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TOLERANCE OF WHITEFISH (COREGONUS LAVARETUS) EARLY LIFE STAGES TO MANGANESE SULFATE IS AFFECTED BY THE PARENTS

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Abstract: European whitefish (*Coregonus lavaretus*) embryos and larvae were exposed to 6 different manganese sulfate ($MnSO_4$) concentrations from fertilization to the 3-d-old larvae. The fertilization success, offspring survival, larval growth, yolk consumption, embryonic and larval Mn tissue concentrations and transcript levels of detoxification-related genes were measured in the long-term incubation. Full factorial breeding design (4 females x 2 males) enabled examining the significance of both female and male effects, and female-male interactions in conjunction with the $MnSO_4$ exposure on the observed endpoints. The $MnSO_4$ exposure reduced the survival of the whitefish early life stages. Also the offspring $MnSO_4$ tolerance was affected by the female parent, and the female-specific mean lethal concentrations (LC50) varied from 42.0 to 84.6 mg $MnSO_4$/L. The larval yolk consumption seemed slightly inhibited at the exposure concentration of 41.8 mg $MnSO_4$/L. The $MnSO_4$ exposure caused a significant induction of metallothionein-A (*mt-a*) and metallothionein-B (*mt-b*) in the 3-d-old larvae, and at the exposure concentration of 41.8 mg $MnSO_4$/L the mean larval *mt-a* and *mt-b* expressions were 47.5 and 56.6 % higher, respectively, than at the control treatment. These results illustrate whitefish reproduction can be impaired in waterbodies that receive Mn and SO_4 in concentrations substantially above the typical levels in boreal freshwaters, but the offspring tolerance can be significantly affected by the parents and in particular the female parent.

Keywords: Aquatic toxicology, Embryonic development of fish, Metal toxicity, Mine effluents Parental effect

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INTRODUCTION

Manganese (Mn) and sulfate (SO_4) occur naturally in the aquatic environment [1, 2]. Median Mn and SO_4 concentrations in the Nordic surface waters range from 3.2 to 65 µg/L and 1.3 to 3.8 mg/L, respectively [3–5]. Even though Mn and sulfur (S) are essential nutrients [1, 2], excessive concentrations of Mn and SO_4 can be toxic to aquatic organisms [6, 7]. Mining and mineral processing is one of the major anthropogenic sources of Mn [1]. Similarly, SO_4 is often a prevalent contaminant in mine water and it can have a substantial contribution to salinization of the waterbodies receiving the mine waters [8]. The metal mining industry has adopted and developed biomining processes, in which micro-organisms are utilized in metal recovery, and biomining is considered to have economic and environmental advantages compared to conventional recovery methods [9]. In Europe, the first commercial application of biomining utilizing the bioheapleaching technology was established in 2008 in North-Eastern Finland [10]. After the mine started to operate, Mn and other metal, as well as SO_4 concentrations in the waterbodies receiving the mine effluents have been elevated and an accidental gypsum pond leakage at the mine in late 2012 deteriorated the nearby water quality [5, 11–13]. This has raised concern about the effects of Mn and SO_4 especially on the commercially important boreal freshwater fish.

The early life stages of fish, larvae in particular, are generally more sensitive to chemical toxicants than the adults [14]. Offspring stress tolerance can depend on their genetic background, and especially on the female parent [15, 16]. Metal exposure during the early development is known to disturb developmental processes, reduce hatching rate and larval body size, and cause both embryonic and larval malformation and mortality [17]. Compared to other metals such as cadmium (Cd), copper (Cu) and zinc (Zn), the toxicity of Mn to aquatic organisms is suggested

to be low [6, 18]. A 25 % inhibition concentration (IC25) of Mn on survival and growth of brown trout (*Salmo trutta*) early life stages have been reported between 4.67 to 8.68 mg Mn/L, Mn being more toxic in soft than hard water [6]. In soft water, Mn concentrations of 0.32 to 0.35 mg/L have already disturbed the mineral uptake and skeletal calcification of brown trout larvae [19]. For SO_4, previously reported IC25 values regarding embryo development of coho salmon (*Oncorhynchus kisutch*), embryo-to-alevin development of rainbow trout (*O. mykiss*) and larval mortality of fathead minnow (*Pimephales promelas*) were 1264 mg/L, 501 mg/L and 933 mg/L, respectively [7]. Mixture toxicity studies on aquatic organisms, such as salmonid embryos, tropical duckweed (*Lemna aequinoctialis*), green hydra (*Hydra viridissima*) and pulmonate snail (*Amerianna cumingi*), focusing on both SO_4 and a cationic metal, such as calcium (Ca^{2+}) or magnesium (Mg^{2+}), suggest that the cation is the toxic cause rather than the SO_4 [20, 21].

External stressors can also activate defense mechanisms in aquatic organisms, and oxidative stress is often associated with a strong stress [22]. Metals and salinity changes are known to modulate oxidative stress responses in fish [23, 24], and the main antioxidant enzymes protecting organisms from oxidative damage are catalase (CAT), glutathione peroxidase (GPx), glutathione-s-transferase (GST) and superoxide dismutase (SOD) [25, 26]. Metal-binding proteins, metallothioneins (MTs) [27], have been considered as suitable biomarkers for metal exposure [25]. Even though the exact role of MTs is still unclear [27], they are known to regulate the availability of essential and non-essential metals [28]. Also, the induction of MT gene transcription can correlate with metal tolerance, as observed with Cd-exposed turbot (*Scophthalmus maximus*) larvae [29].

The present study was designed to specifically assess the critical mixture concentration of Mn and SO_4 that reduces survival of a native boreal fish species, European whitefish (*Coregonus*

lavaretus), embryos and larvae, and disturbs their yolk utilization for growth. We conducted a continuous laboratory-scale manganese sulfate ($MnSO_4$) exposure with whitefish embryos and hatched larvae to investigate 1) the effect of the parental combination on the sensitivity of whitefish early-life stages to $MnSO_4$, 2) the Mn body residues of the whitefish eggs and the 3-d-old larvae, and 3) the transcript abundance of *cat*, *gstt*, *mt-a* and *mt-b* in the embryos and larvae under the $MnSO_4$ exposure. These results bring new information for assessing the effects of SO_4-induced salting and Mn on the reproduction of the European whitefish stocks, and they allow comparison of the species sensitivity to the effects of salting and Mn in freshwaters worldwide.

1.1 MATERIALS AND METHODS

1.1.1 Test species and chemicals

Newly stripped whitefish eggs of 4 females and milt of 2 males (Rautalampi stock from Finnish Game and Fisheries Research Institute, Laukaa, Finland) were transported from the hatchery to the laboratory for fertilization. The eggs were kept in plastic boxes and on ice until fertilization. Milt was transported (40 min) in oxygen filled Minigrip plastic bags and kept on ice. Before fertilization the milt was pipetted into microcentrifuge tubes and placed into a cool block (Echtotherm Chilling/Heating dry bath, Torrey Pines Scientific) to +5 °C temperature.

Manganese sulfate monohydrate, $MnSO_4 \cdot H_2O$ (Emsure, ACS, Reag. Ph Eur, Merck KGaA; purity 98.8 %), was weighed into 4 $MnSO_4 \cdot H_2O$ stock solutions of 6.4 mg/L, 160 mg /L, 4000 mg/L and 100000 mg/L into ultrapure water (Ultra Clear UV UF TM) and the solutions were stored at +4 °C in the dark prior to use. New stock solutions were made twice (6.4–4000 mg $MnSO_4 \cdot H_2O$/L) to 3 (100000 mg $MnSO_4 \cdot H_2O$/L) times during the course of the experiment. Suprapur HNO_3 (65 %, Merck KGaA) was used in the water sample acidification and acid washes were done with analytical grade HNO_3 (Merck KGaA). The reagents (HNO_3 and HCl)

used in tissue sample digestion were of analytical grade (Sigma Aldrich) and only high purity water of 18.2 MΩ cm resistivity produced by a PURELAB Ultra water purification system supplied by Elga was used throughout with the tissue samples.

1.1.2 Test setup and fertilization

Pre-filtered (1 µm, 155383-03, Model BP-410-1, Pentek) Lake Konnevesi (Konnevesi, Central Finland) water was used as a control water and spiked with $MnSO_4$ to 6 nominal $MnSO_4$ exposure concentrations of 0.06 mg/L, 0.29 mg/L, 7.2 mg/L, 35.7 mg/L, 179 mg/L and 893 mg/L, respectively (element specific concentrations are presented in Table 1). The continuous laboratory-scale $MnSO_4$ exposures were started from fertilization and ended 3 d after the hatching of the larvae. The experiment lasted for 160 d, starting on 7 November 2013 and ending on 16 April 2014, when nearly all the embryos had either died or hatched (2 alive but unhatched embryos in concentration 41.8 mg $MnSO_4$/L). During the winter period (experiment days 1–108), the water temperature development followed natural L. Konnevesi water temperature. The water temperature elevation was started on experiment day 109 resulting roughly a 0.2 °C daily mean water temperature increase. The mean, minimum and maximum water temperature of all the pools during the whole experiment period was 3.6 °C, 1.0 °C and 10.3 °C, respectively. The light rhythm followed the local natural light rhythm (Konnevesi, Central Finland) resulting roughly in 7:17-h, 5:19-h, 6:18-h, 9:15-h, 12:12-h and 14:10-h mean monthly light:dark cycles during the experiment time. The embryos were sampled at the end of the winter period to represent the winter time embryonic development, and correspondingly the 3-d-old larvae represent the development during the spring period.

