Effects of exposure to environmentally relevant concentrations of lead (Pb) on expression of stress and immune-related genes, and microRNAs in shorthorn sculpins (Myoxocephalus scorpius)

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Abstract
Old lead–zinc (Pb–Zn) mining sites in Greenland have increased the environmental concentration of Pb in local marine organisms, including the shorthorn sculpin. Organ metal concentrations and histopathology have been used in environmental monitoring programs to evaluate metal exposure and subsequent effects in shorthorn sculpins. So far, no study has reported the impact of heavy metals on gene expression involved in metal-related stress and immune responses in sculpins. The aim of this study was to investigate the effect of exposure to environmentally relevant waterborne Pb (0.73 ± 0.35 μg/L) on hepatic gene expression of metallothionein (mt), immunoglobulin M (igm), and microRNAs (miRNAs; mir132 and mir155) associated with immune responses in the shorthorn sculpin compared to a control group. The mt and igm expression were upregulated in the Pb-exposed group compared to the control group. The transcripts of mir132 and mir155 were not different in sculpins between the Pb-exposed and control group; however, miRNA levels were significantly correlated with Pb liver concentrations. Furthermore, there was a positive correlation between liver Pb concentrations and igm, and a positive relationship between igm and mir155. The results indicate that exposure to Pb similar to those concentrations reported in marine waters around Greenland Pb–Zn mine sites influences the mt and immune responses in shorthorn sculpins. This is the first study to identify candidate molecular markers in the shorthorn sculpins exposed to waterborne environmentally relevant Pb suggesting mt and igm as potential molecular markers of exposure to be applied in future assessments of the marine environment near Arctic mining sites.

Keywords Arctic lead–zinc mines · Dissolved Pb exposure · Gene expression · Greenland sculpin · Immune-related gene · Metal stress-related gene

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Highlights

- mt and igm were upregulated in Pb exposed fish compared to the control group.
- Significant positive correlation was found between Pb concentrations in liver and igm.
- Expression of igm was positively correlated to mir155 expression.
- mir155 expression was positively correlated with severity scores of hepatic lesions.
- Pb levels in the liver were significantly correlated with miRNAs expression.

Introduction

Heavy metal pollution from industrial, mining, and agricultural sources in aquatic ecosystems has been of concern across the world, including the Arctic (Dietz et al. 1998; Evans et al. 2000; Voigt 2003; Chua et al. 2018). Lead (Pb) is a non-essential and toxic heavy metal that is widespread in aquatic environments (Scheuhammer et al. 2008). Exposure to Pb even at low concentrations impairs biological functions, such as reproduction, development, behavior, learning, immune response, and metabolism (Eisler 1988). Pb is particularly harmful to aquatic organisms, including fish, as it bioaccumulates through uptake via gills, dietary consumption, and contaminated sediments (Eisler 1988; Scheuhammer et al. 2008; Mager 2011). In fish, Pb accumulates in liver, spleen, and kidney, as well as the digestive system and gills (Jezielska and Witeska 2006). A wide range of Pb concentrations (1 to 5.15 mg/L) have been demonstrated to activate oxidative stress and cause inflammation in different fish species (Lee et al. 2019; Jing et al. 2020).

Metallothioneins (Mt) are low molecular weight metal binding proteins involved in homeostatic regulation and transportation of essential metals, such as copper (Cu) and zinc (Zn) (Hogstrand and Haux 1990; Coyle et al. 2002; Baird et al. 2006). Mt proteins are also involved in detoxification of non-essential metals, including Pb, to protect tissues from oxidative stress (Hogstrand and Haux 1990; Dallinger et al. 1997; Monteiro et al. 2011). Production of Mt is induced by exposure to heavy metals and Mt is measured to estimate the stress responses to heavy metal exposure, such as Pb, across fish species (Schmitt et al. 2007; Huang et al. 2014; Yin et al. 2018b). Following exposure to varying Pb concentrations (0.07 to 1.16 mg/L), the expression of mt increased and was suggested as biomarker of exposure to Pb (Huang et al. 2014). Expression of mt has been applied as a sensitive biomarker of metal exposure in fish in metal-contaminated environments (Cheung et al. 2004). For example, Wang et al. (2014) showed that expression of mt in the rare minnow (Gobioocypris rarus) was upregulated possibly as a result of heavy metal exposure and oxidative stress.

