similarly, Pb exposed *Bufo arenarum* showed increased total WBC which was claimed as metal mediated induction of proliferation of pluripotent haematopoietic cells (Chiesa et al., 2006).Discrepancy of the present study may be attributed to the combined effect of the metal ions which may pose additive or synergistic effects as immunosuppressants. Other than the total WBC count, changes in proportions of WBCs were reported as a common measure of xenobiotic toxicity in amphibians. *Rana* spp. that lived in polluted water reported increased eosinophil counts and decreased neutrophil counts (Romanova and Romanova, 2003). *B. arenarum* exposed to pesticides showed increased neutrophil counts (Cabagna et al., 2005) while in another study the bullfrog showed no effect of atrazine on its differential WBC populations (Marcogliese et al., 2009).However, higher neutrophil/lymphocytes ratio (NLR) is considered as a general physiological stress response in animals (ForbesMR and Shutler, 2006; DavisAK and Maerz, 2008). Significantly higher NLR suggestive of general inflammation in the metal exposed *E. hexadactylus* under field and laboratory settings in the current study may be indicative of hazardous metal induced stress. Increased neutrophil counts in metal exposed frogs may be yet another indicator/ marker of this phenomenon.

Being one of the most potential tools in immunotoxicology cytokine profiling in relation to immune cell profiling provides a plausible measure of immunity, inflammation, apoptosis, haematopoiesis, and hypersensitivity (Boughton et al., 2011). This study investigated immune cell profiling with comprehensive cytokine profiling inclusive of cytokines associated with all effector Th cell populations including Th1 (IFNγ, TNFα), Th2 (IL10) and regulator of Treg/Th17(IL6) in response to heavy metal mediated immunotoxicity of amphibians.

Basal expression of IFNγ, TNFα, IL10 and IL6 of *E. hexadactylus* reported herein are first time reports for an amphibian species in Sri Lanka or for that matter for any amphibian species globally. Most of the amphibian cytokine studies conducted to date dealt with cytokine gene expression and evolutionary conservation of cytokine genes in comparison to mammalian genes (Zimmerman et al., 2014). *E. hexadactylus* IL6 had a higher level of basal expression reaching 11,540 ± 776pg mL^{-1}in reference frogs while their IFNγ and TNFα were expressed at much lower levels (<1300 pg mL^{-1}). This expression

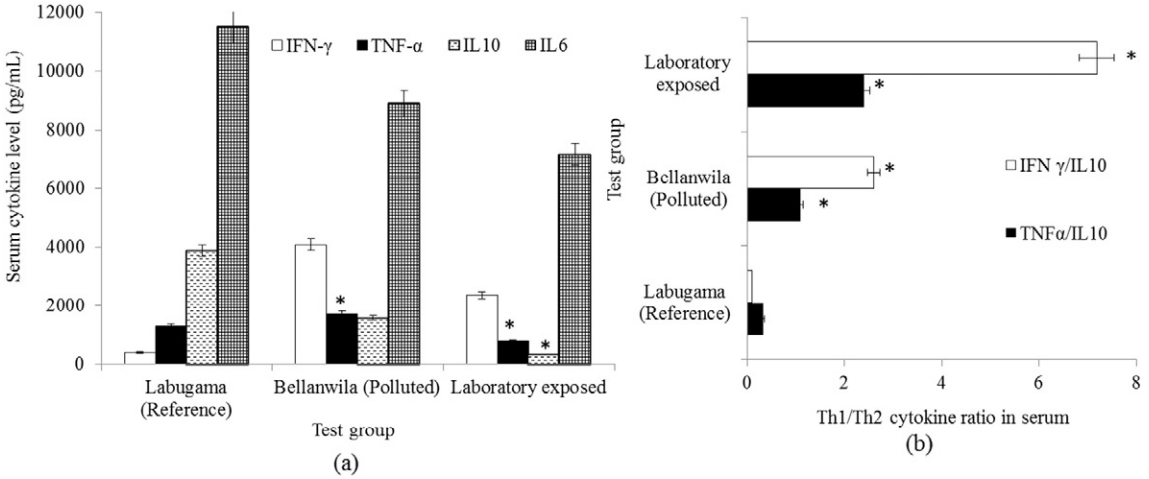


Fig. 4. Serum cytokine concentrations (a) and Th1/Th2 cytokine ratio (b) of adult *E. hexadactylus* ($n = 15$) in different test groups (error bars represent standard error of the mean; *significant alteration compared to reference animals, *t-test*, $P < 0.05$).