A full factorial breeding design was applied by fertilizing the eggs of each female (F1, F2, F3 and F4) with the milt of both males (M1 and M2) separately to produce all 8 different

female-male combinations in 3 replicates for each $MnSO_4$ exposure concentration and the control (Figure 1). The sperm motility of the males was inspected with an Integrated Semen Analysis System (ISASv1 Casa, Proiser) before the fertilizations. Approximately 50 to 200 eggs per replicate were fertilized on plastic Petri dishes with 5 to 10 µL of milt using corresponding exposure or control water as the sperm activation water. The activation water temperature was +5 °C. Few minutes after the fertilization, each replicate egg batch was placed into a plastic box (350 mL, PP, Greiner) containing 100 mL of corresponding exposure or control water and taken immediately into the experiment room at +5 °C temperature. Eggs that were left over from the fertilization were stored for size analysis at -20 °C. Four to 5 d after the fertilization, fertilization success was estimated under light microscope from 10 eggs per replicate and after the investigation those eggs were not returned for further incubation.

Six days after the fertilization, the egg batches were moved into plastic (LE-marked, Robusto, OKT) incubation pools (inside: 565 mm x 365 mm x 220 mm) containing 12.5 L of corresponding test water. There was 1 pool per each $MnSO_4$ exposure and 1 control pool and the eggs were placed into compartment grids, 1 replicate egg batch per 1 randomly selected compartment. The compartment grids (350 mm x 350 mm x 70 mm) containing 36 compartments (50 mm x 50 mm x 40 mm) were made from plexiglass and a 750 µm mesh (PETP, Sefar Petex 07-750/53) glued (Acrifix 192) at the bottom of the compartments. Water depth both underneath and above the eggs was 30 mm (total water depth 60 mm), allowing sufficient water circulation for the eggs. Before the onset of hatching, the compartments were divided into 4 sections with thin plexiglass slides enabling 3-d incubating of the larvae with 24-h accuracy of the individual hatching time. The grids and plexiglass slides were acid washed (10 % HNO_3) and grids and incubation pools were soaked in the corresponding exposure or control

water before placing the eggs into them. Pool waters were aerated with glass Pasteur pipets (10 % HNO_3 acid washed) from 2 opposite sides of the pool into opposite directions to enhance adequate water circulation in the pools. Pools were protected from contamination with loosely placed clear plastic film covers on top and polystyrene covers were placed on the pools for 3 mo in early December to mimic the ice cover typical in boreal regions at that time of the year.

Quality control of the exposure

Interval of water renewals was 3 to 4 d, and 4 L from each pool was changed at a time. In every water renewal time, both new exposure and control waters for the next water renewal were prepared and let to aerate and stabilize to the incubation temperature.

Incubation water temperature, pH (744 pH meter, Metrohm, Professional Plus, YSI and SevenGo pH meter SG2, Mettler Toledo), conductivity (Professional Plus, YSI) and oxygen concentration (ProOdo, YSI) were monitored both at the beginning and end of the experiment and before and after water renewals. Mean water oxygen concentration (± SE) of all the pools during the experiment was 12.6 ± 0.1 mg/L (min 11.0 mg/L and max 15.2 mg/L) and pH was 6.66 ± 0.01 (min 5.46 and max 7.49) (Supplemental Data, Table S1). Degree days (cumulative sum of mean daily temperatures during the whole incubation period) were calculated for each pool with linear interpolation using the water temperature before every water renewal. Dissolved organic carbon of pre-filtered L. Konnevesi water (i.e. newly made control water) was analyzed in the beginning of the experiment and during embryo and larval sampling (TOC-L, Total Organic Carbon Analyzer, Shimadzu), resulting in mean dissolved organic carbon concentration of 7.7 ± 0.2 mg/L. Ammonium concentrations of the pool waters were analyzed in accredited laboratory (FINAS T142; EN ISO/IEC 17025) according to a standard method [30] once after two months of incubation. The mean ammonium concentrations (± SE) of all the pools before

and after the water renewal were 365 ± 28.9 µg/L (min 260 µg/L and max 500 µg/L) and 254 ± 19.3 µg/L (min 180 µg/L and max 340 µg/L), respectively.

Manganese (Mn) and sulfur (S) concentrations in the incubation waters were monitored and common other elements such as aluminum (Al), arsenic (As), cadmium (Cd), calcium (Ca), chrome (Cr), cobalt (Co), copper (Cu), iron (Fe), lead (Pb), magnesium (Mg), nickel (Ni), phosphorus (P), potassium (K), sodium (Na), strontium (Sr), uranium (U) and zinc (Zn) were analyzed as well (Supplemental Data, Table S2). Sulfate concentrations were estimated based on S concentrations, assuming that all the S was present as SO_4 in the well aerated exposure and control waters. Filtered (50 mL sterile syringe BD Plastipak, 25 mm GD/XP syringe filters, 0.45 µm PVDF w/PP, Whatman) and unfiltered water samples from the newly made control and $MnSO_4$ spiked waters were collected twice in the beginning of the experiment and unfiltered pool water samples were collected twice, 1 and 3 d after the placing of the eggs into the pools. Onwards, unfiltered pool water samples were collected right before and after every water renewal and in the end of the experiment. Water samples were collected into metal-free plastic tubes (PP, 50 or 15 mL VWR) and acidified immediately after sampling by adding 6 (50 mL samples) or 2 (15 mL samples) drops of HNO_3 and stored at +4 °C in the dark until the analyses. Total number of analyzed samples is given in the Supplemental Data (Table S2).

The chemical element concentrations of the waters (Supplemental Data, Table S2) were analyzed with ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometry, Optima 8300, Perkin-Elmer) and in a case of low Mn concentrations the Mn analysis was performed with ETAAS (Electrothermal Atomic Absorption Spectrometry, AAnalyst 800, Perkin-Elmer). The water concentration results above the limits of quantification (LOQs) with relative standard

deviation (RSD) below 10 % were accepted. The LOQs were defined according to USEPA Method 200.7 MDL procedure [31].

The control (i.e. background) water mean total concentrations (± SE) of $MnSO_4$, Mn and SO_4 for the whole experiment period were 5.5 (± 0.1) mg/L, 1.5×10^{-2} ($\pm 0.3 \times 10^{-2}$) mg/L and 5.5 (± 0.1) mg/L, respectively (Table 1). The measured total $MnSO_4$, Mn and SO_4 exposure concentrations varied between 100.4 to 107.7 %, 27.9 to 107.6 % and 102.1 to 108.6 % of the nominal concentrations that included the corresponding background levels (Table 1, Supplemental Data, Table S3). During the first month of the experiment, the Mn concentrations in 2 of the lowest exposure concentrations fell below the nominal levels and remained such until the end of the experiment. It is probable, that Mn could have been oxidized in the well-aerated pool water and thus adsorbed onto the pool and compartment grid surfaces and/or onto the developing eggs. In this case, if both the Mn was oxidized and the amount of Mn adsorbed was uniform in each pool, the difference between the measured and the nominal Mn concentration would thus have been the most extreme in the low concentration pools than in the higher concentrations.

Most of the control water Mn was not in dissolved form (mean Mn dissolved 7.5 %), whereas the $MnSO_4$ concentration increase in exposure waters gradually increased the proportion of dissolved Mn (range of the mean Mn dissolved 78–99 %). Sulfur was in dissolved form in the $MnSO_4$ exposure (range of the mean S dissolved 99–100 %) and control (mean S dissolved 99 %) waters.

1.1.3 Mortality and overview of embryo and larval sampling

Dead embryos were counted and removed 3 times per week during the first month of the experiment and twice a week from that onwards. Hatching started 119 d after fertilization and the mean hatching peak of all parent pairs was reached 136 d after fertilization. During the hatching period, hatched and dead larvae were counted daily.

Detailed information of the embryo and larval samples of every sample type are listed in Supplemental Data (Table S4) according to $MnSO_4$ exposure concentration and parent pair. Both embryos and larvae were sampled for tissue element concentration and gene expression analyses, whereas only larvae were sampled for growth and yolk consumption analyses. Embryo samples were collected before the beginning of the water temperature elevation at the end of the winter period, on experiment days 102 (for tissue concentration analyses) and 105 (for gene expression analyses). The hatched larvae were incubated 3 d under exposure conditions before larval sampling. With the 3-d-old larvae, the samples were collected from experiment day 131 to experiment day 146.

Growth, yolk consumption and egg size

One growth and yolk consumption sample from each replicate contained 1 to 10 larvae from the same hatching day and a maximum of 4 larvae per replicate were measured. The samples were collected into 1.5 mL microcentrifuge tubes (StarLab GmbH), excess water was removed and approximately 1 mL of 10 % neutralized formalin (1:9 v/v of 37 % formalin and Na_2HPO_4 3.55 g/L, $NaH_2PO_4 \cdot H_2O$ 7.3 g/L dissolved in ultrapure H_2O) was added. The samples were stored at -20 °C in the dark until analyzed. Thereafter, the samples were thawed on ice, rinsed with ultrapure water (Ultra Clear UV UF TM), then yolk and carcass were separated and

placed into pre-weighed aluminum cups. The samples were dried in +40 °C temperature for 24 h and weighed. The initial egg size of each female was measured by analyzing the dry weight from 16 to 20 eggs per female. The initial egg size samples were stored at -20 °C in the dark without the formalin fixation.

Tissue concentration analysis

The embryo tissue concentration samples were analyzed as whole eggs (no dechorionation) and thus those samples are referred as eggs here. A maximum of 4 eggs (~ 48.4 mg wet weight resulting as 8.7 mg dry weight) from each replicate were collected into each tissue concentration sample. In order to get enough material for the analysis, the egg tissue concentration samples of the 3 replicates for each parent pair in each concentration were pooled (see Supplemental Data, Table S4). With the 3-d-old larvae, the larval samples were replenished until reaching 10 3-d-old larvae per sample per replicate. The sample replicates of the larval tissue concentration were not pooled. The egg and larval tissue concentration samples were collected into 1.5 mL microcentrifuge tubes (StarLab GmbH), washed three times with filtered (50 mL sterile syringe BD Plastipak, 25 mm GD/XP syringe filters, 0.45 µm PVDF w/PP, Whatman) L. Konnevesi water, blotted dry, placed into pre-weighted 1.5 mL microcentrifuge tubes (StarLab GmbH) and stored at -20 °C in the dark prior to analysis. The sampled larvae were anesthesized with sodium hydrogen carbonate containing fruit salt (Samarin, Cederroth AB) and some of the larvae were photographed under a microscope (SteREO, Discovery V8, AxioCam ERc 5s, Zen lite 2011, Zeiss) for malformation investigation before washing and storing. In the course of photographing the larvae at 197.8 mg $MnSO_4$/L were observed to be opaque, but because no proper malformations were observed from any of the larvae at any exposure concentration the malformation data was not analyzed further.

