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Waterborne cadmium and nickel impact oxidative stress responses and retinoid metabolism in yellow perch

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ABSTRACT

In this experiment, we studied the transcriptional and functional (enzymatic) responses of yellow perch (*Perca flavescens*) to metal stress, with a focus on oxidative stress and vitamin A metabolism. Juvenile yellow perch were exposed to two environmentally relevant concentrations of waterborne cadmium (Cd) and nickel (Ni) for a period of 6 weeks. Kidney Cd and Ni bioaccumulation significantly increased with increasing metal exposure. The major retinoid metabolites analyzed in liver and muscle decreased with metal exposure except at high Cd exposure where no variation was reported in liver. A decrease in free plasma dehydroretinol was also observed with metal exposure. In the liver of Cd-exposed fish, both epidermal retinol dehydrogenase 2 transcription level and corresponding enzyme activities retinyl ester hydrolase and lecithin dehydroretinyl acyl transferase increased. In contrast, muscle epidermal retinol dehydrogenase 2 transcription level decreased with Cd exposure. Among antioxidant defences, liver transcription levels of catalase, microsomal glutathione-S-transferase-3 and glucose-6-phosphate dehydrogenase were generally enhanced in Cd-exposed fish and this up-regulation was accompanied by an increase in the activities of corresponding enzymes, except for microsomal glutathione-S-transferase. No consistent pattern in antioxidant defence responses was observed between molecular and biochemical response when fish were exposed to Ni, suggesting a non-synchronous response of antioxidant defence in fish exposed to waterborne Ni. There was a general lack of consistency between muscle transcription level and enzyme activities analyzed. The overall findings from this investigation highlight the usefulness of transcriptional and biochemical endpoints in the identification of oxidative stress and vitamin A metabolism impairment biomarkers and the potential use of multi-level biological approaches when assessing environmental risk in fish.

Abbreviations: ANOVA, Analysis of variance; BHT, Butylated hydroxytoluene; BSA, Bovine serum albumin; CAT, Catalase; CCO, Cytochrome c oxidase; cDNA, Complementary DNA; CDNB, Chloro-2,4-dinitrobenzene; DMSO, Dimethyl sulfoxide; DPPC, Dipalmitoylphosphatidylcholine; dREH, Dehydroretinyl ester hydrolase; DROH, Dehydroretinol; G6PDH, Glucose-6-phosphate dehydrogenase; GPx, Glutathione peroxidase; GST, Glutathione-S-transferase; HCd, High Cd concentration corresponding to waterborne exposure of fish to 8 μg/L (71 nmol/L) of Cd; HNi, High Ni concentration corresponding to waterborne exposure of fish to 600 μg/L (10,200 nmol/L) of Ni; HPLC, High-performance liquid chromatography; Kn, Relative condition index; LARSA, Laboratoire Régional des Sciences Aquatiques; LCd, Low Cd concentration corresponding to waterborne exposure of fish to 0.8 μg/L (7.0 nmol/L) of Cd; LdRAT, Lecithin dehydroretinyl acyl transferase; LNi, Low Ni concentration corresponding to waterborne exposure of fish to 60 μg/L (1020 nmol/L) of Ni; LRAT, Lecithin retinyl acyl transferase; LSI, Liver somatic index; MGST, Microsomal glutathione-S-transferase; mgst-3, Microsomal glutathione-S-transferase-3; MTs, Metallothioneins; NADH, Nicotinamide adenine dinucleotide; PBS, Phosphate saline buffer; RBP, Retinol binding protein; REH, Retinyl ester hydrolase; Rdh-2, Epidermal retinol dehydrogenase 2; ROH, Retinol; ROS, Reactive oxygen species; RT-PCR, Reverse transcription polymerase chain reaction; RQ, Relative quantification; SE, Standard error; SOD, Superoxide dismutase; TCEP, Tris 2-carboxyethyl phosphine; TTR, Transthyretin.

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1. Introduction

Metal pollution is a major environmental hazard affecting the health of freshwater biota. The yellow perch (*Perca flavescens*) is a good candidate as a biomonitor of aquatic metal pollution for several reasons (Giguère et al., 2004). It is widely distributed across North American freshwater ecosystems and is particularly relevant in metal toxicology studies because it tolerates the presence of metals such as cadmium (Cd) and nickel (Ni) at high concentrations tolerated by few other fish (Hontela et al., 1995, 1992; Rajotte and Couture, 2003). The bioaccumulation of metals in its tissues reflects local contamination and corroborates its sedentary behavior (Campbell et al., 2003). Additionally, metal concentrations in yellow perch tissues have been correlated with biological impacts such as metabolic imbalance of vitamin A_2 (Defo et al., 2012), impairment of metabolic capacities (Couture and Kumar, 2003), poor condition and overall health (Couture et al., 2008b; Rajotte and Couture, 2002), elevated metallothionein concentrations (Giguère et al., 2005), and reduction in genetic diversity at both neutral and coding regions of the genome (Bourret et al., 2008; Bélanger-Deschênes et al., 2013).

An emerging priority in biomonitoring is to identify relevant biological impacts in sentinel species providing informative elements about the adverse outcomes induced by environmental stressors (Chandurvelan et al., 2013). Effects of contaminants can be assessed at different levels of biological organization, from molecular to community levels (Ankley et al., 2010). To this end, biomarkers describe biochemical, cellular, physiological or behavioral changes that can be measured in cells, tissues or whole organisms as a result of bioaccumulation or effects of toxicant exposure (Depledge et al., 1995; Tsangaris et al., 2010). Biomarkers can be used as premonitory warning signals of environmental disturbance, by providing the causal link between the presence of toxicants and an ecological effect (Walker et al., 2006).