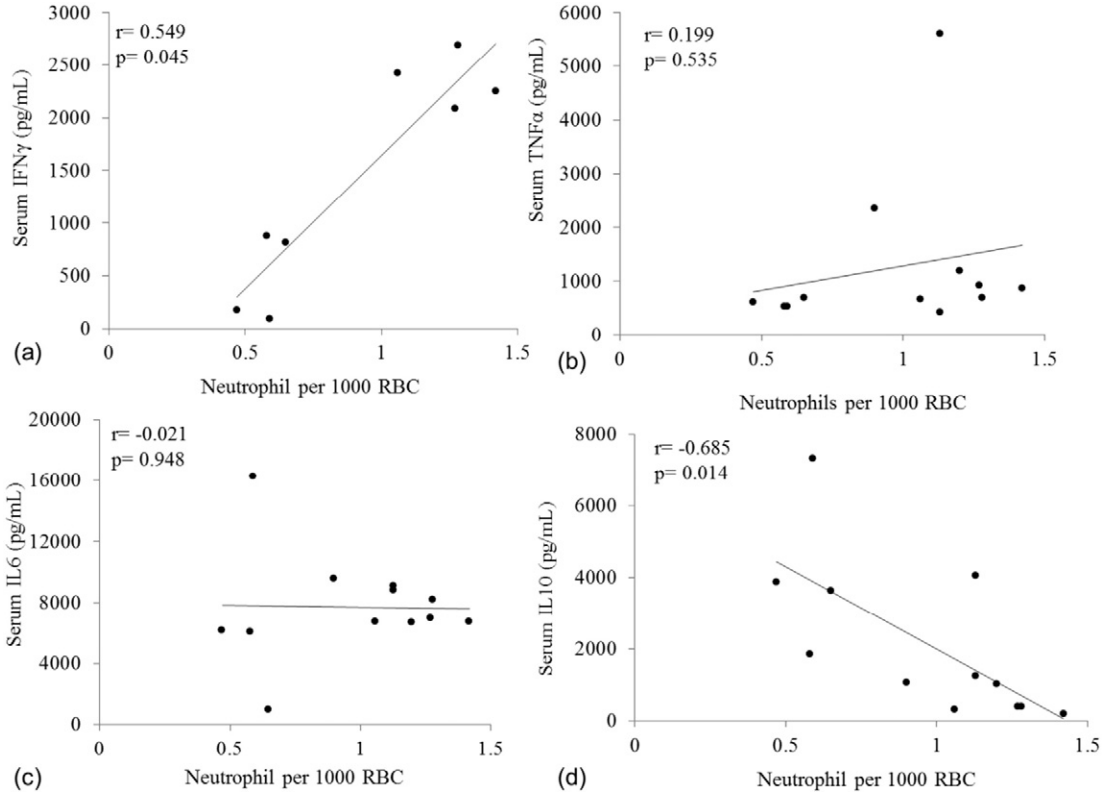


Fig. 5. Correlation between neutrophil counts and the serum cytokine concentrations of IFNγ, TNFα, IL6 and IL10 in adult *E. hexadactylus* ($n = 15$, Pearson correlation) used in the study.

pattern in the amphibian fits the expected levels, based on results from other animal species, under similar environmental perturbations. Considering the large variability of cytokine production, repeated measurements are mandatory (Zimmerman et al., 2014).

Both field and laboratory heavy metal exposed frogs showed significantly elevated serum and hepatic levels of Th1 (pro-inflammatory cytokines; IFNγ and TNFα) than the corresponding Th2 (anti-inflammatorycytokine;IL10), suggesting heavy metal mediated inflammatory responses. This observation was reiterated by the correlation data, which revealed significant positive correlation of serum IFNγ and negative correlation of serum IL10 with the corresponding neutrophil counts. Even though, serum IFNγ increased significantly in heavy metal exposed frogs, this was not reflected in hepatic expression levels of the cytokines. Conversely, hepatic TNFα levels were significantly elevated in heavy metal exposed frogs though not significantly correlated with the serum levels. This tremendous elevation of TNFα is undoubtedly a sign of hepatic necrosis which may be attributed to heavy metal accumulation. Heavy metal mediated necrosis in the liver tissue of *E. hexadactylus* was observed where mild to moderate damage to hepatocytes, bile secretion and haemorrhages were reported compared to reference frogs (unpublished data).Therefore, our data showed that heavy metal accumulation significantly boosted TNFα production in the liver, which was probably not detected systemically due to changes in frog cytokine circulation, metabolism.

Overall, serum and hepatic cytokine responses of *E. hexadactylus* in the study were skewed towards a Th1response that deals with

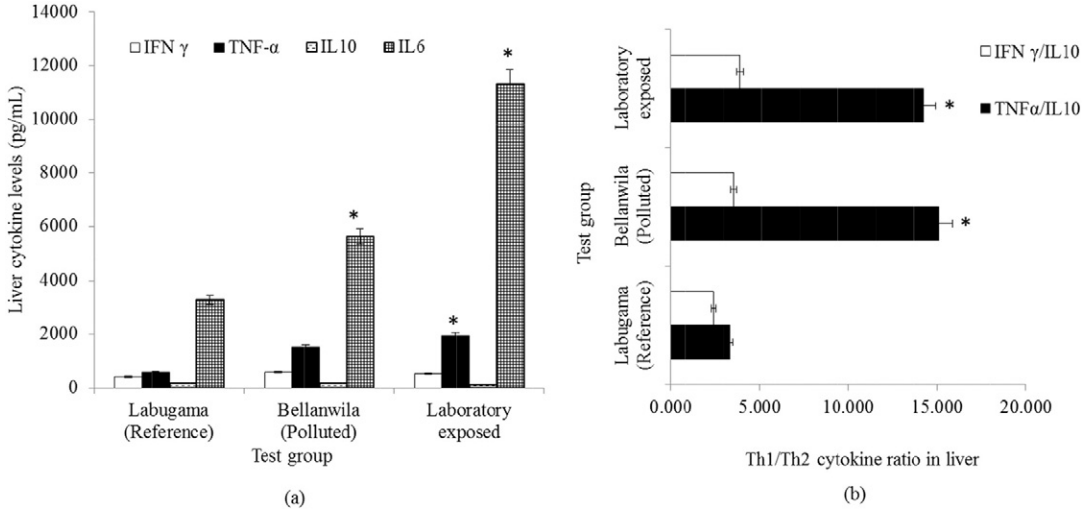


Fig. 6. Liver cytokine concentrations (a) and Th1/Th2 cytokine ratio in liver (b) of *E. hexadactylus* ($n = 15$) in different test groups (error bars represent standard error of the mean; *significant alteration compared to the reference animals, *t-test*, $P < 0.05$).