Heat shock proteins (Hsp), also known as “stress proteins”, are involved in a variety of physiological activities, including protein chaperoning, apoptosis protection, steroidogenesis, and stress resistance (Mahmood et al. 2014). Exposure to heavy metals leads to numerous cellular heat-shock responses, including induction of Hsp to protect cellular functions (Sanders 1993). Hsp, such as Hsp70, are highly conserved proteins in fish and are applied as a potential biomarker to assess cellular stress responses in fish exposed to heavy metals, including Pb (Basu et al. 2002; Kim and Kang 2016). In addition, the expression of hsp can be influenced by a number of factors, including heat and cold shock, xenobiotics, and pathogens (Iwama et al. 1998; Lewis et al. 1999; Basu et al. 2002). Previous studies have shown that the expression of hsp70 in fish was elevated following exposure to heavy metals, including various Pb concentrations (0.05–800 mg/L waterborne Pb) (Yin et al. 2018b; Zhao et al. 2020).

Exposure to Pb alters immune response and induces immunomodulation in fish (Zelikoff 1993; Zelikoff et al. 1995; Luebke et al. 1997; Bols et al. 2001; Qian et al. 2020). Immunoglobulin M (IgM) is the most highly conserved and abundant immunoglobulin isotype in teleosts and is one of the most essential components of the immune system as it mediates humoral adaptive immunity in fish to eliminate invading pathogens (Salinas et al. 2011; Zwollo 2018; Smith et al. 2019). IgM has been used as an indicator of immune response in teleosts (Wester et al. 1994; Lee et al. 2014). Previous studies of rockfish (Sebastes schlegellii) demonstrated that dietary exposure to Pb activated an immune response, increasing plasma IgM concentration (Kim and Kang 2016). In contrast, Zhao et al. (2020) showed that waterborne Pb exposure decreased serum IgM concentration in the northern snakehead (Channa argus). MicroRNAs (miRNAs) are important regulators of the immune response and expression of immune associated miRNAs can be modulated in many different species by exposure to environmental pollutants (O’Connell et al. 2007; Mehta and Baltimore 2016; Andreassen and Høyheim 2017; Li et al. 2019; Badry et al. 2020; Sun et al. 2021). Recent research found that miRNAs, such as mir132 and mir155, play critical roles in regulating inflammation, suggesting they are crucial regulators of immune responses (Rodriguez et al. 2007; Roy and Sen 2010; He et al. 2014; Ma et al. 2018; Zhao et al. 2022). There is some evidence that the alteration of mir155 expression could be a novel biomarker of exposure to pollution (Huang et al. 2016; Badry et al. 2020). For example, mir155 was downregulated in adult zebrafish (Danio rerio) after exposure to an insecticide fipronil (Huang et al. 2016). In addition, miRNAs, including mir132, have been identified as important...
miRNAs associated with responses to exposure to metals (Pellegrini et al. 2016).

Although previous studies have proposed molecular markers for assessing the effects of exposure to metals on stress and immune responses in many fish species, the study of molecular endpoints to identify candidate molecular markers remains a knowledge gap in benthic species, including the sculpins.