Fishes are considered to be sensitive to metal-induced oxidative stress (Kubrak et al., 2012; Souid et al., 2013), resulting from an imbalance between the amount of cellular antioxidants and that of ROS (reactive oxygen species) (Defo et al., 2014; Vertuani et al., 2004). Yet, studies have reported that yellow perch living in metal impacted areas possess efficient biochemical defence mechanisms (Giguère et al., 2005). Depletion of cellular antioxidants, or an overproduction of ROS, can disturb this equilibrium leading to oxidative stress (Scandalios, 2005), which can lead to an impairment of cellular repair mechanisms (Dorval and Hontela, 2003). Both non-enzymatic antioxidant biomarkers such as vitamin A metabolites and enzymatic antioxidant biomarkers including SOD (superoxide dismutase), CAT (catalase), GST (glutathione-S-transferase) and G6PDH (glucose-6-phosphate dehydrogenase) interact together to minimize the damaging effects of ROS in fish (Scandalios, 2005).

As argued by Ankley et al. (2010), linking the responses to chemical interactions at different biological organization levels provides a critical basis for predictive approaches in environmental risk assessment. Cadmium and Ni are both non-essential metals for fish. These metals are known to be toxic at low levels and can cause oxidative stress in aquatic organisms (Kubrak et al., 2013; Vertuani et al., 2004). They can be found in sediments, the water column, and biota at elevated levels as a consequence of anthropogenic activities (Luoma and Rainbow, 2008), such as mining and smelting. In a previous study on yellow perch chronically exposed to metals in the field, we reported a negative correlation between hepatic Cd concentrations and liver transcription levels of genes encoding for enzymes involved in the metabolism of retinoids (Pierron et al., 2011). Subsequent research showed that the percentage of hepatic free dehydroretinol decreased, suggesting that Cd inhibits the enzymes and the binding proteins involved in retinoid homeostasis (Defo et al., 2012). Yellow perch from Cd-contaminated lakes had significantly higher concentrations of liver dehydroretinol and dehydroretinyl esters than did fish from reference lakes. To our knowledge, the only other study reporting simultaneous molecular and biochemical responses to metal exposure on antioxidant enzymes in fish is that of Henrik Hansen et al. (2007). Trout (*Salmo trutta*) from an environment with low metal levels were transferred to a polluted river with higher Cd and Zn concentrations for 15 days. Although a significant correlation between Cd accumulation and the transcription level of some antioxidant genes was reported, the changes of enzymes activities and gene transcription levels were inconsistent (Henrik Hansen et al., 2007), suggesting that changes in gene transcription levels do not necessarily lead to modifications in the activity of corresponding enzymes (Giuliani et al., 2013; Regoli et al., 2011).

In our investigation of the mechanisms of toxicity of Cd and Ni in yellow perch, we have identified transcriptional signatures specific to Cd and Ni exposure. The results indicate that Cd and Ni affect the transcription level of genes involved in several metabolic pathways including oxidative stress and vitamin metabolism (Bougas et al., 2013). The general objective of the present study was to investigate at two biological organization levels (molecular and biochemical) the effects of waterborne Cd and Ni exposure on oxidative stress response biomarkers in yellow perch. Renal metal concentrations were used as indicators of metal accumulation. Our specific objectives were: (i) to examine changes in liver and muscle transcription levels of a set of toxicologically-relevant genes involved in oxidative stress response in yellow perch exposed to Cd and Ni; and (ii) to compare gene expression levels to corresponding biochemical endpoints (enzyme activities and retinoid storage) in order to determine if metal-induced changes in gene transcription levels lead to significant physiological effects. This study will contribute to a better understanding of the role of perturbations in retinoid metabolism and oxidative stress in chronic metal toxicity.

2. Materials and methods

2.1. Fish and maintenance conditions

Juvenile yellow perch were obtained from a private fish supplier (Trois-Rivières, Québec, Canada) between September and October 2011, and transferred to the LARSA (Laboratoire Régional des Sciences Aquatiques) at Université Laval (Québec). Fish were acclimated in a flow-through holding tank (1 m^3) for 4 weeks with aerated, dechlorinated water kept at 18 °C and photoperiod set at 12-h light:12-h dark. One week prior to the beginning of exposures, fish were randomly selected and transferred to aquaria (40 L), where acclimation continued. Water of the same chemical composition was used for both the acclimation phase and the experimental treatments: pH 7.2–7.3; Ca 0.35 mmol/L; Mg 0.073 mmol/L; Na 0.70 mmol/L; K 0.036 mmol/L; Cl 0.48 mmol/L; SO_4 0.22 mmol/L; alkalinity 0.22 mmol/L. Temperature was adjusted to 20 °C and oxygen maintained at 100% saturation.

2.2. Experimental treatments

Each exposure group (aquarium) consisted of 15 fish with an average total biomass of 250 g per aquarium. Perch were exposed to low and high Cd and Ni nominal water concentrations as follows: control (0 μg/L; 0 nmol/L), low Cd concentration (LCd, 0.8 μg/L; 7 nmol/L), high Cd concentration (HCd 8 μg/L; 71 nmol/L), low Ni concentration (LNi, 60 μg/L; 1020 nmol/L), and high Ni concentration (HNi, 600 μg/L; 10,200 nmol/L). Each condition was

replicated twice. Inorganic speciation calculations carried out with the MINEQL chemical equilibrium model (Schecher and McAvoy, 1992) showed that both metals were present almost entirely as the free metal ions (>93% Cd^{2+}, >96% Ni^{2+}). Low Cd and Ni concentrations represent environmental concentrations respectively found in some lakes in the mining regions of Rouyn-Noranda (Québec, Canada) and Sudbury (Ontario, Canada) (Couture et al., 2008a). The higher concentrations of Cd and Ni were chosen in order to obtain tissue metal concentrations commonly measured in tissues of yellow perch living in contaminated areas. At the beginning of exposures, fish were fed frozen *Artemia salina* daily with a ratio of 2% of their body weight, increasing to 3% at the end of exposure. Every week, water metal concentrations were monitored and adjusted as necessary and the exposures were conducted with continuous renewal mode.