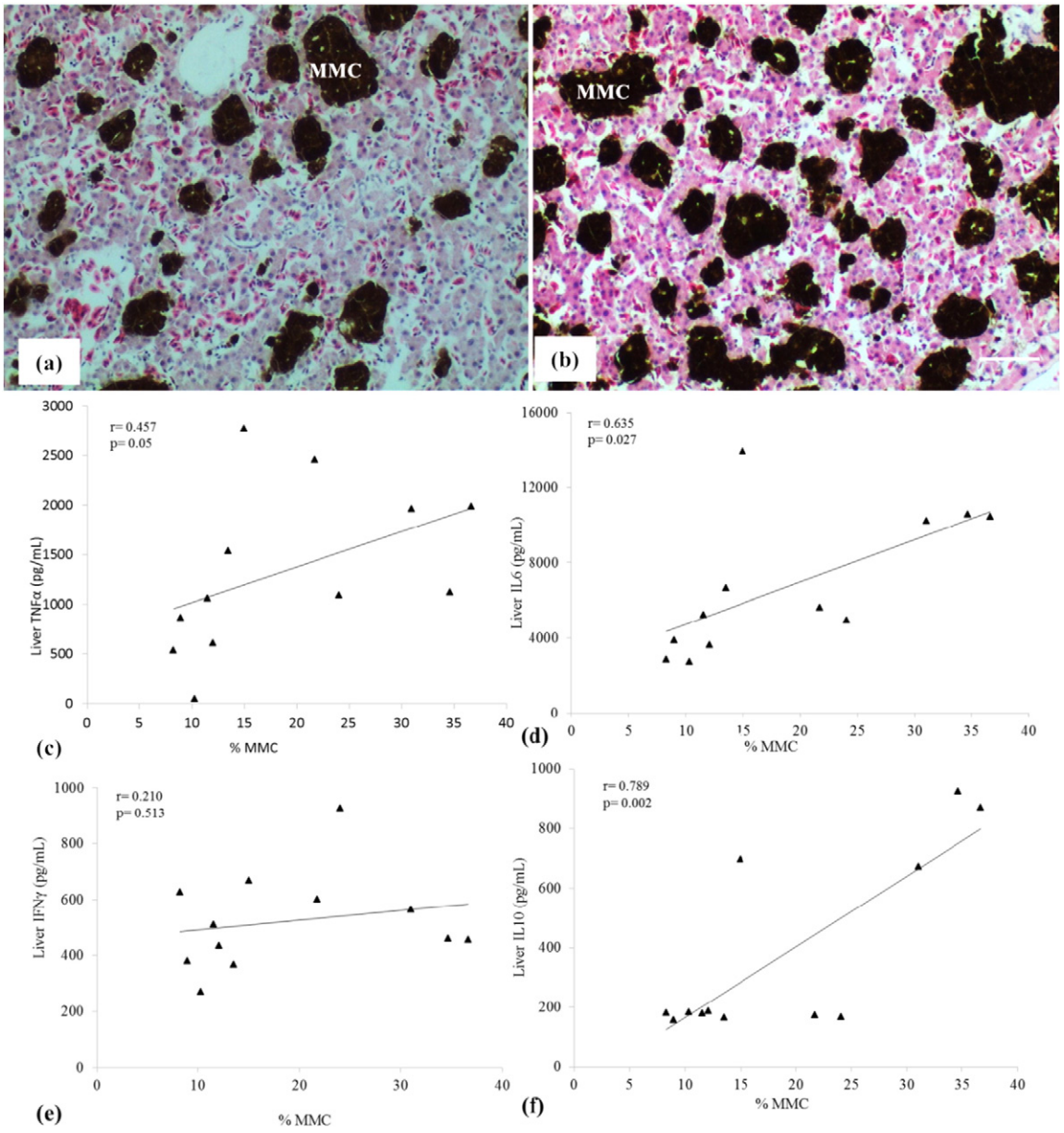


Fig. 7. Correlation of liver cytokine expression and percentage melanomacrophage aggregation (%MMC). (a) Reference liver cross section with low % MMC (~9.5%), (b) metal exposed liver showing high % MMC (~25%), (c) liver TNFα and % MMC, (d) liver IL6 and % MMC, (e) liver IFNγ and %mmc, (f) liver IL10 and % MMC. Significant positive correlations in TNFα, IL6 and IL10 (Pearson correlation, CI 95%).

intracellular pathogens and pro-inflammatory responses. Further, a shift towards Th1 cytokine response upon metal exposure may have resulted due to higher numbers of phagocytic cells in the circulation, in particular neutrophils as affirmed by the elevation of neutrophils/lymphocyte ratio and the correlation data. The balance between Th1: Th2 or pro- and anti-inflammatory cytokines are generally maintained to maximize parasite elimination and to minimize host cell damage (Brunet, 2001). Moreover, these Th1 and Th2 cytokines are directly responsible, respectively, for the up- and down-regulation of anti-oxidant enzymes such as NO synthase, which maintain reactive oxygen (ROS) and nitrogen species (RNS) in the body. Reactive oxygen species lead to oxidative stress resulting in cellular damage unless these are neutralized by antioxidant enzymes. Conversely, increased phagocytosis of the innate immune response further generates ROS and RNS which can damage host cells. Oxidative stress caused by heavy metal exposure depletes anti-oxidant provisions and challenge the body's ability to endure stress. Consequently, heavy metal mediated oxidative burst triggers a battery of pro-inflammatory cytokines, enhancing overall body inflammatory responses and local tissue damage, particularly in tissues associated with heavy metal accumulation or metabolism. Serum and liver tissue homogenate levels of NO (nitric oxide) in heavy metal exposed *E. hexadactylus* showed marked elevation compared to their corresponding reference values (unpublished data). Therefore, oxidative burst triggered by heavy metal exposure may posit to be the mechanism by which the Th1-Th2 balance in *E. hexadactylus* is achieved.