Freeze-dried and weighed egg and larval samples were digested in aqua regia (HNO_3:HCl, 1:3 v/v solution) by ultra sound (ELMA Model Transonic 820/H (650 W, 35 kHz) or Bandelin Sonorex RK 512/H (400 W, 35 kHz)). Eggs were digested in 1.5 mL microcentrifuge (StarLab GmbH) tubes by moisturizing the sample with ultrapure water and adding 8 drops of aqua regia, followed by sonication in 2 to 3 min cycles at +30 to 40 °C. Larval samples were digested in 13 mL tubes (PP, Sarstetd) by moisturizing the sample with ultrapure water and adding 10 drops of aqua regia, followed by sonication in 3 min cycles at +55 °C. The sonication was repeated 2 to 12 times until the samples were fully digested. Samples were shaken and sample tube pressure was released by opening the caps between each sonication cycle. The digested samples were filtered (Whatman No. 41, GE Healthcare Life Sciences) and filled to final volume of 5 mL with ultrapure water.

The element concentrations of the eggs (Supplemental Data, Table S5) and the larvae (Supplemental Data, Table S6) were analyzed with ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometry, Optima 8300, Perkin-Elmer). The same LOQ and RSD limit requirements were used for both the water and the tissue concentrations and the tissue concentration results are presented as upper bound concentrations (values below LOQ and/or RSD > 10 % are replaced with LOQ), unless mentioned otherwise.

1.1.4 Gene expressions

Gene expression samples were collected at the incubation temperature in order to avoid sudden temperature changes that may affect the gene expressions of the embryos and the larvae. A maximum of 3 embryos per sample from each replicate were collected into 1.5 mL microcentrifuge tubes (StarLab GmbH), washed 3 times with filtered (50 mL sterile syringe BD Plastipak, 25 mm GD/XP syringe filters, 0.45 µm PVDF w/PP, Whatman) L. Konnevesi water,

blotted dry and placed into 1.5 mL microcentrifuge tubes (StarLab GmbH). With the 3-d-old larvae, 1 sample from each replicate contained 5 larvae from the same hatching day. The larvae were sampled into 1.5 mL microcentrifuge tubes (StarLab GmbH) and the excess water was removed with a needle and a syringe. Both the embryonic and larval samples were immediately frozen in liquid nitrogen and stored at -80 °C until analyzed.

The embryonic and larval expressions of target genes *mt-a*, *mt-b*, *gstt* and *cat* were analyzed with quantitative reverse transcription PCR (qRT-PCR) using ribosomal protein L2 (*rl2*) and *beta actin* as reference genes (Supplemental Data, Table S7). The selected reference genes had the most stable expression among treatments from all the tested reference genes. The *mt-a* gene was obtained from Hansen et al. [32]. Other target gene primers were designed with Primer3 4.0.0 (http://bioinfo.ut.ee/primer3/) [33, 34] and the reference gene primers with AmplifX 1.5.4 (http://crn2m.univ-mrs.fr/pub/amplifx-dist) [35]. The specificity of the genes was checked with Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) [36]. The RNA extraction and integrity analysis, DNase treatment, cDNA synthesis and amplification reactions were done as described in Vehniäinen and Kukkonen [37]. The qPCR run was done using CFX96 Real-Time PCR cycler (Bio-Rad) and the protocol was +95 °C for 3 min; 40 cycles of +95 °C (10 s), +58 °C (10 s), and +72 °C (30 s); at +95 °C for 10 s and melt curves from +65 to +95 °C with 0.5 °C intervals. Samples were run in duplicates using clear 96-well PCR plates (Bio-Rad). A no template control (NTC) was always run for each gene.

1.1.5 Mortality calculations and statistics

Fertilization loss Z_F, (Equation 1) as well as instantaneous mortality of the winter period Z_W, (Equation 2), spring period Z_S, (Equation 3) and total mortality Z_T, (Equation 4) excluding the fertilization loss of the cross-fertilizations were estimated according to equations:

$$Z_F = -LN(N_F/N_0) \quad (1)$$

$$Z_W = -LN(N_W/N_F + 0.01) \quad (2)$$

$$Z_S = -LN(N_L/N_S + 0.01) \quad (3)$$

$$Z_T = Z_W + Z_S \quad (4)$$

N_0 is number of live eggs before fertilization, N_F is number of successfully fertilized eggs, N_W is number of embryos survived by the end of the winter period right before embryo sampling, N_S is number of embryos in the beginning of the spring period right after embryo sampling, N_L is number of live larvae three days after hatching and 0.01 is a constant added to avoid zero values when taking the natural logarithm LN. Instantaneous mortality (Z) was assumed to be a normally distributed variable. A full-factorial general linear model (GLM), including the main effects of male, female and $MnSO_4$ exposure and their interactions was used to test for the effect of $MnSO_4$ exposure on Z_F and Z_T.

The highest $MnSO_4$ concentration in which the female-specific total survival did not differ significantly from the control values (NOEC) were tested with ANOVA and depending on the homogeneity of variances the pairwise comparisons were done with one-sided Dunnett's test or Dunnett's T3 test. The lethal $MnSO_4$ concentrations resulting in 50 % mortality of the offspring (LC50) with 95 % confidence limits were calculated for each female separately by pooling the data of both males per female. The female-specific LC50 values for $MnSO_4$ were

calculated according to total offspring mortality and the mean measured total $MnSO_4$ concentrations of each pool during the whole experiment period using each female's mean total mortality in the control pools as natural response rates. Data analyses were made with Probit model using a LN-transformed covariate. A heterogeneity factor was used in the confidence limit calculations since the significance level of the Pearson goodness-of-fit chi-square test was less than 0.15.

A full-factorial GLM, including the main effects of male, female and $MnSO_4$ exposure, their interactions and degree days as a covariate, was used to test for the effect of $MnSO_4$ exposure on growth and yolk consumption of the 3-d-old larvae. The mean carcass and yolk dry mass of the larvae in each replicate were used in the analyses.

A full-factorial GLM, including the main effects of female and $MnSO_4$ exposure and their interactions, was used to test for the effect of $MnSO_4$ exposure on the egg and larval Mn and S tissue concentrations. Due to mortality, the number of samples was small and thus the male effect was not included in the data analysis. The concentrations below the LOQ or with RSD above 10 % were replaced with upper bound concentrations.

The critical body residues of Mn taken up by the eggs and larvae causing 50 % mortality of the observed individuals (CBR50) with 95 % confidence limits were calculated separately for eggs and egg-to-larvae stages. Due to mortality, the number of samples was small and thus the CBR50 values could not be distinguished according to the parent pairs or females. In all the egg samples, the Mn concentrations were above LOQ with RSD less than 10 %, whereas for the larvae, an upper bound (concentrations below the LOQ or with RSD above 10 % replaced with LOQ) estimate for the CBR50 value was calculated. Data analyses were made with Probit model using a LN-transformed covariate. A heterogeneity factor was used in the confidence limit

calculations since the significance level of the Pearson goodness-of-fit chi-square test was less than 0.15. Since the egg tissue concentration replicates of each exposure concentration and control were pooled according to the parent pair, the corresponding mean winter time embryonic mortality values were used in the egg Mn CBR50 analysis, and the mean mortality of the control samples was used as a natural response rate. The total mortality values were used in the egg-to-larval analysis, and thus the egg-to-larval CBR50 values represent the entire exposure period from the fertilization until the larvae were 3-d-old. The natural response rate in the egg-to-larvae analysis was the mean total mortality of the control samples.

Embryonic and larval gene expression differences of 3 and 2 parent pairs, respectively, were analyzed and thus the female and male effect could not be tested. The pair selection was based on the offspring total mortality: a pair with low (F4 x M2), intermediate (F2 x M1) and high (F3 x M2) offspring total mortality. The degree days of the analyzed replicates were taken into account and thus no effects of developmental differences were expected. The gene expression differences between the parent pairs and treatments were analyzed with LOG10 transformed normalized expressions using the GLM full factorial effect model structure. Additionally, to distinguish the differences in the embryonic gene expressions between the 3 parent pairs, the exposure concentration was excluded from the factors and only the parent pairs were compared with one-way ANOVA including Tukey HSD or Tamhane T2 post hoc tests depending on the homogeneity of the variances. Also, the differences in the gene expressions of the offspring of each parent pair separately were analysed for both embryos and larvae with one-way ANOVA, including Tukey HSD or Tamhane T2 post hoc test depending on the homogeneity of the variances.

All the statistical analyses were done with SPSS (IBM SPSS Statistics 22).

1.2 RESULTS

Fertilization, mortality, NOEC and LC50 values

The Z_F of the whitefish eggs was significantly affected by both the $MnSO_4$ exposure concentration and the female, but male effect or interactions between the variables were not found. (Table 2, Figure 2A). Similarly, the $MnSO_4$ exposure and female, but not the male, had significant effect on the Z_T (Figure 2B). The significant interaction between the $MnSO_4$ exposure and female indicates that some of the females had produced more $MnSO_4$-tolerant embryos than others, and this was evident from the female-specific NOEC and LC50 values as well (Table 3). Even though there was a significant interaction between the $MnSO_4$ exposure and male as well, the differences between the males were not as clear as between the females in different concentrations.