2.3. Sampling

After 6 weeks of exposure, fish in each experimental aquarium were sacrificed and dissected. A total of 30 adult fish were sampled per condition. Length (12.4 ± 1.3 cm) and weight (14.3 ± 4.2 g) were recorded (mean ± SE, standard error) and sex determined. Following the method described in Defo et al. (2012), blood was collected from the dorsal artery of the caudal peduncle using a heparinized needle. After centrifugation, the plasma was immediately frozen on dry ice and stored at −80 °C in heparinized tubes until analysis. Livers were dissected and weighed (0.07 ± 0.02 g) for molecular and biochemical analyses. Muscle (molecular and biochemical analyses) as well as kidney (metal analyses) and pyloric cæca (biometric analyses) were also dissected. Tissues were immediately frozen and stored in liquid nitrogen. Tissues were then stored at −80 °C until used for analyses. The fish manipulation protocol was approved by Université Laval's Animal Care Committee.

2.4. Calculation of fish condition index, liver somatic index and corrected pyloric cæca weights

Kn (relative condition index) was calculated according to the method cited in Defo et al. (2012) using the equation $Kn = (Wf/L^{3.13}) \times 100$ (where "Wf" is fish weight, "L" is the total fish length). LSI (liver somatic index) was calculated as (Liver weight/Wf) × 100 (Miller et al., 2009). The corrected pyloric cæca weights were calculated according to the method described in Defo et al. (2012) with 14.3 g as the standard fish weight and 3.0 as the allometric exponent.

2.5. Measurement of metal concentrations in aquarium water and yellow perch kidney

Since the yellow perch used in this study were juveniles, the mass of liver tissue available for analyses was limited. Certain analyses (retinoids, enzymes and qRT-PCR) were prioritized for the liver, in addition to its use in a parallel study conducted with a cDNA (complementary DNA) microarray (see Bougas et al., 2013); metal analyses were therefore only performed on kidney samples. After acidification of unfiltered aquarium water samples (5% ultrapure HNO_3), Cd and Ni analyses were performed on water samples and kidney tissues according to Defo et al. (2012), using an inductively coupled-plasma atomic emission spectrophotometry (ICP-AES). Internal standards were within 10% of nominal values in all cases and mean metal recoveries for the reference material analyzed (TORT-2, lobster hepatopancreas, National Research Council of Canada, Ottawa, ON) were 88 ± 2% for Cd and 75 ± 5% for Ni (mean ± SE).

2.6. RNA isolation and cDNA synthesis

The analysis of gene transcription levels was performed in accordance with methods and technical standardization procedures listed in Bustin et al. (2009). According to the manufacturer's instructions, total RNA from a single perch was extracted from portions of liver and muscle tissue using PureLink™ Micro-to-Midi™ Total RNA Purification System Mini Kits. Excess collagen fibres and lipids, particularly in muscle tissue, were removed with Trizol reagent (Invitrogen, Carlsbad, CA) prior to RNA extraction. To determine the RNA concentration and to ensure the integrity and the quality of RNA, samples were analyzed by electrophoresis on a 1% agarose gel and with a NanoDrop spectrophotometer 2000 (Thermo Scientific, Wilmington, DE); the ratio (260 nm/280 nm and 260 nm/230 nm) of all homogenates yielded values above 1.8. In order to remove residual genomic DNA, purified RNA was subjected to DNAse I (Invitrogen, Carlsbad, CA) treatment according to the manufacturer's instructions. Briefly, a sample of 1 μg RNA was treated with DNAse I (1 U/μL) at room temperature. After 30 min, the reaction was stopped by adding 1 μL of EDTA solution (25 mM) and the samples were then heated for 10 min at 65 °C and stored at −80 °C until reverse transcription analysis. First-strand cDNA was then synthesized from treated total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Briefly, reverse transcription reactions were performed in a total reaction volume of 50 μL containing equal volumes of RT master mix and treated RNA sample. The master mix included 5 μL random primers, 2 μL dNTP, 5 μL RT buffer, 2.5 μL reverse transcriptase and 10.5 μL of nuclease free water. Depending on the RNA concentration, a specific volume of treated RNA (0.5 μg/μL) was diluted with nuclease free water up to final volume of 25 μL. After a brief centrifugation of the plate to spin down the contents and to eliminate air bubbles, amplification reactions were carried out using the following temperature and time profiles: 10 min at 25 °C, 120 min at 37 °C, 5 min at 85 °C, and at 4 °C for an undefined duration. Synthetized cDNA was then stored at −20 °C until RT-qPCR analysis.

2.7. Quantitative RT-PCR (reverse transcription polymerase chain reaction)

Based on published yellow perch cDNA sequences (Bougas et al., 2013), partial coding sequences (primers) used in this study were designed with Primer blast (http://www.ncbi.nlm.nih.gov) (Table 1). Real-time qPCR reactions were performed after parameter optimization (cDNA concentration and primer specificity). cDNA from muscle and liver were 2- and 3-fold diluted with nuclease free water respectively. Reactions were performed in triplicate and each well contained a total reaction volume of 12.5 μL composed of 6.25 μL of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 0.25 μL of specific primer pairs (forward and reverse) at a final concentration of 300 nM, 3.25 μL of nuclease free water and 2.5 μL of cDNA. After a quick centrifugation of the plate, the reactions were performed in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions (95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s).