The current study revealed a comprehensive profile of hepatic and serum cytokine changes due to heavy metal mediated oxidative stress and to liver damage. Involvement of different cytokines in oxidative stress management in the liver is complicated. For instance, TNFα and IL6 are known for the activation of stellate cells (macrophages) which engage in fundamental steps involved in hepatic inflammation (Knittel et al., 1997). Because the hepatic TNFα and IL6 levels were elevated and were significantly higher in heavy metal exposed frogs than in their reference site counterparts, it may be suggested that these play a role in hepatic inflammatory processes. Expansion of

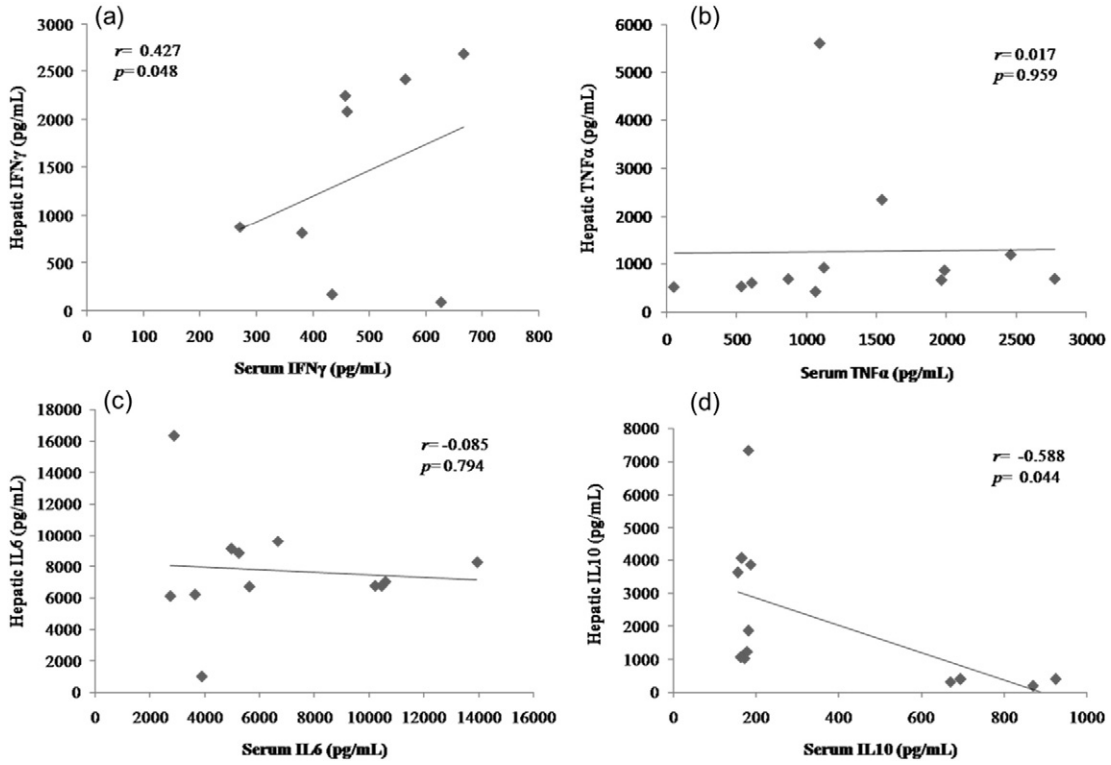


Fig. 8. Correlation between serum and hepatic cytokine concentrations of IFNγ, TNFα, IL6 and IL10 in adult *E. hexadactylus* ($n = 15$, Pearson correlation) used in the study.

melanomacrophage centres as we experienced in metal exposed *E. hexadactylus* was a common phenomenon in acute and chronic liver injuries, where this aggregation was attributed to the influx of peripheral monocytes rather than augmentation of tissue-resident macrophages (Zimmermann et al., 2010). Stimulation of melanomacrophages through xenobiotic or any other cue enhanced the binding of toll-like receptor complexes which are engaged in several downstream transcriptional factors such as NF-kB and STAT-1 which eventually upregulate the synthesis of proinflammatory mediators (Abu-Amara et al., 2010). Counter balancing cytokines such as IL6 and IL10 have shown to play a compensatory role in liver injury by abrogating deleterious signals of TNFα (Abu-Amara et al., 2010). This explains the positive correlation of TNFα, IL6 and IL10 with the increasing MMCs in the liver. However, since the net effect of cytokines was Th1 biased the protective role of counteracting cytokines seem elusive. Moreover, this study reports antagonism of cytokine expression between liver and serum levels of cytokine suggesting a cytokine elimination role in the liver. In our study serum IFNγ level of the laboratory exposed frogs correlated with corresponding hepatic levels. A similar observation was reported where serum IFNγ showed positive correlation with the hepatic IFNγ m-RNA as the severity of hepatic inflammation increases (McGuiness et al., 2000).We previously observed that heavy metal exposed frogs showed significantly elevated levels of liver enzymes alanine transaminase [ALT], aspartate aminotransferase [AST]), total bilirubin and lipid peroxidation indices (thiobarbituric acid reactive substances [TBARS]), which was consistent with hepatic inflammation and injury, elicited even by histopathological investigation(unpublished data). As liver products, albumin and total protein levels also decreased, suggestive of hepatocellular damage, which in turn, presumably, impaired hepato-productivity. Several studies reported that, during the acute phase reaction IL6 stimulates hepatocytes to produce acute phase proteins such as C-reactive protein (CRP), fibrinogen and simultaneously suppresses albumin production (Castell et al., 1988; Naka et al., 2002). The present study reiterated this as the albumin production decreased significantly (data not presented) with the significantly increasing IL6 levels under heavy metal exposure.