1.2.1 Growth and yolk consumption

The carcass dry weight of the 3-d-old whitefish larvae was affected significantly by the $MnSO_4$ exposure and female (Table 4). The male effect was not significant, but the interaction between the exposure and male was. Degree days, $MnSO_4$ exposure and female had a significant effect on the yolk dry weight of the 3-d-old larvae (Table 4). The interactions between female and $MnSO_4$ exposure, and between parent pair and $MnSO_4$ exposure were also significant. On average, the larvae of each female had more yolk left in 41.8 mg $MnSO_4$/L concentration compared to the control larvae (Figure 3).

1.2.2 Manganese and S concentrations in the eggs and the larvae

The Mn concentrations of the eggs were affected significantly by the $MnSO_4$ exposure (GLM, $F = 207.137$, $df = 6$, $p < 0.001$) and the Mn concentrations of the eggs of the different

females differed significantly as well (GLM, $F = 3.098$, $df = 3$, $p = 0.049$) (Table 5, Supplemental Data, Table S8). The $MnSO_4$ exposure had also a significant effect on the larval Mn concentrations (GLM, $F = 58.647$, $df = 5$, $p < 0.001$), but the effect of the female was not significant (GLM, $F = 1.102$, $df = 3$, $p = 0.354$). Significant interaction between the $MnSO_4$ exposure and female were not found either with the eggs or the larvae (GLM, $p > 0.05$, Supplemental Data, Table S8). Compared to the eggs, the 3-d-old larvae had roughly 8 to even 60 times lower Mn body burdens. The winter time egg CBR50 value for Mn was 9.08 µmol/g dry weight with 95 % confidence limits of 7.13 to 12.81 µmol/g dry weight with natural response rate of 30.5 %. For the egg-to-larvae period, the CBR50 value was 0.88 µmol/g dry weight with 95 % confidence limits of 0.56 to 2.07 µmol/g dry weight with the natural response rate of 29.1 %. Sulfur was concentrated neither into the eggs nor to the larvae (GLM, $p > 0.05$, Supplemental Data, Table S8).

1.2.3 Gene expressions

The $MnSO_4$ exposure did not have a significant effect on the transcript abundance of any of the target genes in the embryos (Figure 4, Table 6). However, the *cat*, *mt-a* and *mt-b* expressions differed significantly between the embryos of the 3 different parent pairs (Table 6). As the exposure concentration was excluded from the analysis and only the embryonic gene expression differences between the parent pairs were analyzed, the significant differences in *cat*, *mt-a* and *mt-b* expressions between the parent pairs were still observed (ANOVA, *cat*: $p = 0.001$, $F = 8.060$, $df = 2$; *gstt*: $p = 0.062$, $F = 2.945$, $df = 2$; *mt-a*: $p = 0.010$, $F = 5.052$, $df = 2$; *mt-b*: $p = 0.027$, $F = 3.925$, $df = 2$). The pairwise comparisons showed that *cat* expression of the embryos of the parent pair with high offspring total mortality (F3 x M2) was significantly higher than with the embryos of the two other pairs (Tukey HSD, F2 x M1: $p = 0.015$ and F4 x M2: $p = 0.001$).

The *mt-a* expression of the embryos of the parent pair with the lowest offspring total mortality (F4 x M2) was significantly lower than in the embryos of the two other parent pairs (Tukey HSD, F2 x M1: $p = 0.028$ and F3 x M2: $p = 0.018$), whereas the *mt-b* expression of the embryos of the parent pair with intermediate offspring total mortality (F2 x M1) was significantly lower than with the embryos of the parent pair with high offspring total mortality (F3 x M2) (Tukey HSD, $p = 0.028$). According to the individual parent pair analyses, only the pair F2 x M1 had significant differences (ANOVA, $p = 0.023$, $F = 4.344$, $df = 5$) in their offspring's *cat* expressions. However, only in $MnSO_4$ concentration of 5.6 mg/L the embryonic *cat* expression was significantly lower than in the control (Tamhane T2, $p = 0.011$).

The 3-d-old larvae showed induction of both *mt-a* and *mt-b* under $MnSO_4$ exposure (Figure 4, Table 6). According to the GLM results, the *mt-b* was differently induced among the 2 pairs, F2 x M1 and F4 x M2, and the joint effect between the $MnSO_4$ exposure and the parent pair was significant as well (Table 6). Even though the GLM results revealed significant differences only in the *mt-b* induction between the parent pairs, the individual parent pair analyses showed that both *mt-a* and *mt-b* were significantly induced only in the larvae of the pair with the lowest offspring total mortality (F4 x M2) (ANOVA, *mt-a*: $F = 10.569$, $df = 4$, $p = 0.003$; *mt-b*: $F = 7.176$, $df = 4$, $p = 0.009$), whereas the larvae of the pair with an intermediate offspring total mortality (F2 x M1) had no metallothionein induction (ANOVA, *mt-a*: $F = 0.931$, $df = 4$, $p = 0.484$; *mt-b*: $F = 1.038$, $df = 4$, $p = 0.435$). In pairwise comparisons, the larval *mt-a* and *mt-b* expressions of the pair F4 x M2 were significantly higher in $MnSO_4$ exposures of 5.9 mg/L (*mt-a*: Tamhane T2, $p = 0.035$; *mt-b*: Tukey HSD, $p = 0.038$) and 41.8 mg/L (*mt-a*: Tamhane T2, $p = 0.043$; *mt-b*: Tukey HSD, $p = 0.020$) than in the control larvae. However, also the *mt-b* expressions of the larvae reared in 12.8 mg $MnSO_4$/L concentration differed

significantly from the ones reared in 41.8 mg $MnSO_4$/L (Tukey HSD, $p = 0.032$). According to the GLM results, the $MnSO_4$ exposure or parent pair did not have a significant effect on *gstt* or *cat* expressions of the larvae (Table 6), and the individual parent pair analyses did not reveal any differences either. The number of valid replicates for both embryos and larvae for each target gene and parent pair are listed in Supplemental Data (Table S9).

1.3 DISCUSSION

Under experimental conditions, early life stages of whitefish were sensitive to $MnSO_4$, and the variation in their tolerance was significantly affected by the parent fish, the female in particular. The female had a significant effect on the fertilization success, the offspring total mortality and the growth and yolk consumption of the larvae, whereas male alone did not have a significant effect on those end points. The $MnSO_4$ exposure had caused a significant induction of *mt-a* and *mt-b*, but only in the larvae of the pair with the lowest total offspring mortality.

The female-dependent differences in the offspring tolerance to toxic chemicals or unfavorable environmental conditions could be due to genetic or environmentally induced variability. In methylmercury-exposed mummichog (*Fundulus heteroclitus*) embryos, some females living in an unpolluted environment produced more tolerant offspring than other females of the same population, and the variability in offspring methylmercury tolerance was suggested to be linked to genetic differences between the females [15]. Also, the survival of freshwater-adapted European whitefish embryos under chronic osmotic stress has been shown to depend significantly on their female parents [16]. However, there is evidence that if the parent fish is exposed to metals before spawning, the metal tolerance can be maternally transferred to the offspring as well. For example, female fathead minnows that had been exposed to Cu produced larvae with a higher Cu tolerance [38]. In the present study, however, the parent fish were

hatchery-reared and thus likely had a uniform life history, and had been reared in an unpolluted environment. Thus, the female effect observed in the present study was more likely caused by natural individual variation. The male effect was not observed or remained vague due to a low number of males.

The higher $MnSO_4$-tolerance of the offspring of F4 could also be related to the larval *mt-a* and *mt-b* induction observed in parent pair F4 x M2. Previously, the ability of Cd-exposed turbot larvae to induce MT gene transcription correlated to their Cd tolerance [29] and thus the activation of detoxification processes could have been the reason for the higher $MnSO_4$-tolerance of the offspring of F4 as well. However, the offspring gene expressions of the parent pair F4 x M1 were not investigated, and only the larvae had induced *mt-a* and *mt-b*, whereas with the embryos the observed differences in gene expressions between the parent pairs did not seem to be connected to offspring survival. Also, the induction of the *mt-a* and *mt-b* in the whitefish larvae did not show a consistent concentration-related pattern. Previously, a concentration-related pattern of MT induction has been observed from several tissue types of Cd-exposed juvenile river pufferfish (*Takifugu obscurus*) [39]. On the other hand, the expression patterns of MT have also been shown to depend on the exposure duration [39], and different metals can cause very different induction patterns as well [40].

The $MnSO_4$ exposure inhibited slightly the yolk consumption of all the larvae in the 41.8 mg $MnSO_4$/L exposure concentration and with nearly all the females, the exposure had reduced the dry weight of larval carcass as well. Metal exposure can cause inability to utilize yolk reserves as demonstrated with Cu-exposed common carp (*Cyprinus carpio*) larvae [41] and the larval growth of brown trout has shown to be reduced under Mn exposure [6]. Also, increased osmoregulatory cost can reduce larval length of whitefish [16].

Since the Mn accumulation in the eggs was substantially higher than in the larvae, it is most likely that Mn had been blocked by the chorion and/or perivitelline fluid, as previously demonstrated with Cd [42] and Cr [43] exposed fish embryos. Also, the finding that none of the target genes with the embryos were significantly affected by the $MnSO_4$ exposure supports that conclusion. The observed difference between the Mn concentrations of the eggs of the different females is most likely due to the differences in the size of the offspring as the F3 had the largest eggs and larvae, but those nearly always had accumulated the least Mn. This was most likely because the surface area to body mass -ratio was smaller for the eggs and larvae of F3 than with the other females. Thus, those female-related differences also back up the view that Mn had been accumulated into the chorion and/or pervitelline fluid.