The RQ (relative quantification) of the transcription level of each gene of interest was normalized against the transcription level of β-actin, a RT-qPCR relevant reference gene for yellow perch (Pierron et al., 2009, 2011). To diminish the variability during the analysis, sufficient numbers of biological replicates were used (Bustin et al., 2009).

Table 1
Nucleotide sequences of specific partial coding sequence (primer) pairs and PCR products used in RT-qPCR analysis.

Gene abbreviations	Name and functions	Specific primers	PCR products
β-actin	Reference gene	F: 5′-GCCTCTCTGTCCACCTTCCA-3′ R: 5′-GGGCCGGACTCATCGTACT-3′	From Pierron et al. (2009)
rdh-2	Photoreceptor associated retinol dehydrogenase 2 (retinoid and antioxidant system)	F: 5′-AGTCAAGCAGTGCATCAACAAT-3′ R: 5′-CATGCGAACAACACCAAAGAAG-3′	151
Cat	Catalase (antioxidant system)	F: 5′-GTCTTTCTTGTTCAGCGATCGA-3′ R: 5′-GTAGAAACGTTCACCATCAGCA-3′	106
mgst-3	Microsomal glutathione S transferase-3 (antioxidant system)	F: 5′-CCTTCCTCTACAGCTGGATCAT-3′ R: 5′-TGAATACCTGCTCCTTGTCACT-3′	114
g6pdh	Glucose 6 phosphate dehydrogenase (Pentose phosphate pathway and antioxidant system)	F: 5′-ACGAGAGGCTGATATTGGATGT-3′ R: 5′-TCCATATGTGTAAGGGATGGGG-3′	146

F, forward primer; R, reverse primer.

2.8. Biochemical analysis

2.8.1. Plasma, liver and muscle retinoid analysis

Retinoids were analyzed using a slightly modified version of the protocol used by Defo et al. (2012). Analyses were conducted with minimal light in order to prevent molecule isomerisation. Briefly, 100 μL of thawed yellow perch plasma were vortexed with an equal volume of ethanol and ultrapure water to achieve protein precipitation and dissociate retinoid–protein complexes. Extraction was achieved by adding 200 μL of hexane followed by phase separation by centrifugation (13,000 × *g*, 10 min). A 160 μL aliquot of the organic phase was evaporated to dryness under nitrogen (at 50 °C). The extract was re-dissolved in 50 μL of ethanol and a 40-μL volume was injected for HPLC (high-performance liquid chromatography). Once prepared and prior to analysis, the extracts were held for maximum 24 h at −20 °C to minimize retinoid loss.

Partially thawed yellow perch liver (0.01 g) and muscle (0.05 g) were dehydrated by grinding with anhydrous Na_2SO_4 and the resulting powder was extracted with 1000 μL hexane for 10 min using a rotary mixer. After centrifugation (2000 × *g* for 2 min), an 800-μL aliquot of the organic phase was transferred to a Pyrex glass tube (15 mL) and evaporated to dryness under nitrogen (at 50 °C). The residue was dissolved in 50 μL of acetonitrile and 40 μL samples were collected for HPLC analysis.

2.8.2. High-performance liquid chromatography

All solvents used were HPLC grade. The HPLC system consisted of a Spectra SYSTEM P4000 model pump, a model of Spectra SYSTEM AS3000 injector and a model Spectra SYSTEM UV 2000 detector. Data acquisition and instrument control were achieved with the ChromQuest 5.0 computer program (Thermo Scientific Wilmington, DE). Retinoids were separated on a Bio Basic C-18 analytical column (5 mm particle size, 300 Å pore, 4.6 mm × 250 mm, Thermo Scientific Wilmington, DE). The detector was set at 354 nm, corresponding to the maximum light absorbance of all-trans-3,4-dehydroretinoids, the major retinoids in yellow perch tissues (Defo et al., 2012). Under isocratic conditions of 90% methanol and 10% water and a flow rate of 1 mL/min plasma dROH (dehydroretinol) and ROH (retinol) eluted at 7.3 and 8.6 min respectively. Plasma samples were injected every 12 min.

For hepatic retinoids, peak separation occurred under a gradient condition with a flow rate maintained constant at 1 mL/min. The initial mobile phase (acetonitrile/water (90:10)) changed linearly between 9.5 and 12 min to the second mobile phase composed of tetrahydrofuran/methanol/water solvents in the proportions of 36:57:7 respectively. Under these conditions, retention times were dROH 5.7 min, ROH 7.0 min, dROH-myristate 14.7 min, ROH-myristate 15.4 min, dROH-palmitate 16.1 min, ROH-palmitate 16.8 min, dROH-stearate 17.7 and ROH-stearate 18.1 min. Samples could be injected every 25 min after the elution of retinoids and column solvent re-equilibration. Peak identification was determined by comparing their elution time and their absorption spectra with those of certified standards (ROH and ROH-palmitate) and based on other studies (Defo et al., 2012; Doyon et al., 1998). For muscle retinoids, HPLC conditions were the same as for the liver, but only dROH and dROH-stearate were eluted. The major retinoids were quantified using a ROH and ROH-palmitate standard curve. Quantification of dROH was reported on the ROH standard curve multiplied by 1.26, a correction factor corresponding to the ratio of extinction coefficients for ROH and dROH (1832/1450). Also, dROH-ester concentrations were calculated with the standard curve of ROH-palmitate multiplied by 1.26 (Doyon et al., 1998). To take into account the overall tissue retinoid storage, the concentrations of individual ROH-esters and dROH-esters were summed. The mean recovery of retinoids was 75.0 ± 6.5%.