Hepatic expression of IL10 was relatively consistent in all three exposure groups, though the serum levels of IL10 in reference frogs were comparatively higher. Moreover, circulating IL10 levels were negatively correlated with the corresponding hepatic expression. Excessive production of pro-inflammatory cytokines in the liver, particularly TNFα may have suppressed IL10 expression. However, IL10 reported potent anti-inflammatory and anti-fibrotic properties on hepatocytes (Nelson et al., 2000) which produce negative regulating effects on the production of collagen, promoting collagenase activity. Therefore, patients with alcoholic cirrhosis and fibrosis in the liver have decreased serum IL10 expression compared to healthy individuals (Daniluk et al., 2001). Since the patterns of serum cytokine changes and hepatic expressions were inconsistent, cytokine kinetics in the liver may have dynamic and specific roles for hepatocytes. In most of the studies serum IFNγ levels did not correlate with their hepatic expression levels (Hsieh et al., 2011). However, the present study demonstrates that for serum IFNγ, there was a significant positive correlation with the corresponding hepatic levels. This condition is comparable with patients with chronic liver diseases where IFNγ levels are reported to increase both locally and systemically (Hsieh et al., 2011).

The underlying mechanism for this Th1 biased alteration in frog cytokines remains obscure, but NFκB activation due to oxidative stress governed by Fenton mechanisms of heavy metals may provide a plausible explanation for both overall serum and hepatic expression dynamics. Heavy metal mediated depletion of natural antioxidants such as glutathione (GSH) in hepatocytes may have sensitized the hepatocytes to TNF induced apoptosis where NFκB is the major regulator of TNF susceptibility (Marí et al., 2008). Goetz and colleagues (Goetz et al., 2004) reported involvement of similar kinetics in NFκB activated TNFα production in both fishes and mammals. Therefore, similar transcriptional changes may be anticipated in the amphibian model as well. Furthermore, signal transduction pathways altered by NFκB are considered critical for hepatocellular toxicity (Deng and Chai, 2009), suggesting their role in liver necrosis.

Neuro-endocrine and immune system interaction is fundamental to understand the efficiency of immune function and energy trade-offs

between other costly physiological processes. Stress hormones in vertebrates can enhance or suppress immune functions (McEwen et al., 1997). Chronic stress, characterized by long-term, high levels of glucocorticoids is known to suppress the immune system (McEwen et al., 1997), while these hormones may serve as immunostimulators in short-term exposure (Dhabhar, 2009).This suppression or stimulation vary with varying components of the immune system, where increased glucocorticoids tend to suppress Th1 (cell-mediated immunity) but stimulate Th2 (humoral immunity) responses (Dhabhar, 2009). However, elevated glucocorticoids from acute stressors are known to induce inflammatory responses and cell mediated immunity with accompanied B-cell and T-cell proliferation (Dhabhar, 2009). Therefore, neuroendocrine and immune system co-regulation is dependent upon the nature of stress as acute stress stimulates immune responses but chronic stress can be suppressive (Dhabhar, 2009). Heavy metal exposure is often associated with chronic release of glucocorticoids which in turn suppress the immune system skewing the Th1:Th2 balance towards Th2. However, the results of the current study revealed Th1 polarisation, suggestive of acute response behaviour of the neuro-endocrine and immune system co-regulation.

IL6 is produced by Th17 cells, macrophages, and/or target organ chronic inflammation and is synchronized with TNFα while is suppressed by IFNγ (Asarch et al., 2008). IL6 activates IL6 receptor complexes, such as IL6R and gp130, which phosphorylate associated Janus Kinases (JAK) and then induce the cytoplasmic transcriptional factors such as STAT1 and STAT3 (Asarch et al., 2008). However, IFNγ inhibits this development in a STAT1 dependent manner (Ihle and Kerr, 1995). Therefore, in the present study continuous production of IFNγ due to Th1 biased responses may have inhibited IL6 production through STAT1 inhibition. As a consequence of this suppression, IL6 levels in the serum of heavy metal exposed frogs under natural and laboratory exposure reported relatively low levels compared to that of the reference group. However, this reduction of IL6 level is crucial for the animal to reactivate TGFβ mediated Treg cell differentiation which modulates their immune system.