Also, the egg and egg-to-larval Mn CBR50 values (95 % confidence limits) of 9.08 (7.13–12.81) µmol/g dry weight and 0.88 (0.56–2.07) µmol/g dry weight, respectively, suggested that the chorion and/or perivitelline fluid had protected the developing embryo from Mn. Also, according to the CBR50 values, the eggs seemed to be far more tolerant to Mn compared to the whole development period from egg to 3-d-old larva. However, we used the total offspring mortality instead of just larval mortality when estimating the egg-to-larval CBR50 value, since the actual larval mortality during the 3-d rearing was rather negligible. The egg-to-larval CBR50 value thus represent the entire exposure period from the fertilization until the larvae were 3-d-old, which may represent the real conditions in the waterbodies affected by the mining effluents better.

With the present experiment it cannot be distinguished if the observed responses were caused by Mn or SO_4 alone or by their interaction. However, if assumed that the mixture toxicity effect of $MnSO_4$ in the exposures would have been solely additive of the 2 substances, and so

back calculate the $MnSO_4$ NOEC and LC50 values to the respective Mn and SO_4 exposure concentrations, we can enable some comparison to the previously reported toxicity values of Mn and SO_4. The female-specific NOEC range for Mn and SO_4 would thus result as 0.04 to 12.5 mg/L and 5.8 to 29.3 mg/L, respectively. The female-specific $MnSO_4$ LC50 values varied from 42.0 to 84.6 mg/L with the 95 % confidence limit variation being from 33.9 to 147.1 mg/L. Those values are within the $MnSO_4$ exposure concentration range of 12.8 to 197.8 mg $MnSO_4$/L, and the mean proportions of Mn and SO_4 in the water samples of those exposure concentrations were 28 % and 72 %, respectively. Those proportions thus result in LC50 values of 11.8 to 23.7 mg Mn/L and 30.2 to 60.9 of mg SO_4/L and the 95 % confidence limit range values of 9.5 to 41.2 mg Mn/L and 24.4 to 105.9 mg SO_4/L. Compared to previously reported Mn [6, 18] and SO_4 [7], toxicity values of the early life stages of other fish species, whitefish embryos and larvae seemed to be slightly more or equally sensitive to Mn than those of other fish species, whereas for SO_4 the whitefish early life stages were far more sensitive. However, the difference in the water hardness between the different experiments makes the comparing of the toxicity values uncertain.

The test chemical was $MnSO_4$ with 98.8 % purity and especially in the highest exposure concentration elevated levels of Cu, Ni and Pb were measured, and Cr concentration was elevated already at 41.8 mg $MnSO_4$/L (Supplemental Data, Table S2). However, compared to previous experiment with rainbow trout embryos and larvae [44], the effect of the Cu impurity in the present experiment was likely negligible, as well as the effect of Ni when estimated according to the Annual Average Environmental Quality Standard (AA-EQS) concentration for Ni (20.0 µg/L) [45]. Also, the egg and larval tissue concentrations of Cu did not increase as the exposure concentration increased and the tissue concentrations of Ni were below the LOQs.

Exposure water Pb concentrations above the AA-EQS (7.2 µg/L) [45] were observed once from the 41.8 mg $MnSO_4$/L, and increasingly from the 197.8 mg $MnSO_4$/L and 965.0 mg $MnSO_4$/L concentrations, but the egg and larval Pb concentrations were below the LOQ. With Cr, a 499.2 µg/L concentration has been reported to increase the larval mortality of common carp [43] and the mean water Cr concentration (±SE) in the highest $MnSO_4$ exposure concentration (422.2 ± 45.0 µg Cr/L) was close to that value. However, according to other studies, only a Cr concentration of several mg/L is toxic to fish embryos and larvae [46, 47]. However, in the present study the egg Cr concentrations were elevated in four of the highest exposure concentrations. Thus, the possible interference of Pb and Cr especially in the highest exposure concentration cannot be completely excluded. Additionally, approximately after 1 mo of incubation, brown precipitates and/or bacterial growth was observed on the walls of some of the pools and compartment grids (visually observed from pools containing 5.9–41.8 mg/L of $MnSO_4$), and that may have affected on the Mn balance of those pools.

Finally, even though the present study does not enable distinguishing the individual causation of Mn or SO_4, the results give relevant insight to the field conditions regarding freshwaters under the impact of metal mining, since the concentrations of both Mn and SO_4 are often elevated in waterbodies receiving mining effluents [5, 48]. In the case of the bioheapleaching mine in North-Eastern Finland, approximately from 2010 onwards the annual mean Mn and SO_4 concentrations had been increased in the waterbodies impacted by the mine, the highest SO_4 concentrations being even several thousand mg/L and the highest Mn concentrations several hundred mg/L [5, 12]. Whitefish has been still caught from some of the less impacted lakes, and in those lakes the highest annual mean values of Mn and SO_4 have been 2022 µg/L and 257 mg/L, respectively [12, 49]. Comparing those concentrations to the findings

of the present study, the observed concentrations of the impacted lakes can have adverse effects on the early-life-stages of whitefish. However, as has been demonstrated in earlier experiments, fish populations can also adapt to chemical stressors [50, 51].

1.4 CONCLUSIONS

Continuous exposure to $MnSO_4$ decreased whitefish embryonic survival in relation to the $MnSO_4$ exposure concentration. The offspring tolerance to $MnSO_4$ exposure depended on the female parent in particular, resulting in substantial differences in offspring instantaneous total mortality already at moderate $MnSO_4$ concentrations. Also, the larval expressions of metal-regulating genes indicated that better offspring $MnSO_4$ tolerance is linked to the induction of metal-regulating genes. The present study has shown that whitefish reproduction success can be impaired in populations under waterborne $MnSO_4$ concentrations of approximately 40 mg/L in boreal soft waters. As a relevant continuum to the present experiment, we will continue with a corresponding field application to assess the implications of mining effluents on whitefish reproductive success in field conditions.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx

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Data availability—Readers can access the data and associated metadata by contacting the corresponding author directly (hanna.e.arola@jyu.fi).

1.5 REFERENCES

1. Howe PD, Malcolm HM, Dobson S. 2004. Manganese and its compounds: Environmental aspects. WHO. Concise International Chemical Assessment Document 63. World Health Organization, Geneva.

2. Meays C, Nordin R. 2013. British Columbia ambient water quality guidelines for sulphate. Technical Appendix. Ministry of Environment, Water Protection & Sustainability Branch, Environmental Sustainability and Strategic Policy Division. Victoria, BC, Canada.

3. Henriksen A, Skjelkvåle BL, Mannio J, Wilander A, Jensen JP, Moiseenko T, Harriman R, Traaen T, Fjeld E, Vuorenmaa J, Kortelainen P, Forsius M. 1997. Results of national lake surveys (1995) in Finland, Norway, Sweden, Denmark, Russian Kola, Russian Karelia, Scotland and Wales. Report sno 3645-97. Norwegian Institute for Water Research.

4. Lydersen E, Löfgren S, Arnesen LT. 2002. Metals in Scandinavian surface waters: Effects of acidification, liming, and potential reacidification. *Environ Sci Technol* 32: 3–2: 73–295.

5. Kauppi S, Mannio J, Hellsten S, Nystén T, Jouttijärvi T, Huttunen M, Ekholm P, Tuominen S, Porvari P, Karjalainen A, Sara-Aho T, Saukkoriipi J Maunula M. 2013. Assessment of the potential impacts on water environment caused by the gypsum pond leakage at the Talvivaara mine. Reports of the Finnish Environment Institute 11/2013. 90 p (In Finnish).

6. Stubblefield WA, Brinkman SF, Davies PH, Garrison TD, Hockett JR, McIntyre MW. 1997. Effects of water hardness on the toxicity of manganese to developing brown trout (*Salmo trutta*). *Environ Toxicol Chem* 16: 2082–2089.

7. Elphick JR, Davies M, Gilron G, Canaria EC, Lo B, Bailey HC. 2011. An aquatic toxicological evaluation of sulfate: the case for considering hardness as a modifying factor in setting water quality guidelines. *Environ Toxicol Chem* 30: 247–253.

8. Bowell RJ. 2000. Sulphate and salt minerals: the problem of treating mine waste. *Min Environ Manage* 5: 11–13.

9. Johnson DB 2013. Development and application of biotechnologies in the metal mining industry. *Environ Sci Pollut Res* 20: 7768–7776.

10. Riekkola-Vanhanen M. 2013. Talvivaara mining company - From a project to a mine. *Miner Eng* 48: 2–9.

11. Anon. 2013. Lausunto vesistön pilaantumisen merkittävyyden arvioimiseksi (in Finnish). Finnish Environment Institute, Helsinki, Finland.

12. Anon. 2016. Terrafame Oy. 2015. Terrafame Mine Monitoring report. Part V surface water quality. Ramboll Oy, Finland. (in Finnish).

13. Salmelin J, Leppänen MT, Karjalainen AK, Vuori K-M, Gerhardt A, Hämäläinen H. 2016. Assessing ecotoxicity of biomining effluents in stream ecosystems by *in situ* invertebrate bioassays: a case study in Talvivaara, Finland. *Environ Toxicol Chem* (in press). DOI: 10.1002/etc.3511.

14. Hutchinson TH, Solbé J, Kloepper-Sams PJ. 1998. Analysis of the ecetoc aquatic toxicity (EAT) database III – comparative toxicity of chemical substances to different life stages of aquatic organisms. *Chemosphere* 36: 129–142.

15. Weis JS, Weis P, Heber M. 1982. Variation in response to methylmercury by killifish (Fundulus heteroclitus) embryos. In: Pearson JG, Foster R and Bishop WE, (eds,). Aquatic toxicology and hazard assessment: Fifth Conference, ASTM STP 766, American society for testing materials, Philadelphia, PA, pp. 109–119.