2.8.3. Preparation of microsomes

Liver microsomes from single individuals were prepared as described elsewhere with minor modifications (Boily et al., 2005; Ndayibagira and Spear, 1999). Briefly, 0.3 g of tissue was homogenized in 1.2 mL Tris–KCl buffer (0.15 M KCl, 50 mM Tris–HCl, pH 7.4). The resulting homogenates were centrifuged at 10,000 × *g* for 20 min at 4 °C followed by an ultracentrifugation of the supernatant at 100,000 × *g* for 60 min at 4 °C. The pellets were resuspended in 108 μL of sucrose buffer (0.25 M sucrose, 0.15 M KCl, 50 mM Tris, pH 7.0) and stored at −80 °C until used for REH (retinyl ester hydrolase) and LRAT (lecithin retinyl acyl transferase) assays. A 10-μL aliquot of the microsome preparation was immediately used for mGST (microsomal glutathione-S-transferase) activity analysis.

2.8.4. Liver lecithin dehydroretinol acyl transferase and retinyl ester hydrolase assays

Prior to analysis, preliminary tests were conducted to optimize pH, incubation time, substrate concentration (dROH and ROH-palmitate) and protein concentration (quantity of microsomes) (data not shown). Liver REH activity was higher in acid conditions (pH 4.2) and optimal after a 45 min incubation. 5 μL of ROH palmitate stock solution and 5 μL of microsomes were used to measure REH activity. Liver LdRAT (lecithin dehydroretinyl acyl transferase) activity was higher in alkaline condition (pH 11.0) with an incubation time of 60 min. An aliquot of 2.5 μL of dROH combined with 5 μL of microsomes were used to measure LdRAT activity.

For LdRAT analysis, a 250-μM stock solution of DPPC (Dipalmitoylphosphatidylcholine) containing 2.5% of BSA (bovine serum albumin) was prepared in Tris buffer (0.1 M, pH 9.0). After sonication on ice for 45 min, the mixture was divided into aliquots and stored at -80◦C. Meanwhile a 0.1 mL of a stock solution of dROH (0.1 mg/mL) prepared in hexane was evaporated under nitrogen

and re-suspended in 150 μL of DMSO (dimethyl sulfoxide). A 2.5 μL of aliquot of dROH in DMSO was added to 50 μL of thawed DPPC stock solution and vortexed for 1 min. The mixture was once more vortexed at room temperature for 1 min with 137.5 μL of Tris–HCl buffer (0.1 M, pH 11.0) and pre-incubated at 9 °C for 10 min. To start the reaction, 5 μL of microsomes (previously thawed and pre-incubated with 2 mM of TCEP (tris 2-carboxyethyl phosphine) for 10 min at room temperature) were added to the mixture, vortexed for 1 min and kept for 60 min in a shaking stirrer placed in an incubator set at 9○C. To stop the reaction, 250 μL of ice-cold ethanol were added to the mixture followed by 1 min of vortexing. Then 100 μL of distilled water were added and the tube was once more vortexed for 30 s. DROH–palmitate was extracted twice using 800 μL of hexane-BHT (butylated hydroxytoluene; 0.01%) then 1400 μL of the organic phase was evaporated and resuspended in 50 μL of acetonitrile containing BHT for HPLC analysis. To correct for endogenous dROH-palmitate, blank incubations without dROH were also analyzed.

For REH analysis, 5 μL of stock solution of ROH-palmitate were prepared in hexane and evaporated under nitrogen gas, then 75 μL of buffer containing 0.2% Triton X-100, 50 mM Tris, 0.15 M KCl, pH 4.2 were added to the precipitated ROH-palmitate and vortexed for 5 s. The reaction was started by adding 5 μL of thawed microsomes and the mixture was kept in a shaking stirrer placed in an incubator set at 9 °C. After 45 min, the reaction was stopped by adding 250 μL of ice-cold ethanol. ROH was extracted twice with hexane (1600 μL) containing 0.01% of BHT, centrifuged and 1400 μL of supernatant were evaporated under nitrogen. The extract was re-suspended in 50 μL of ethanol-BHT for HPLC analysis. Parallel samples without microsomes and without ROH-palmitate were analyzed in order to correct for endogenous ROH and potential impurities respectively.

2.8.5. CAT, SOD, G6PDH, total GST, mGST and protein assays

The activities of CAT (EC 1.11.1.6), SOD (EC 1.15.1.1.), G6PDH (EC 1.1.1. 49), and GST (EC 2.5.1.18), as well as protein determinations, were measured in triplicate using a spectrophotometer (Varian 50 Bio) coupled to a microplate reader system. The optimal substrate concentrations as well as sample dilution used in all enzyme activity analyses were determined in preliminary experiments. Partially ice thawed liver and white muscle tissue samples were weighed after rinsing with PBS (phosphate saline buffer), pH 7.4, in order to remove blood and clots. Tissues were then diluted (1:10 tissue–buffer ratios) in a homogenization buffer (HEPES 20 mM, EDTA 1 mM, Triton X-100 0.1%, pH 7.5) and mechanically mixed on ice with a homogenizer (Ultra Turrax® T25; Janke and Kunkel). Samples were then divided into three aliquots. For SOD activity, homogenates were centrifuged at 1500 × *g* for 5 min at 4 °C and for CAT activity the homogenates were centrifuged at 10,000 × *g* for 15 min also at 4 °C. After homogenization, supernatants were collected, stored on ice until analysis or frozen at −80 °C for a maximum of 1 month. The remaining homogenates were used for assaying total GST and G6PDH activities as well as total protein determination.