The role of sentinels in ecotoxicology is to be among the first to experience the xenobiotic mediated challenges and express easily detectable biological responses, which can be considered as biomarker/s. As amphibian development occurs without maternal influences, they may serve as a valuable model to study adverse effects of xenobiotics during early development (Rollins-Smith et al., 2004). *Xenopus laevis* as a non-mammalian comparative model species has been utilised extensively for evolutionary, comparative, and developmental studies of the immune system (Robert and Ohta, 2009; Rollins-Smith et al., 2004). Cytokines may serve as potential biomarkers that could be detected in non-destructive ways as it requires only a small amount of serum/tissue fluid, which can be obtained easily without sacrificing the animal. However, to date there are very few cytokine/chemokine expression and functional studies on amphibians. Owing to the emerging issue of xenobiotic pollution on global dimension, more fundamental and comparative research on sentinels and biomarkers is required. Therefore, extensive studies on the amphibian immune system may provide valuable insights to understand the role of xenobiotic stressors on higher vertebrates including human. In this quest, the full genome sequence of amphibian species *X. tropicalis* and its significant genetic conservation with mammals in concert with genome mapping and mutagenesis studies provide a better insight for immunological studies (Robert and Cohen, 2011).

In conclusion, heavy metal mediated inflammatory response and liver injury of the amphibians is associated with Th1 skewed upregulation of pro-inflammatory cytokines, particularly IFNγ in blood circulation and TNFα in the liver, suggesting cytokines as a novel immunotoxicological biomarker for heavy metal mediated toxicity in amphibians. Elevated levels of pro inflammatory cytokines significantly correlated with blood neutrophil counts and % MMC in the liver indicative of general inflammation, possibly due to metal mediated toxicity in adult *E. hexadactylus*. Thus, neutrophil counts in relation to systemic expression of cytokines may possibly serve as a biomarker of metal toxicity. Certain cytokines can only be correlated with the systemic expression, signifying the multifaceted functions of cytokines under physiological stress. As environmental sentinels in wetland ecosystems which suffer unprecedented levels of environmental stresses, an in-depth understanding of amphibian ecoimmunology is fundamental. Potentially important, but poorly understood factors underlying the variation of amphibian cytokine activity in response to environmental pollutants may provide better perception on current challenges faced by these sensitive species.

Acknowledgements

We acknowledge financial assistance through grants from the Ministry of Higher Education, Sri Lanka (HETC/CMB/QIGW3/SCI/OS/2012/02) and the University of Colombo (AP/3/2/2012/RG/SC/04). The valuable comments and suggestions by Dr Reinier Mann, Centre for Environmental Sustainability (CEnS), University of Technology, Sydney (UTS) to improve the manuscript are much appreciated.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.05.171.

References

Abu-Amara, M., Yang, S.Y., Tapuria, N., Fuller, B., Davidson, B., Seifalian, A., 2010. Liver ischemia/reperfusion injury: processes in inflammatory networks–a review. Liver Transpl. 16, 1016–1032.
Aggarwal, B.B., Puri, R.K., 1995. Human Cytokines: Their Role in Disease and Therapy. Blackwell Science.
Agius, C., Roberts, R.J., 2003. Melano-macrophage centres and their role in fish pathology. J. Fish Dis. 26 (9), 499–509.
Asarch, A., Barak, O., Loo, D.S., Gottlieb, A.B., 2008. Th17 cells: a new therapeutic target in inflammatory dermatoses. J. Dermatol. Treat. 19, 318–326.
ASTM, 2003. Standard guide for conducting acute toxicity tests on test materials with fishes, macro invertebrates, and amphibians (E729–96 [2002]). Annual Book of ASTM Standards vol. 11.05. ASTM, West Conshohocken, PA, pp. 179–200.
Biron, C.A., Sen, G.C., 2001. Interferons and other cytokines. Fields Virology. 1, pp. 321–351.
Boughton, R.K., Joop, G., Armitage, S.A., 2011. Outdoor immunology: methodological considerations for ecologists. Funct. Ecol. 25 (1), 81–100.
Brunet, L.R., 2001. Nitric oxide in parasitic infections. Int. Immunopharmacol. 1, 1457–1467.
Burlibaşa, L., Gavrilă, L., 2011. Amphibians as model organisms for study environmental genotoxicity. Appl. Ecol. Environ. Res. 9 (1), 1–15.
Cabagna, M.C., Lajmanovich, R.C., Stringhini, G., Sanchez-Hernandez, J.C., Pelzer, P.M., 2005. Hematological parameters of health status in the common toad *Bufo arenarum* in agroecosystems of Sante Fe Province, Argentina. Appl. Herpetol. 2, 373–380.
Castell, J.V., Gomez-Lechon, M.J., David, M., Hirano, T., Kishimoto, T., Heinrich, P.C., 1988. Recombinant human interleukin-6 (IL-6/BSF-2/HSF) regulates the synthesis of acute phase proteins in human hepatocytes. FEBS Lett. 232, 347–350.
Chiesa, M.E., Rosenberg, C.E., Fink, N.E., Salibián, A., 2006. Serum protein profile and blood cell counts in adult toads *Bufo arenarum* (Amphibia: Anura: Bufonidae): effects of sublethal lead acetate. Arch. Environ. Contam. Toxicol. 50 (3), 384–391.
Daniels, M.J., Ménache, M.G., Burleson, G.R., Graham, J.A., Selgrade, M.K., 1987. Effects of NiCl and CdCl on susceptibility to murine cytomegalovirus and virus-augmented natural killer cell and interferon responses. Fundam. Appl. Toxicol. 8 (4), 443–453.
Daniluk, J., Szuster-Ciesielska, A., Drabko, J., Kandefer-Szerszeń, M., 2001. Serum cytokine levels in alcohol-related liver cirrhosis. Alcohol 23 (1), 29–34.
Davis, A.K., Maney, D.L., Maerz, J.C., 2008. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Funct. Ecol. 22, 760–772.
Demas, G., 2004. The energetics of immunity: a neuroendocrine link between energy balance and immune function. Horm. Behav. 45, 173–180.
Deng, H.P., Chai, J.K., 2009. The effects and mechanisms of insulin on systemic inflammatory response and immune cells in severe trauma, burn injury, and sepsis. Int Immunopharmacol. 9 (11), 1251–1259.
Dhabhar, F., 2009. Enhancing versus suppressive effects of stress on immune function: implications for immunoprotection and immunopathology. Neuroimmunomodulation 16, 300–317.
Edwards, S.W., 1994. Biochemistry and Physiology of the Neutrophil. Cambridge University Press, New York.
Elenkov, I.J., Iezzoni, D.G., Daly, A., Harris, A.G., Chrousos, G.P., 2005. Cytokine dysregulation, inflammation and well-being. Neuroimmunomodulation 12 (5), 255–269.
Ezemonye, L.I., Enuneku, A.A., 2011. Haematological changes in *Bufo maculatus* treated with sublethal concentrations of cadmium. J. Xenobiotics 1 (1), 7.