Previous field studies on the impact of metal pollution at historic Pb–Zn mining sites in Greenland used shorthorn sculpins (Myxoceophilus scorpius) as a sentinel species to assess aqueous exposure and effects of toxic elements, including Pb, on bioaccumulation (e.g., resulting in Pb residues of 0.01–0.94 μg/g in liver and 0.01–0.69 μg/g in muscle) and histology (Sonne et al. 2014; Dang et al. 2017, 2019; Nørregaard et al. 2018; Hansson et al. 2020). M. scorpius is a relatively sedentary and benthic marine fish species that lives in the North Atlantic coast and the Arctic Ocean (Luksenburg and Pedersen 2002; Thorsteinson and Love 2016). In Greenland, M. scorpius is abundant at both western and eastern Greenland mine sites, and easy to catch by angling near mine sites (Søndergaard and Mosbech 2022). Recently, the effects of Pb exposure on shorthorn sculpin, under controlled laboratory conditions, have corroborated field observations, including bioaccumulation in organs and blood, and histopathology of liver and gills (Jantawongsri et al. 2021). However, there has been no research on the effects of exposure to Pb on stress and immune responses in shorthorn sculpin, or any other species in this genus. Thus, our aim was to investigate the expression of stress-related and immune-related genes in shorthorn sculpins exposed to Pb concentrations that are relevant for the marine environment adjacent to Greenland Pb–Zn mines. Following a controlled laboratory experiment, hepatic expression of mt, igm, hsp70, and miRNAs were investigated in control and Pb-exposed fish. The aim was to assess the potential of these stress and immune-related genes as molecular markers of Pb exposure in sculpins around Pb–Zn mines in the Arctic, including Greenland.

Methods

Experimental design

For a detailed description of the experiment, see Jantawongsri et al. (2021). Briefly, wild-caught sculpins (15 fish in each of two Pb-exposed tanks and 15 fish in each of two control tanks) were exposed to an environmentally-relevant concentration of dissolved Pb (0.73 ± 0.35 μg/L (mean ± standard deviation, SD)) consistent with a previous report on seawater near the former Black Angel Pb–Zn mine in Maarmorilik, West Greenland (0.46 μg/L of dissolved Pb; Søndergaard et al. 2011). At the end of the experiment, a liver sample was collected from each fish and fixed in RNAlater (Ambion, Austin, TX, USA), incubated at 4 °C overnight and then stored at −20 °C. As there were no significant variations in biometrics, age, or residues of other elements (excluding Pb concentrations) between control and exposed sculpins caught in the same area, it was assumed there was no background difference between the fish before the experiment (Jantawongsri et al. 2021). After 28 days of exposure, liver residues of Pb were significantly higher in Pb-exposed sculpins (0.50 ± 0.23 μg/g dry weight) than in control fish (0.13 ± 0.10 μg/g dry weight) (p < 0.001; Jantawongsri et al. 2021).

RNA isolation and cDNA synthesis

RNA extraction and cDNA synthesis were performed on 22 control sculpins and 20 Pb-exposed sculpins following the method of Castaño-Ortiz et al. (2019). RNA was extracted from the liver samples (approx. 50 mg) using the miRNeasy Mini Kit (Qiagen, Oslo, Norway) as per manufacturer’s instructions. cDNA synthesis was performed using 500 ng of RNA and the miRCURY LNA™ RT Kit (Qiagen, Oslo, Norway) as per manufacturer’s instructions.

Partial isolation of candidate genes and qPCR primer design

Target genes in this study represented (1) metal-ion binding protein (mt), (2) immune-related (igm), (3) heat shock protein (hsp70), and (4-5) miRNAs associated with immune response (mir132 and mir155) (Table 1). To amplify fragments of mt, igm, and hsp70 gene from M. scorpius, the mRNA nucleotide sequences from shorthorn sculpin-related species retrieved from GenBank® database (NCBI) were aligned using the Clustal Omega multiple sequence alignment tool (https://www.ebi.ac.uk/Tools/msa/clustalo/) and degenerate oligonucleotide primers were designed from the conserved regions (Pankhurst et al. 2011) (Table S1). mir132 and mir155 primers were commercially designed by miRCURY LNA™ miRNA PCR Assays (Qiagen, Oslo, Norway).

PCR amplification for mt, igm, and hsp70 was carried out using Taq PCR Core Kit (Qiagen, VIC, Australia) according to the manufacturer’s specifications with 10 μM of each primer. Amplification was performed on Bio-Rad C1000™ thermal cycler using the following cycling conditions: 3 min at 94 °C, then 40 cycles of 94 °C for 30 s, 56 °C (mt) or 57 °C (igm) or 52 °C (hsp70) for 30 s and 72 °C for 30 s, followed by a final extension at 72 °C for 10 min.