Liver G6PDH was measured at 340 nm under the following conditions: 0.5 mM β-NADH (nicotinamide adenine dinucleotide) prepared in imidazole buffer (50 mM imidazole, 20 mM $MgCl_2$, 70 mM KCl, pH 7.5) and 100 mM glucose-6-phosphate (omitted for control). A 10 μL portion of 100-fold-diluted homogenate was used in this analysis. Liver mGST was measured by adding 6 mM of reduced glutathione (omitted for control) to 2 mM CDNB (chloro-2,4-dinitrobenzene); for total muscle GST, 1 mM CDNB was used. The reaction was monitored in potassium phosphate buffer (50 mM KH_2PO_4, 50 mM K_2HPO_4, 1 mM EDTA, pH 6.5) for at least 3 min at 340 nm.

Yellow perch tissue CAT activity was determined using Cayman's catalase assay kit (item no. 707002; Ann Arbor, MI, USA). The kit measures at 540 nm the conversion of oxygen peroxide to oxygen and water. A 20 μL portion of the homogenate was diluted 5000- or 50-fold for liver and muscle CAT analysis, respectively. One unit of CAT activity is the amount of enzyme needed to cause the formation of 1 nmol of formaldehyde/min.

Liver and muscle SOD activities were analyzed using Cayman's Superoxide Dismutase assay kit (item no. 706002; Ann Arbor, MI, USA). The kit uses tetrazolium salt to detect at 440–460 nm the superoxide radicals generated by the xanthine oxidase and hypoxanthine reaction; 10 μL of 1000- and 50-fold diluted liver homogenate were used for liver and muscle SOD analysis, respectively. SOD activity is expressed as U/mg protein, where one unit represent the amount of enzyme needed to Exhibit 50% dismutation of the superoxide radical.

The total protein content of each homogenate was determined using BSA as a standard.

2.9. Data analysis

Results were expressed as means ± standard error. Differences in biometric parameters, metal concentrations, gene transcription levels, enzyme activities analysis and vitamin A levels among experimental conditions were compared using one-way ANOVA (analysis of variance), after checking the assumption of normality (Levene test, $p > 0.05$). When the probability was significant ($p < 0.05$), multiple comparison tests were used to identify differences between two conditions. When the assumption of normality was not met, and no transformation of data using $\log_{10}$ or Box-Cox (Peltier et al., 1998) was effective to normalize the distribution, non-parametric paired Wilcoxon tests were applied. For vitamin A determination, statistical analyses were performed without considering sex as an explanatory variable. A probability of $p < 0.05$ indicates a statistically significant difference from the control experiment condition. Computations were performed using JMP 9.0 program.

3. Results

3.1. Metal concentrations in aquarium water and yellow perch kidney

Aqueous metal concentrations measured in aquarium water were very close to targeted nominal concentrations except for high Cd, where the measured concentration was half of the nominal concentration, likely due to adsorption on filters and aquarium glass. Measured aqueous metal concentrations ($N = 6$; mean ± SE) for each condition were: control (0 μg/L; 0 nmol/L), low Cd concentration (LCd, 0.86 ± 0.09 μg/L; 8.0 ± 1.0 nmol/L), high Cd concentration (HCd 3.65 ± 0.21 μg/L; 32.0 ± 2.0 nmol/L), low Ni concentration (LNi, 68.5 ± 6.8 μg/L; 1167 ± 116 nmol/L), and high Ni concentration (HNi, 542 ± 16 μg/L; 9230 ± 273 nmol/L). After 6 weeks of metal exposure, kidney Cd concentration had significantly increased with increasing Cd exposure (Fig. 1). Renal Cd concentrations varied from 3.7 to 10.2 μg/g dry weight (32–91 nmol/g) in controls and HCd, groups, respectively (Fig. 1). Renal Ni concentrations also showed a clear concentration gradient among the conditions and varied from 1.69 to 17.2 μg/g dry weight (29–294 nmol/g) in controls and HNi groups, respectively.

3.2. Biometric parameters

Exposure to low or high aqueous Ni or Cd concentrations did not affect Kn, LSI or corrected pyloric cæca weight (data not shown).

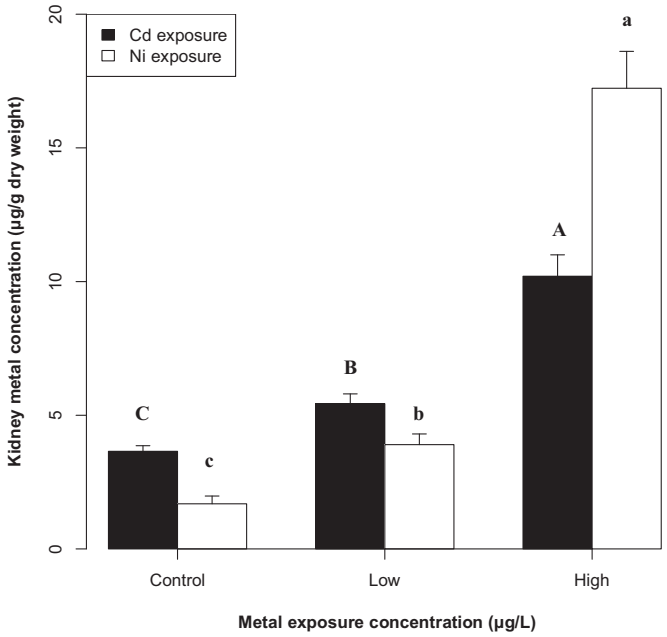

Fig. 1. Total metal concentrations in yellow perch kidney after 6 weeks Cd or Ni exposure. Low and high categories refer to exposure concentrations (see Section 3.1). Values are means ± SE ($27 \leq N \leq 30$/condition). Means designated with different letters for a given metal exposure are significantly different (p-value < 0.05). Capital letters represent comparisons among Cd exposures and control, and lowercase letters indicate comparison among Ni exposures and control.