16. Papakostas S, Vasemägi A, Vähä J-P, Himberg M, Peil L, Primmer GR. 2012. A proteomics approach reveals divergent molecular responses to salinity in populations of European whitefish (*Coregonus lavaretus*). *Mol Ecol* 21: 3516–3530.

17. Jezierska B, Ługowska K, Witeska M. 2009. The effects of heavy metals on embryonic development of fish (a review). *Fish Physiol Biochem* 35: 625–640.

18. Lewis M. 1978. Acute toxicity of copper, zinc and manganese in single and mixed salt solutions to juvenile longfin dace *Agosia chrysogaster*. *J Fish Biol* 13: 695–700.

19. Reader JP, Everall NC, Sayer MDJ, Morris R. 1989. The effects of eight trace metals in acid soft water on survival, mineral uptake and skeletal calcium deposition in yolk-sac fry of brown trout, *Salmo trutta* L. *J Fish Biol* 35: 187–198.

20. Ketola HG, Longacre D, Greulich A, Phetterplace L, Lashomb R. 1988. High calcium concentration in water increases mortality of salmon and trout eggs. *Prog Fish Cult* 50: 129–135.

21. van Dam RA, Hogan AC, McCullough CD, Houston MA, Humphrey CL, Harford AJ. 2010. Aquatic toxicity of magnesium sulfate, and the influence of calcium, in very low ionic concentration water. *Environ Toxicol Chem* 29: 410–421.

22. Lushchak VI. 2011. Environmentally induced oxidative stress in aquatic animals. *Aquat Toxicol* 101: 13–30.

23. Choi CY, An KW, An MI. 2008. Molecular characterization and mRNA expression of glutathione peroxidase and glutathione S-transferase during osmotic stress in olive flounder (*Paralichthys olivaceus*). *Comp Biochem Phys A* 149: 330–337.

24. Woo S, Yum S, Park H-S, Lee T-K, Ryu J-C. 2009. Effects of heavy metals on antioxidants and stress-responsive gene expression in Javanese medaka (*Oryzias javanicus*). *Comp Biochem Phys C* 149: 289–299.

25. Di Giulio RT, Meyer JN. 2008. Reactive oxygen species and oxidative stress. In Di Giulio RT, Hinton DE, eds, The toxicology of fishes. CRC Press, Taylor & Francis Group, Boca Raton, FL, USA, pp. 273–324.

26. Sevcikova M, Modra H, Slaninova A, Svobodova Z. 2011. Metals as a cause of oxidative stress in fish: a review. *Veterinarni Medicina* 11: 537–546.

27. Coyle P, Philcox JC, Carey LC, Rofe AM. 2002. Metallothionein: The multipurpose protein (Review). *Cell Mol Life Sci* 59: 627–647.

28. Roesijadi G. 1992. Metallothioneins in metal regulation and toxicity in aquatic animals - Review. *Aquat Toxicol* 22: 81–114.

29. George SG, Hodgson PA, Tytler P, Todd K. 1996. Inducibility of Metallothionein mRNA Expression and Cadmium Tolerance in Larvae of a Marine Teleost, the Turbot (*Scophthalmus maximus*). *Fund Appl Toxicol* 33: 91–99.

30. Finnish Standard Association. 1976. Determination of ammonia-nitrogen of water. SFS 3032:1976. (In Finnish).

31. United States Environmental Protection Agency. 2001. US-EPA METHOD 200.7, Revision 4.4, Cincinnati.

32. Hansen BH, Rømma S, Garmo ØA, Pedersen SA, Olsvik PA, Andersen RA. 2007. Induction and activity of oxidative stress-related proteins during waterborne Cd/Zn-exposure in brown trout (*Salmo trutta*). *Chemosphere* 67: 2241–2249.

33. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. 2012. Primer3—new capabilities and interfaces. *Nucleic Acids Res* 40:15: e115 1–12.

34. Koressaar T, Remm M. 2007. Enhancements and modifications of primer design program Primer3. *Bioinformatics* 23: 1289–1291.

35. AmplifX 1.5.4. Jullien N. CNRS, Aix-Marseille Université. http://crn2m.univ-mrs.fr/pub/amplifx-dist

36. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden T. 2012. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 13:134: 1–11.

37. Vehniäinen E-R, Kukkonen JVK. 2015. Multixenobiotic resistance efflux activity in *Daphnia magna* and *Lumbriculus variegatus*. *Chemosphere* 124: 143–149.

38. Peake EB, Locke JC, Tierney LL, Kolok AS. 2004. Copper tolerance in fathead minnows: II. Maternal transfer. *Environ Toxicol Chem* 23: 208–211.

39. Kim J-H, Wang S-Y, Kim I-C, Ki J-S, Raisuddin S, Lee J-S, Han K-N. 2008. Cloning of a river puff erfish (*Takifugu obscurus*) metallothionein cDNA and study of its induction profile in cadmium-exposed fish. *Chemosphere* 71: 1251–1259.

40. Cheung APL, Lam TH-J, Chan KM. 2004. Regulation of *Tilapia* metallothionein gene expression by heavy metal ions. *Mar Environ Res* 58: 389–394.

41. Stouthart XJHX, Haans JLM, Lock RAC, Wendelaar Bonga SE. 1996. Effects of water pH on copper toxicity to early life stages of the common carp (*Cyprinus carpio*). *Environ Toxicol Chem* 15: 376–383.

42. Michibata H. 1981. Uptake and distribution of cadmium in the egg of the teleost, *Oryzias latipes*. *J Fish Biol* 19: 691–696.

43. Stouthart AJHX, Spanings FAT, Lock RAC, Wendelaar Bonga SE. 1995. Effects of water pH on chromium toxicity to early life stages of the common carp (*Cyprinus carpio*). *Aquat Toxicol* 32: 31–42.

44. Stasiūnaitė P. 2005. Toxicity of copper to embryonic development of rainbow trout (Oncorhynchus Mykiss). *Acta Zoologica Lituanica* 15:3: 259–265

45. Directive 2008/105/EC of the European Parliament and of the Council of 16 December 2008 on environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 2000/60/EC of the European Parliament and of the Council. Official Journal of the European Union. 51.

46. Nguyien LTH, Jansen CR. 2001. Comparative sensitivity of embryo–larval toxicity assays with African catfish (*Clarias gariepinus*) and zebra fish (*Danio rerio*). *Environ Toxicol* 16: 566–571.

47. Domingues I, Oliveira R, Lourenço J, Grisolia CK, Mendo S, Soares AMVM. 2010. Biomarkers as a tool to assess effects of chromium (VI): Comparison of responses in zebrafish early life stages and adults. *Comp Biochem Phys C* 152: 338–345.

48. Olías M, Nieto JM, Sarmiento AM, Cerón JC and Cánovas CR. 2004. Seasonal water quality variations in a river affected by acid mine drainage: the Odiel River (South West Spain). *Sci Total Environ* 333: 267–281.

49. Anon. 2016. Terrafame Oy. 2015. Terrafame Mine Monitoring report. Part VII fisheries monitoring. Ramboll Oy, Finland. (in Finnish).

50. Weis JS, Weis P, Heber M, Vaidya S. 1981. Methylmercury tolerance of killifish (*Fundulus heteroclitus*) embryos from a polluted vs non-polluted environment. *Mar biol* 65: 283–287.

51. Angus RA. 1983. Phenol tolerance in populations of mosquitofish from polluted and nonpolluted waters. *T Am Fish Soc* 112: 794–799.

Figure 1. A schematic illustration of the experiment setup with a timeline and procedures. The exposures were started at fertilization and continued until all larvae were hatched. Fertilization success was estimated 4 to 5 days after the fertilization from 10 eggs/replicate from each exposure concentration and the control. In spring period, water temperature was elevated gradually and hatching started at experiment day 119. On average, 50 % of all the embryos had hatched on experiment day 136 and larval samples were collected after 3-d incubation of the larvae. In the highest exposure concentration, none of the embryos hatched. See Supplemental Data Table S4 for more detailed information about the number of samples of each sample type in each concentration and parent pair.

Figure 2. Mean instantaneous fertilization loss (Z_F ± SE, *n*=6 per female and concentration), (A) and mean instantaneous total mortality (Z_T ± SE, *n*=6 per female and concentration) (B) for each female (F1–F4) in L. Konnevesi control water and the different $MnSO_4$ exposure concentrations (note: y-axes are LOG10 scale). The Z values of 1.0 and 4.6 are approximately equal to 63.2 % and 100 % mortality, respectively.

Figure 3. Mean larval carcass and yolk dry weights (mg) (± SE, *n*=6 per female and concentration, except with F3 in 41.8 mg $MnSO_4$/L *n*=2) of each female (F1–F4) in L. Konnevesi control water and the three different $MnSO_4$ exposure concentrations.

Figure 4. Mean embryonic (*n*=6–9) and larval (*n*=3–6) *cat*, *gstt*, *mt-a* and *mt-b* normalized expressions ± SE under different $MnSO_4$ exposure concentrations after subtracting the mean expressions of the control samples. The number of valid observations is given in more detail in Supplemental Data Table S9.

Table 1. The nominal and the mean measured $MnSO_4$, Mn and SO_4 concentrations (mg/L, ±SE and n > LOQ and RSD < 10 %/n total) of the whole experiment period. The mean proportion (% ±SE and n) of the measured $MnSO_4$, Mn and SO_4 exposure concentrations compared to the nominal concentrations including the background levels of the corresponding time are presented in parentheses.