PCR products were separated via gel electrophoresis in 2% agarose gel and purified from the gel by using
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| Gene     | Primer sequence (5' → 3') | Amplicon size (bp) | Tm (°C) | NCBI/miRBase accession number | E  |
|----------|---------------------------|---------------------|---------|-------------------------------|----|
| hsp70    | F: GGT GTC CAA CGC AGT CAT C
R: CCG TCG GCT CGT TGA TGA T | 119 | 64.4 | OK668366 | 1.86 |
| igm      | F: TAT TTC GTG GGA GAA CCA GG
R: GGG TGT CTT AAG TGG TAC CAT CC | 178 | 63.7 | OK668365 | 1.92 |
| mt       | F: GAG GAT CCA CCA CCT GCA A
R: GTG TCG CAC GTC TCC CCT TT | 124 | 66.6 | OK668364 | 1.95 |
| mir132   | ola-miR-132 (5' UAA CAG UCU ACA GCC AUG G) amplified by miRCURY LNA™ miRNA PCR Assays, catalog number: YP02103600 (Qiagen, Oslo, Norway) | | | MIMAT0022617 | 1.92 |
| mir155   | dre-miR-155 (5' UUA AUG CUA AUC GUG AUA GGG G) amplified by miRCURY LNA™ miRNA PCR Assays, catalog number: YP02102917 (Qiagen, Oslo, Norway) | | | MIMAT0001851 | 1.72 |

bp base pairs, Tm melting temperature, E efficiency

ISOLATE II PCR and Gel Kit (Bioline, NSW, Australia). Purified PCR products were quantified using Qubit® dsDNA BR Assay Kits (Thermo Fisher Scientific, VIC, Australia) then sent to Griffith University DNA Sequencing Facility (GUDSF; Griffith University, Nathan, QLD, Australia) for Sanger sequencing. Sequencing data was assessed using Chromas Version 2.6.6 (Technelysium, QLD, Australia) and sequences were submitted to GenBank. These sequences were used to design qPCR primers (Primer-BLAST, https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to have a melting temperature between 63–67 °C and produce an amplicon between 100–200 bp (Table 1).

### qPCR procedure

Each qPCR reaction for mt, igm, and hsp70 contained: 5 μL SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, NSW, Australia), 100 nM each primer, 4 ng of cDNA template, and water to a final volume of 10 μL. Each mir132 and mir155 reaction contained: 5 μL miRCURY LNA™ SYBR® Green PCR Kits (Qiagen, Oslo, Norway), 10 μM each primer, 2.5 ng of cDNA template, and water to a final volume of 10 μL. All qPCRs were performed in duplicate.

qPCRs for mt, igm, and hsp70 were performed on CFX96™ real-time PCR detection system (Bio-Rad, NSW, Australia). A touch-down qPCR protocol was used according to the guidelines of Zhang et al. (2015) as follows: one cycle 95 °C for 3 min and four cycles 95 °C for 20 s, 66 °C for 10 s by decreasing 3 °C per cycle, followed by 40 cycles of 95 °C for 15 s, 58–60 °C for 15 s. All primers were evaluated for specificity at the end of cycle 40 using melt curve analysis, which comprised of a 1 °C per 5 s temperature gradient from 60–94 °C. qPCRs for mir132 and mir155 were conducted on Roche LightCycler® 96 (Roche Diagnostics, Basel, Switzerland) with the following running conditions: 2 min at 95 °C, two steps cycling at 10 s at 95 °C and 60 s at 56 °C for 40 cycles followed by a melt curve (as per Table S2). Duplicate no template controls (NTCs) were used in each qPCR plate and no contamination was detected.

The efficiency of each individual sample was calculated from the slopes of amplification curves and averaged for each gene using a window-of-linearity approach in LinRegPCR software (version 2020.2) (Ramakers et al. 2003; Ruijter et al. 2009). qPCR primers were considered acceptable based on the following criteria: (1) the estimated efficiency was between 1.7 and 2.0 (Wilkerson et al. 2013; Kim et al. 2017b), (2) the melting curve presented one single peak, and (3) no primer-dimers formed in reactions containing template (Rodríguez et al. 2015).