3.3. Plasma, liver and muscle retinoid levels

Unsurprisingly, HPLC analysis revealed that all-trans-3,4-didehydroretinoids (vitamin A_2) are the predominant form of vitamin A in yellow perch plasma, liver and muscle. Total dROH concentrations in plasma extracts of fish exposed to both Cd and Ni decreased at the end of exposure (Fig. 2). Compared to control fish, the concentration of plasma dROH in fish exposed to low and high Ni was significantly lower ($p < 0.05$). However for Cd-exposed fish, such a decrease was only significant for fish exposed to the high concentration. Also as expected, the major vitamin A_2 stored in yellow perch liver was dROH palmitate, with lesser amounts of ROH palmitate, dROH myristate, ROH myristate, dROH stearate and ROH stearate (Table 2).

Liver concentrations of specific free (i.e. unesterified) dROH, dehydroretinyl esters, and total vitamin A esters generally differed significantly between metal-exposed and control fish. Fish exposed to Ni and Cd exhibited a reduction in liver dehydroretinyl ester concentrations compared to control fish. However the reduction was not significant in fish exposed to high Cd (Table 2). For example, the concentrations of dROH palmitate were 15.4, 2.7, 1.5 and 17.9 times lower in fish from LCd, LNi, HCd and HNi, respectively, compared to control fish. The same trend was observed for retinyl esters stored in liver. For example, concentrations of ROH palmitate were 11, 4, 1.5 and 18.7 times lower, respectively, in fish from LCd, LNi, HCd and HNi, respectively, compared to control fish (Table 2).

Concentrations of the free form of vitamin A in liver for both ROH and dROH decreased in metal-exposed fish, although the decrease was only significant in fish exposed to the low Cd and Ni concentrations, except for free dROH in HNi fish which was higher compared to controls (Table 2). Interestingly, the percentage of free ROH increased in metal-exposed fish. The increase was 7.6, 2, 2.4 and 13.8 times greater than the control, for LCd, LNi, HCd and HNi exposures respectively (Table 2). However the percentage of free dROH remained unaffected by either Cd exposure or Ni exposures when compared to the control fish (Table 2).

Table 2
Liver vitamin A concentrations (ηmol/g liver wet weight; mean ± SE) determined in yellow perch ($6 \leq N \leq 8$/condition) sampled after 6 weeks waterborne exposure to Cd or Ni. Values within a row and designated with different letters are significantly different (p-value < 0.05). Capital letters represent comparisons among Cd exposures and control and lowercase letters indicate comparisons among Ni exposures and control. Abbreviations in column headings and exposure conditions are listed in the abbreviation list.

Exposure conditions	Free retinol (ROH)	Free dehydroretinol (dROH)	ROH-palmitate	ROH-myristate	ROH-stearate	dROH-palmitate	dROH-myristate	dROH-stearate	Total vitamin A esters	Free ROH (%)	Free dROH (%)
Control	0.91 ± 0.23^{Aa}	479.7 ± 2.5^{Aa}	24.7 ± 8.3^{Aa}	163.8 ± 2.5^{Aa}	12.5 ± 3.6^{Aa}	1012.6 ± 316.3^{Aa}	391.3 ± 117.9^{Aa}	97.3 ± 30.9^{Aa}	1702.2 ± 511.3^{Aa}	0.7 ± 0.2^{Ca}	35.0 ± 9.5^{Aa}
LCd	0.36 ± 0.11^{B}	27.3 ± 14.7^{B}	2.2 ± 1.0^{B}	23.4 ± 12.0^{B}	1.65 ± 0.9^{B}	65.6 ± 40.9^{B}	46.2 ± 22.1^{B}	10.2 ± 4.5^{B}	138.3 ± 65.7^{B}	5.3 ± 2.5^{A}	21.0 ± 5.2^{A}
HCd	0.70 ± 0.08^{AB}	337.5 ± 86.6^{A}	16.2 ± 11.4^{A}	106.0 ± 62.8^{A}	7.2 ± 3.8^{A}	647.8 ± 428.0^{A}	250.4 ± 138.3^{A}	65.0 ± 41.3^{A}	1092.6 ± 685.2^{A}	1.7 ± 0.5^{B}	40.2 ± 7.5^{A}
LNi	0.30 ± 0.10^{b}	65.7 ± 22.4^{b}	5.9 ± 2.6^{b}	47.9 ± 22.4^{b}	5.0 ± 2.5^{b}	377.0 ± 215.5^{b}	162.2 ± 89.6^{a}	37.8 ± 21.8^{a}	635.8 ± 352.8^{a}	1.4 ± 0.5^{b}	20.7 ± 6.4^{a}
HNi	0.60 ± 0.10^{ab}	37.6 ± 27.8^{b}	1.32 ± 0.50^{c}	8.6 ± 4.0^{c}	0.6 ± 0.4^{c}	56.6 ± 25.8^{c}	29.0 ± 12.8^{b}	6.4 ± 2.9^{b}	102.5 ± 44.9^{b}	9.7 ± 2.9^{a}	22.9 ± 5.7^{a}