Nominal concentration added to control			Measured concentrations		
$MnSO_4$	Mn	SO_4	$MnSO_4$	Mn	SO_4[a]
Control	Control	Control	5.5 ± 0.1	1.5[b] ± 0.3[b]	5.5 ± 0.1
			47/55	47/55	55/55
5.7[b]	2.1[b]	3.6[b]	5.6 ± 0.1	1.0[b] ± 0.2[b]	5.6 ± 0.1
			46/46	46/46	46/46
			(102.2 ± 0.9)	(27.9 ± 4.1)	(102.1 ± 0.8)
			(41)	(41)	(46)
28.6[b]	10.4[b]	18.2[b]	5.9 ± 0.1	4.5[b] ± 0.6[b]	5.8 ± 0.1
			50/50	50/50	50/50
			(102.0 ± 0.8)	(35.1 ± 4.9)	(102.9 ± 0.8)
			(42)	(42)	(47)
7.2	2.6	4.6	12.8 ± 0.1	2.1 ± 0.1	10.7 ± 0.1
			44/44	44/44	44/44
			(102.5 ± 1.7)	(81.6 ± 5.0)	(107.4 ± 1.5)
			(36)	(36)	(41)
35.7	13.0	22.7	41.8 ± 0.6	12.5 ± 0.3	29.3 ± 0.5

			48/48	48/48	48/48
			(100.4 ± 1.8)	(96.2 ± 2.9)	(102.7 ± 2.0)
			(38)	(38)	(43)
178.7	65.0	113.7	197.8 ± 1.4	70.2 ± 0.4	127.6 ± 1.2
			56/56	56/56	56/56
			(106.2 ± 0.7)	(107.6 ± 0.6)	(105.9 ± 0.9)
			(44)	(44)	(51)
893.4	325.0	568.4	965.0 ± 5.9	341.6 ± 1.6	623.4 ± 5.5
			49/49	49/49	49/49
			(107.7 ± 0.7)	(105.1 ± 0.5)	(108.6 ± 1.0)
			(42)	(42)	(49)

[a] Calculated from S

[b] $\times 10^{-2}$

Table 2. The GLM-analysis results of the $MnSO_4$ exposure concentration, female and male significance on fertilization loss (Z_F), and total mortality (Z_T).

Z	*df*	*F*	*p*
Z_F, Concn.	6	4.478	< 0.001
Z_F, F	3	206.356	< 0.001
Z_F, M	1	0.117	0.733
Z_F, F*Concn.	18	1.329	0.183
Z_F, M*Concn.	6	0.274	0.948
Z_F, F*M	3	2.253	0.086
Z_F, F*M*Concn.	18	0.998	0.467
Z_T, Concn.	6	358.180	< 0.001
Z_T, F	3	9.182	< 0.001
Z_T, M	1	1.341	0.249
Z_T, F*Concn.	18	5.419	< 0.001
Z_T, M*Concn.	6	2.375	0.034
Z_T, F*M	3	1.183	0.319
Z_T, F*M*Concn.	18	1.665	0.056

Concn.: $MnSO_4$ exposure concentration

F: Female

M: Male

Table 3. The female-specific NOEC and LC50 values of $MnSO_4$ (mg/L) according to total mortality of the offspring for each female, the natural response rate according to the control mean total mortality of the female's offspring.

Female	NOEC	$LC50_{MnSO4}$	95 % confidence limits	Natural response rate (%)
1	12.8	45.6	39.1 - 53.0	28.1
2	5.9	42.0	33.9 - 50.9	27.6
3	41.8	76.3	36.1 - 147.1	57.8
4	12.8	84.6	71.0 - 97.9	21.2

Table 4. The GLM-analysis results of the $MnSO_4$ exposure concentration, female and male significance on the growth and yolk consumption analysis of the 3-d-old larvae.

	df	*F*	*p*
DW C, Degree days	1	1.074	0.304
DW C, Concn.	3	8.740	< 0.001
DW C, F	3	27.761	< 0.001
DW C, M	1	0.174	0.678
DW C, Concn.*F	9	1.894	0.070
DW C, Concn.*M	3	3.037	0.036
DW C, F*M	3	1.537	0.214
DW C, Concn.*F*M	8	1.808	0.093
DW Y, Degree Days	1	38.902	< 0.001
DW Y, Concn.	3	17.611	< 0.001
DW Y, F	3	19.163	< 0.001
DW Y, M	1	2.315	0.133
DW Y, Concn.*F	9	3.691	0.001
DW Y, Concn.*M	3	1.732	0.170
DW Y, F*M	3	1.193	0.320
DW Y, Concn.*F*M	8	3.290	0.004

DW C: Carcass dry weight

Concn.: $MnSO_4$ exposure concentration

F: Female

M: Male

DW_Y: Yolk dry weight

Table 5. Mean Mn tissue concentrations ± SE of the eggs and the 3-d-old larvae of each female in different $MnSO_4$ exposure concentrations and the control. All embryo samples were above the LOQ with RSD < 10 %. The larval samples of each female in each concentration are presented as upper bound values and if none of the replicates exceeded the LOQ and/or had RSD above 10 %, they are presented as < LOQ.

Eggs	Mn mg/kg dry weight			
$MnSO_4$ (mg/L)	Female 1 (n=1–2)	Female 2 (n=1–2)	Female 3 (n=1–2)	Female 4 (n=2)
Control (5.5)	10.7 ± 1.0	11.1 ± 1.7	9.5 ± 2.3	18.1 ± 1.8
5.6	17.4	10.8 ± 0.05	12.8 ± 1.8	19.0 ± 6.1
5.9	36.8 ± 3.2	36.0	33.4 ± 7.0	53.2 ± 3.5
12.8	131.5 ± 19.0	112.5 ± 8.5	74.6 ± 5.7	137.0 ± 7.4
41.8	210.5 ± 4.3	223.0 ± 41.2	201.8	299.1 ± 18.9
197.8	474.2 ± 57.0	394.1 ± 1.1	354.8	397.5 ± 4.3
965.0	-	-	-	661.9 ± 67.3
Larvae	Mn mg/kg dry weight			
$MnSO_4$ (mg/L)	Female 1 (n=5–6)	Female 2 (n=5–6)	Female 3 (n=1–2)	Female 4 (n=3–6)
Control (5.5)	0.3 ± 0.1	0.5 ± 0.3	< LOQ	< LOQ
5.6	< LOQ	0.5 ± 0.3	< LOQ	< LOQ
5.9	< LOQ	0.6 ± 0.3	1.0 ± 0.7	< LOQ
12.8	3.3 ± 0.8	2.4 ± 0.3	1.6 ± 0.7	3.0 ± 0.6

41.8	23.2 ± 3.7	21.2 ± 2.7	18.7	33.9 ± 5.1
197.8	-	-	-	46.7 ± 11.3
965.0	-	-	-	-

- No samples due to mortality

LOQ: Limit of quantification

Table 6. The GLM-analysis results of the $MnSO_4$ exposure concentration and parent pair significance on the gene expressions of the embryos and the 3-d-old larvae.

	Embryos			Larvae		
Gene	*df*	*F*	*p*	*df*	*F*	*p*
cat, Concn.	5	1.553	0.206	4	1.214	0.336
cat, PP	2	8.987	0.001	1	0.277	0.605
cat, Concn. * PP	10	1.174	0.348	4	0.996	0.435
gstt, Concn.	5	0.583	0.712	4	0.480	0.751
gstt, PP	2	2.655	0.085	1	0.005	0.946
gstt, Concn. * PP	10	0.375	0.949	4	0.462	0.763
mt-a, Concn.	5	0.388	0.853	4	3.794	0.021
mt-a, PP	2	3.867	0.031	1	1.245	0.279
mt-a, Concn. * PP	10	0.343	0.962	4	1.413	0.270
mt-b, Concn.	5	0.620	0.685	4	3.805	0.021
mt-b, PP	2	3.582	0.040	1	5.559	0.030
mt-b, Concn. * PP	10	0.339	0.963	4	3.028	0.045

cat: Catalase

Concn: $MnSO_4$ exposure concentration

PP: Parent pair

gstt: Glutathione S-transferase T

mt-a: Metallothionein-A

mt-b: Metallothionein-B

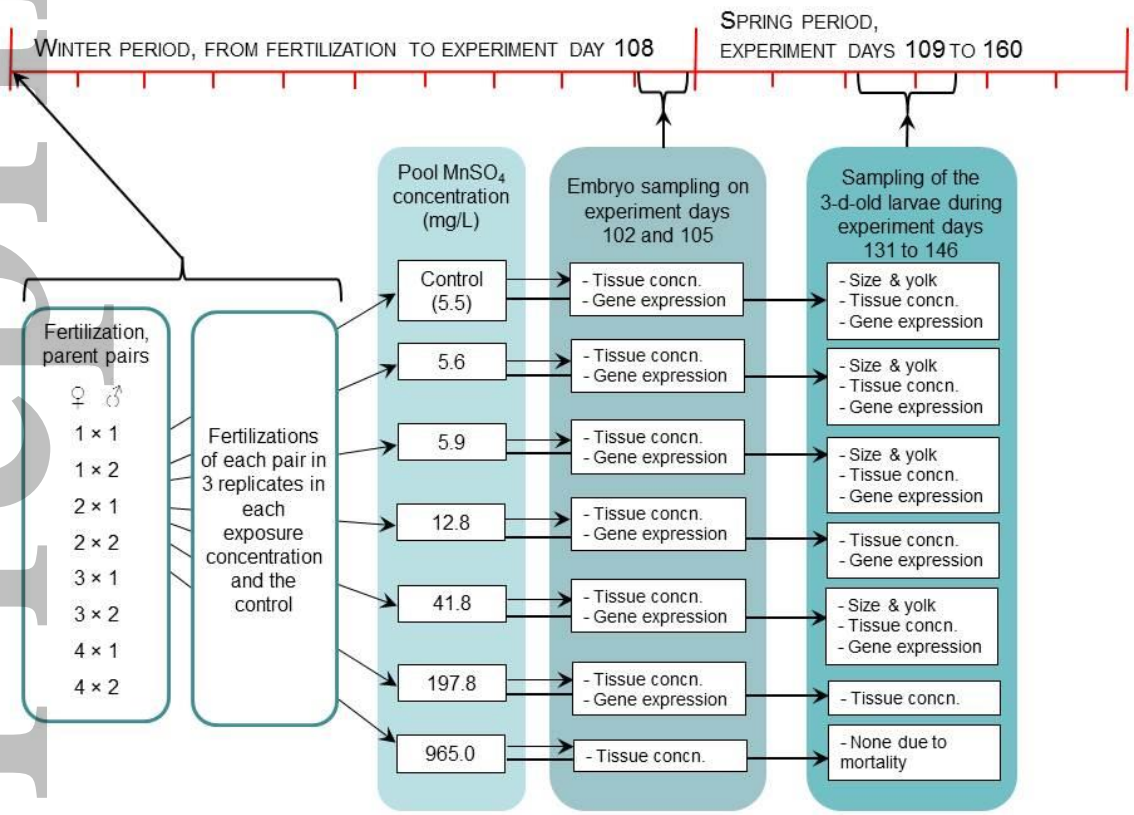

Figure 1.