### Data analysis

A Bayesian Markov Chain Monte Carlo (MCMC) chain algorithm was conducted to evaluate the response of target mRNA/miRNA to experimental factors. The unit of biological replication used was an individual fish, so the replication level was \( n = 9–11 \) per tank and \( n = 20–22 \) per treatment. Hepatic gene expression levels were determined using a reference gene-free approach and the MCMC.qpcr package, implemented in R Version 3.6.1 (R Core Team 2021) following the procedures proposed by Matz et al. (2013). In MCMC, a two-way design “naïve” model was fitted to estimate the expression of target genes in response to fixed effects of “treatment” (control and Pb exposure) and “tank” (2 control and 2 Pb exposure tanks), and random effect (sample) as follows:

\[
\text{ln}(\text{rate}) \sim \text{gene} + \text{gene} : \text{Treatment} + \text{Gene} : \text{Tank} : \text{Treatment} + [\text{sample}]
\]

Gene expression data were reported as log2 transcript abundances in posterior mean (model estimates) with 95% credible intervals (CIs). The credible intervals are the Bayesian analog of confidence intervals. The statistical significance of

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changes in expression were evaluated using MCMC, with a significance threshold of $p < 0.05$ (Matz et al. 2013).

Transcript abundances (normalized data) from individual sculpin for all target genes were used to analyze the relationship between gene expression (this study) and data previously reported by Jantawongsri et al. (2021); i.e., body mass, length, liver mass, condition factor, hematocrit, and blood. For a detailed description of the histology and metal analyses, see Jantawongsri et al. (2021). Spearman’s rank correlation was then analyzed using the Hmisc package (Harrell 2015) and stats package in R (R Core Team 2021), and correlation coefficients ($r_s$) with $p < 0.05$ were considered significant.

Results

Hepatic gene expression

Hepatic expression levels of $mt$ mRNA were significantly greater in the Pb-exposed sculpins compared to those control sculpins (1.24-fold change, $p = 0.030$; Fig. 1 and Table S3). Similarly, $igm$ mRNA levels were significantly higher in Pb-exposed fish than in control fish (1.53-fold change, $p = 0.028$; Fig. 1 and Table S3). In contrast, $hsp70$ mRNA levels and the transcripts of $mir132$ and $mir155$ did not differ significantly between the Pb-exposed and control sculpins ($p > 0.05$; Fig. 1 and Table S3). The replicate tanks were pooled to compare Pb-exposed and control sculpin as no significant differences were observed in hepatic mRNA levels of $mt$, $igm$, and $hsp70$, and transcripts levels of $mir132$ and $mir155$ in the sculpins among the tanks ($p > 0.25$; Table S3).

Relationships between gene expression and other parameters

Transcript levels of $hsp70$ of all sculpins were positively correlated with body mass ($r_s = 0.58$, $p = 0.019$, $n = 23$; Fig. 2) and age ($r_s = 0.61$, $p = 0.004$, $n = 21$; Fig. 2). A significant positive correlation was observed between $igm$ and $mir155$ expression in all sculpins ($r_s = 0.66$, $p = 0.001$, $n = 39$; Fig. 2). Expressions of $igm$ ($r_s = 0.54$, $p = 0.038$, $n = 20$; Fig. 2) and $mir155$ ($r_s = 0.82$, $p = 0.008$, $n = 22$; Fig. 2) were positively correlated with hepatic Pb concentrations of all sculpins. There were statistically significant positive correlations between expression of $mt$ and number of mucous cells/ILU in the gills ($r_s = 0.43$, $p = 0.036$, $n = 42$; Fig. 2) and severity score of gill lesions, including: hyperplasia ($r_s = 0.66$, $p = 0.012$, $n = 42$; Fig. 2) and complete lamellar fusion ($r_s = 0.57$, $p = 0.014$, $n = 42$; Fig. 2). Moreover, transcript of $mir155$ of all fish was positively correlated with severity scores of hepatic lesions, including megalocytic hepatosis ($r_s = 0.67$, $p = 0.002$, $n = 42$; Fig. 2), necrosis ($r_s = 0.53$, $p < 0.001$, $n = 42$; Fig. 2), granuloma ($r_s = 0.42$, $p = 0.007$, $n = 42$; Fig. 2) and hepatic neoplasm ($r_s = 0.87$, $p = 0.001$, $n = 42$; Fig. 2), but negatively correlated with condition factor ($r_s = -0.8738$, $p = 0.02$, $n = 41$; Fig. 2). There was significant negative correlation between expression of $mir132$ and Pb concentrations in liver ($r_s = -0.75$, $p = 0.008$, $n = 22$; Fig. 2).