Forbes MR, McRuer DL, Shutler D. 2006. White blood cell profiles of breeding American toads (*Bufo americanus*) relative to sex and body size. CompClinPath15:155–159.
Forson, D.D., Storfer, A., 2006. Atrazine increases ranavirus susceptibility in the tiger salamander, *Ambystoma tigrinum*. Ecol. Appl. 16, 2325–2332.
Funkhouser, S.W., Martinezmaza, O., Vredevoe, D.L., 1994. Cadmium inhibits IL-6 production and IL-6 mRNA expression in a human monocytic cell line, THP-1. Environ. Res. 66 (1), 77–86.
Goetz, F.W., Planas, J.V., MacKenzie, S., 2004. Tumor necrosis factors. Dev. Comp. Immunol. 28 (5), 487–497.
Hardie, L.J., Chappell, L.H., Secombes, C.J., 1994. Human tumor necrosis factor α influences rainbow trout *Oncorhynchus mykiss* leucocyte responses. Vet. Immunol. Immunopathol. 40 (1), 73–84.
Haynes, L., Cohen, N., 1993. Transforming growth factor beta (TGFβ) is produced by and influences the proliferative response of *Xenopus laevis* lymphocytes. Clin. Dev. Immunol. 3 (3), 223–230.
Hsieh, H.G., Huang, H.C., Lee, F.Y., Chan, C.Y., Lee, J.Y., Lee, S.D., 2011. Kinetics of cytokine expression in cirrhotic rats. J. Chin. Med. Assoc. 74 (9), 385–393.
Hudson, L., Hay, F.C., Hudson, L., 1989. Practical Immunology. Blackwell Scientific.pp, Oxford, p. 507.
Ihle, J.N., Kerr, I.M., 1995. Jaks and Stats in signaling by the cytokine receptor superfamily. Trends Genet. 11, 69–74.
Jørgensen, J.B., Lunde, H., Jensen, L., Whitehead, A.S., Robertsen, B., 2000. Serum amyloid A transcription in Atlantic salmon (*Salmo salar* L.) hepatocytes is enhanced by stimulation with macrophage factors, recombinant human IL-1β, IL-6 and TNFα or bacterial lipopolysaccharide. Dev. Comp. Immunol. 24 (6), 553–563.
Kaiser, P., Rothwell, L., Avery, S., Balu, S., 2004. Evolution of the interleukins. Dev. Comp. Immunol. 28 (5), 375–394.
Kimura, A., Kishimoto, T., 2010. IL-6: regulator of Treg/Th17 balance. Eur. J. Immunol. 40 (7), 1830–1835.
Knittel, T., Müller, L., Saile, B., Ramadori, G., 1997. Effect of tumour necrosis factor-α on proliferation, activation and protein synthesis of rat hepatic stellate cells. J. Hepatol. 27 (6), 1067–1080.
Kushibiki, S., 2011. Tumor necrosis factor-α-induced inflammatory responses in cattle. Anim. Sci. J82 (4), 504–511.
Mann, R.M., Hyne, R.V., Choung, C.B., Wilson, S.P., 2009. Amphibians and agricultural chemicals: review of the risks in a complex environment. Environ. Pollut. 157 (11), 2903–2927.
Marcogliese, D.J., King, K.C., Salo, H.M., Fournier, M., Brousseau, P., Spear, P., Champoux, L., McLaughlin, J.D., Boily, M., 2009. Combined effects of agricultural activity and parasites on biomarkers in the bullfrog, *Rana catasbeiana*. Aquat. Toxicol. 91, 126–134.
Marí, M., Colell, A., Morales, A., Caballero, F., Moles, A., Fernández, A., et al., 2008. Mechanism of mitochondrial glutathione-dependent hepatocellular susceptibility to TNF despite NF-κB activation. Gastroenterology 134 (5), 1507–1520.
McEwen, B.S., Biron, C.A., Brunson, K.W., Bulloch, K., Chambers, W.H., Dhabhar, F.S., Goldfarb, R.H., Kitson, R.P., Miller, A.H., Spencer, R.L., Weiss, J.M., 1997. The role of adrenocorticoids as modulators of immune function in health and disease: neural, endocrine and immune interactions. Brain Res. Rev. 23, 79–133.
McGuiness, P.H., Painter, D., Davies, S., McCaughan, G.W., 2000. Increases in intrahepatic CD68 positive cells, MAC387 positive cells, and proinflammatory cytokines (particularly interleukin 18) in chronic hepatitis C infection. Gut 46 (2), 260–269.
Nagano, T., Yamamoto, K., Matsumoto, S., Okamoto, R., Tagashira, M., Ibuki, N., et al., 1999. Cytokine profile in the liver of primary biliary cirrhosis. J. Clin. Immunol. 19 (6), 422–427.