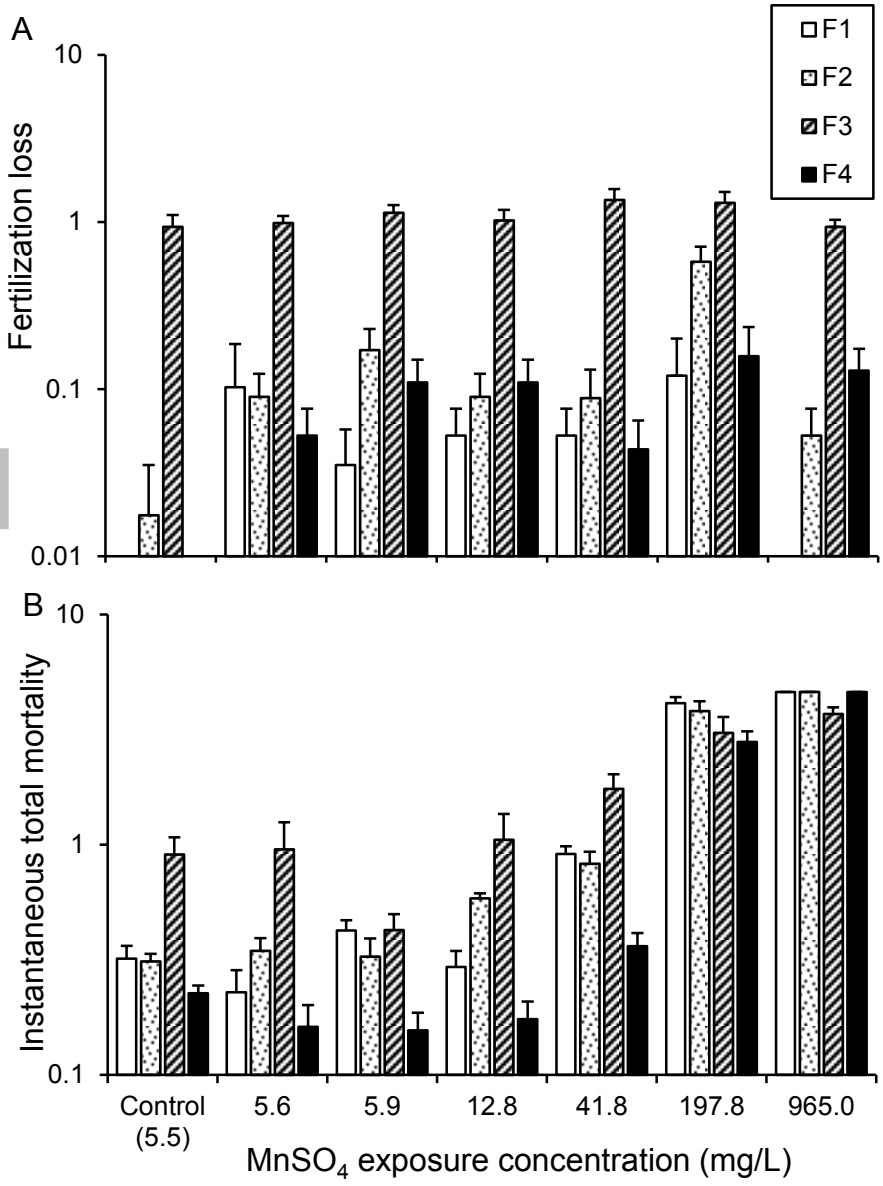

Figure 2.

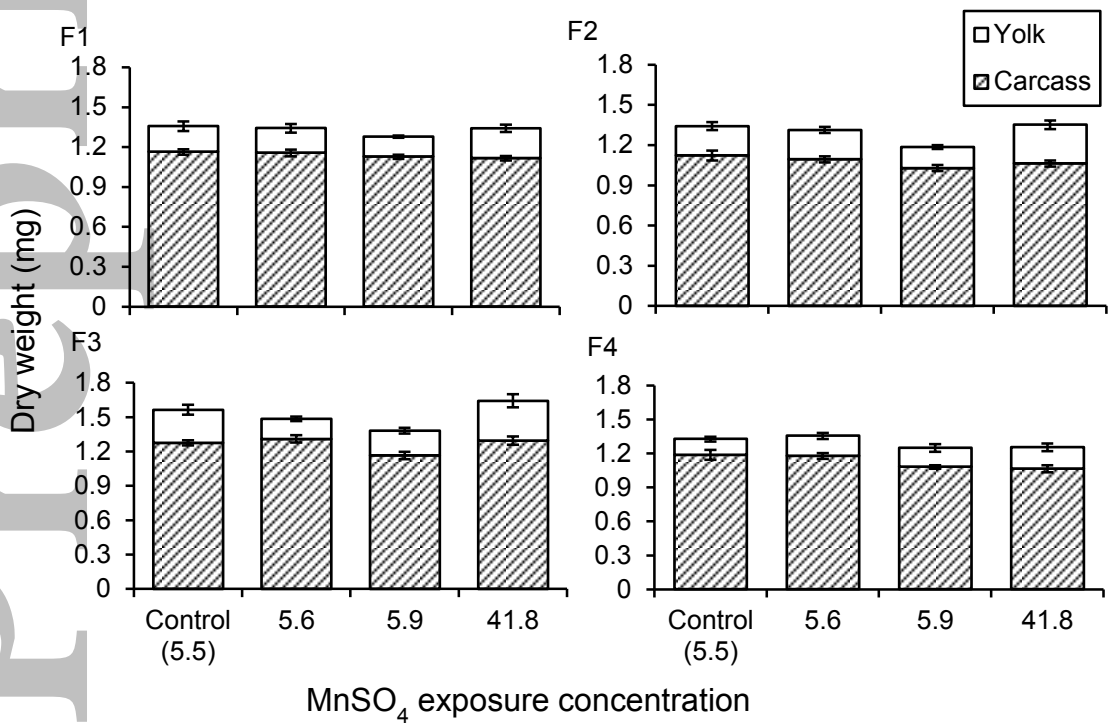

Figure 3.

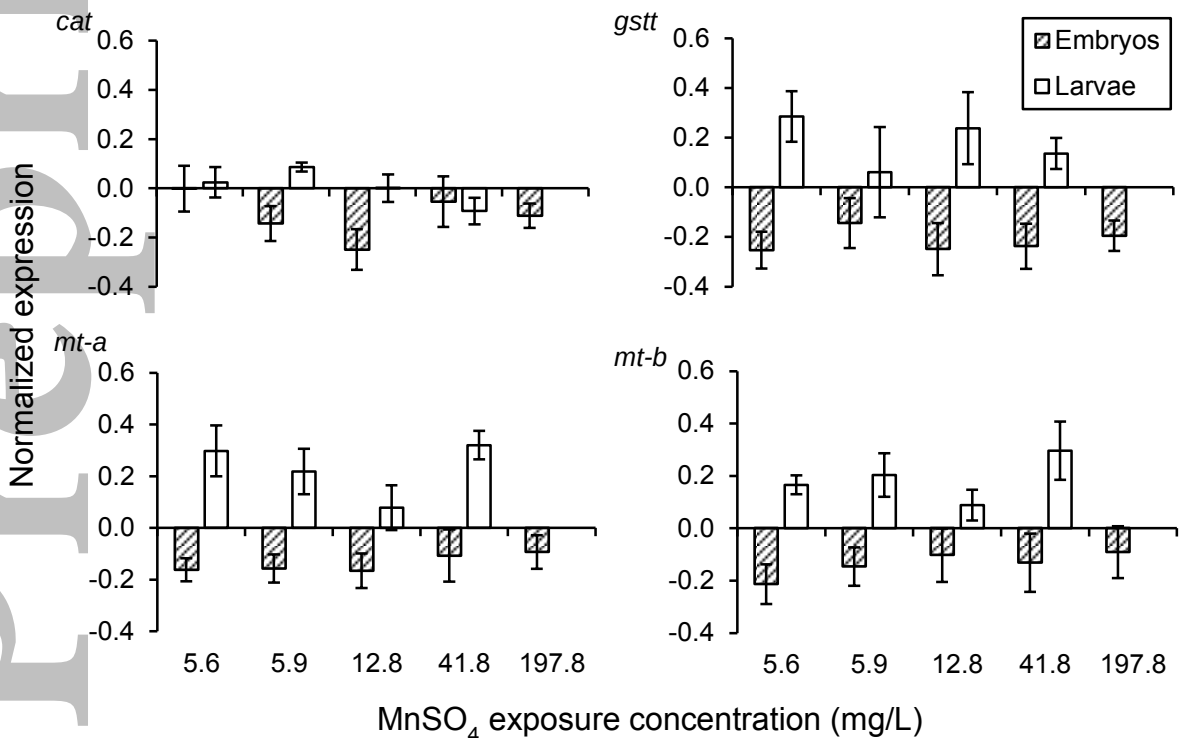

Figure 4.