Discussion

In teleosts, Mt induction, and hence increased expression of $mt$, could occur in response to oxidative stress caused by exposure to heavy metals such as Cu, cadmium (Cd), mercury (Hg), nickel (Ni), Pb, and Zn (Schlenk et al. 1999; Cheung et al. 2004; Tom et al. 2004; Schmitt et al. 2007). In the present study, significant up-regulation of hepatic $mt$
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(TNF-α) in head kidney of Asian carp (Jing et al. 2020). Expression of mir132 was also found to be negatively associated with liver Pb levels in this study. However, there are few reports on how mir132 regulates fish immune responses. A previous study on miuy croaker (Miichthys miuy) showed that mir132 is a negative regulator of fish inflammatory cytokine production implicated in the immune response induced by lipopolysaccharides (LPS) (Dong et al. 2021). Further research to determine the specific target genes of miRNAs and their function related to the immune response particularly in sculpin with regards to heavy metals exposure is necessary to understand the underlying regulatory processes of miRNA expression.

Conclusions

In conclusion, the present study evaluated the potential toxicity of Pb exposure on gene expression associated with stress (mt and hsp70) and immune response (igm, mir132, and mir155) in the shorthorn sculpin, M. scorpius. The results demonstrated that exposure of shorthorn sculpin to environmentally relevant dissolved Pb concentration induced an increase in hepatic mt and igm expression. Expression of igm was positively correlated to Pb concentration in the liver. There were positive correlations between mir155 and igm and hepatic Pb concentration in liver, while mir132 was negatively correlated with Pb. Prior to this study, there was no information on effect of metal exposure on gene expression in marine sculpin. This study was the first to report that Pb exposure can affect expressions of hepatic metal homeostasis and immune response-related genes in the shorthorn sculpin. Overall, our results suggest that up-regulation of hepatic mt and igm has a potential as a biomarker of exposure to Pb which could improve the assessment of impacts of mining in the Arctic, including Greenland. However, further research is needed to evaluate their applications.

Data availability

Data are available from the corresponding author.

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Author contributions Conceptualization: RDN, LB, RD, CS, CAW, RE, and KA; Data curation: KJ and RDN; Formal analysis: KJ; Funding acquisition: KJ, RDN, CS, RE, BN, and KA; Investigation: KJ, RDN, KJØ, SL, TMC, BMJ, CAW, RE, and KA; Methodology: KJ, TMC, BMJ, CAW, and KA; Project administration: CS and BN; Resources: RDN, CS, KJØ, SL, CAW, and KA; Supervision: LB, CS, CAW, RE, BN, and KA; Visualization: KJ; Writing—original draft: KJ; Writing—review and editing: KJ, LB, RD, CS, TMC, BMJ, CAW, RE, BN, KA.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Ethical approval The waterborne Pb exposure experiment was conducted at the Blue Planet, National Aquarium, Copenhagen, Denmark, using shorthorn sculpins caught in the southern Kattegat, Denmark (license numbers for the experiment: 2015-15-0201-00692 (Dyrforøstgøtislyset), Ministry of Environment and Food of Denmark approved 28 September 2015).

Consent to publish All authors read and approved the final manuscript to be published in its present form.

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