Naka, T., Nishimoto, N., Kishimoto, T., 2002. The paradigm of IL-6: from basic science to medicine. Arthritis Res. 4 (Suppl. 3), S233–S242.
Nelson, D.R., Lauwers, G.Y., Lau, J.Y., Davis, G.L., 2000. Interleukin 10 treatment reduces fibrosis in patients with chronic hepatitis C: a pilot trial of interferon non-responders. Gastroenterology 18, 655–660.
OECD, 2008. (Organisation for economic co-operation and development). The Amphibian Metamorphosis Assay, Guideline for the Testing of Chemicals. OECD, Paris, France.
Priyadarshani, S., Madhushani, W.A.N., Jayawardena, U.A., Wickramasinghe, D.D., Udagama, P.V., 2015. Heavy metal mediated immunomodulation of the Indian green frog, *Euphlyctis hexadactylus* (Anura: Ranidae) in urban wetlands. Ecotoxicol. Environ. Saf. 116, 40–49.
Robert, J., Cohen, N., 2011. The genus *Xenopus* as a multispecies model for evolutionary and comparative immunobiology of the 21st century. Dev. Comp. Immunol. 35 (9), 916–923.
Robert, J., Ohta, Y., 2009. Comparative and developmental study of the immune system in *Xenopus*. Dev. Dyn. 238 (6), 1249–1270.
Robertsen, B., 2006. The interferon system of teleost fish. Fish Shellfish Immunol. 20 (2), 172–191.
Rollins-Smith, L.A., Hopkins, B.D., Reiner, L.K., 2004. An amphibian model to test the effects of xenobiotic chemicals on development of the hematopoietic system. Environ. Toxicol. Chem. 23 (12), 2863–2867.
Romanova, E.B., Romanova, O.Y., 2003. Peculiarities of leukocytic formula of peripheral blood of green frogs under conditions of anthropogenic load. J. Evol. Biochem. Physiol. 39, 480–484.
Rosenberg, C.E., Salibia'n, A., Fink, N.E., 2002. An enzyme linked immunosorbent assay for measuring anti-sheep red blood cells antibodies in lead-exposed toads. J. Pharmacol. Toxicol. Methods 4, 121–128.
Roy, D., 2002. Amphibians as environmental sentinels. J. Biosci. 27 (3), 187–188.
Samuel, C.E., 2001. Antiviral actions of interferons. Clin. Microbiol. Rev. 14 (4), 778–809.
Savan, R., Ravichandran, S., Collins, J.R., Sakai, M., Young, H.A., 2009. Structural conservation of interferon gamma among vertebrates. Cytokine Growth Factor Rev. 20 (2), 115.
Simpson, K.J., Lukacs, N.W., Colletti, L., Strieter, R.M., Kunkel, S.L., 1997. Cytokines and the liver. J. Hepatol. 27 (6), 1120–1132.
Snyder, C.A., Udasin, I., Waterman, S.J., Taioli, E., Gochfeld, M., 1991. Reduced IL-6 levels among individuals in Hudson County, New Jersey, an area contaminated with chromium. Arch. Environ. Health 51 (1), 26–28.
Van der Schalie, W.H., Gardner Jr., H.S., Bantle, J.A., De Rosa, C.T., Finch, R.A., Reif, J.S., Stokes, W.S., 1999. Animals as sentinels of human health hazards of environmental chemicals. Environ. Health Perspect. 107 (4), 309.
Van Snick, J., 1990. Interleukin-6: an overview. Annu. Rev. Immunol. 8 (1), 253–278.
Wang, J.Y., Wicklund, B.H., Gustilo, R.B., Tsukayama, D.T., 1996. Titanium, chromium and cobalt ions modulate the release of bone-associated cytokines by human monocytes/macrophages in vitro. Biomaterials 17 (23), 2233–2240.
Watkins, D., Parsons, S.C., Cohen, N., 1987. A factor with interleukin-1-like activity is produced by peritoneal cells from the frog, *Xenopus laevis*. Immunology 62 (4), 669.
Wright, K.M., 2001. Amphibian hematology. Amphibian Medicine and Captive Husbandry, pp. 129–146.
Zimmerman, L.M., Bowden, R.M., Vogel, L.A., 2014. A vertebrate cytokine primer for ecoimmunologists. Funct. Ecol. 28 (5), 1061–1073.
Zimmermann, H.W., Seidler, S., Nattermann, J., Gassler, N., Hellerbrand, C., Zernecke, A., Tischendorf, J.J., Luedde, T., Weiskirchen, R., Trautwein, C., Tacke, F., 2010. Functional contribution of elevated circulating and hepatic nonclassical CD14, CD16 monocytes to inflammation and human liver fibrosis. PLoS ONE 5, e11049. http://dx.doi.org/10.1371/journal.pone.0011049.