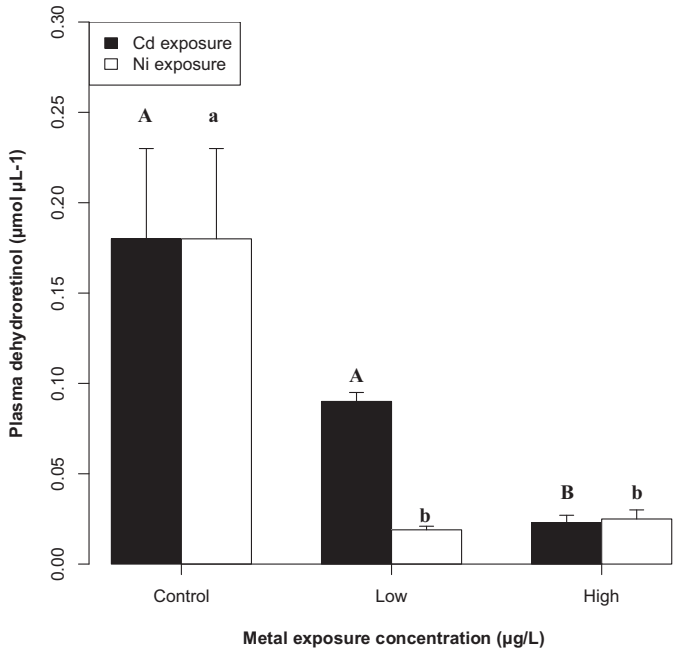

Fig. 2. Plasma dehydroretinol concentrations in yellow perch sampled after 6 weeks waterborne exposure to Cd or Ni. Low and High categories refer to exposure concentrations (see Section 3.1). Bars indicate means and standard errors (SE). Bars that do not share a common letter in the same metal exposure test are significantly different (p-value < 0.05). Capital letters represent comparison among Cd exposure and control, and lowercase letters indicate comparison among Ni exposure and control. The same control fish was used to compare exposure to Cd and exposure to Ni.

Analysis of muscle extracts revealed that the major component of esterified retinoids was dROH stearate (Table 3). The dROH stearate tended to decrease with metal exposure, although the decrease was not significant in high Ni exposed fish. The concentration of dROH, the free form of retinoids found in muscle, was also lower in fish exposed to metals. However the reduction was only significant in fish exposed to the higher metal concentrations (Table 3). The percentage of muscle free dROH decreased in HNi fish. In contrast, the percentage of muscle free dROH increased in Cd-exposed fish, although the increase was not significant in the HCd group (Table 3).

3.4. Effects of metals on liver REH and LdRAT enzyme activities

Fig. 3 illustrates the effects of metal exposure on REH (Fig. 3A) and LdRAT activities (Fig. 3B). No measurable effect of Ni on liver REH and LdRAT activities was detected for either level of exposure. However a significant increase in liver REH and LdRAT activities was observed in fish exposed to high Cd at the end of exposure. The increase (p < 0.005) was 3- and 3.5-fold greater for the hydrolysing (REH) and the esterification (LdRAT) activities, respectively.

3.5. Effects of metals on the transcription level of the gene involved in retinoid metabolism (rdh-2, epidermal retinol dehydrogenase-2)

The liver *rdh-2* transcription level was higher in fish exposed to Cd at both concentrations (Fig. 4A). In contrast to the observations for liver, the muscle *rdh-2* transcription level decreased with increasing Cd concentrations (Table 3). Indeed, liver and muscle *rdh-2* transcription levels were inversely correlated to kidney Cd concentrations after 6 weeks (data not shown).

Table 3
Muscle mean ± SE gene transcription level of *cat*, *mgst-3*, *g6pdh* and *rdh-2*; enzyme activities of CAT, SOD and GST; retinoid concentration of dROH, dROH-stearate and percentage of free dROH of yellow perch ($6 \leq N \leq 8$/condition) sampled after 6 weeks exposure to Cd or Ni. Means designated with different letters within a row are significantly different (p-value < 0.05). Capital letters represent comparisons among Cd exposures and control and lowercase letters indicate comparisons among Ni exposures and control. Abbreviations in column headings and exposure conditions are listed in the abbreviation list.

Exposure conditions	Transcription level[#]					Enzyme activities			Retinoid metabolism	
	Cat	*mgst-3*	*g6pdh*	*rdh-2*	CAT[†]	SOD[‡]	GST[‡]	dROH[*]	dROH-stearate[*]	Free dROH (%)
Control	2.1 ± 0.2^{Aa}	2.2 ± 0.2^{Aa}	0.8 ± 0.3^{Ba}	5.9 ± 0.5^{A}	0.0150 ± 0.0070^{Ba}	16.9 ± 4.8^{Ab}	0.0140 ± 0.0050^{Aa}	0.032 ± 0.008^{Aa}	0.077 ± 0.009^{Aa}	28.1 ± 2.9^{Ba}
LCd	2.4 ± 0.2^{A}	1.7 ± 0.1^{A}	1.6 ± 0.3^{A}	3.2 ± 0.3^{B}	0.0300 ± 0.0200^{A}	13.4 ± 3.3^{A}	0.0070 ± 0.0010^{AB}	0.026 ± 0.002^{A}	0.047 ± 0.004^{B}	35.8 ± 3.1^{A}
HCd	2.0 ± 0.2^{A}	2.1 ± 0.3^{A}	0.5 ± 0.2^{B}	2.6 ± 0.3^{B}	0.0020 ± 0.0003^{B}	23.9 ± 4.1^{A}	0.0057 ± 0.0004^{B}	0.022 ± 0.003^{B}	0.055 ± 0.005^{B}	28.6 ± 3.0^{AB}
LNi	1.7 ± 0.3^{a}	1.8 ± 0.1^{a}	0.4 ± 0.1^{a}	–	0.0013 ± 0.0004^{b}	51.6 ± 11.9^{a}	0.0090 ± 0.0010^{a}	0.025 ± 0.003^{a}	0.055 ± 0.005^{b}	31.5 ± 2.0^{a}
HNi	1.9 ± 0.3^{a}	2.1 ± 0.4^{a}	0.7 ± 0.2^{a}	–	0.0060 ± 0.0003^{a}	183.3 ± 104.9^{a}	0.0071 ± 0.0007^{a}	0.021 ± 0.003^{a}	0.070 ± 0.009^{a}	23.6 ± 1.7^{b}

[*] Expressed in ηmol/g muscle wet weight.
[†] Expressed in U mg protein^{-1}.
[‡] Expressed in U g protein^{-1}.
[#] Expressed in relative quantification (RQ).

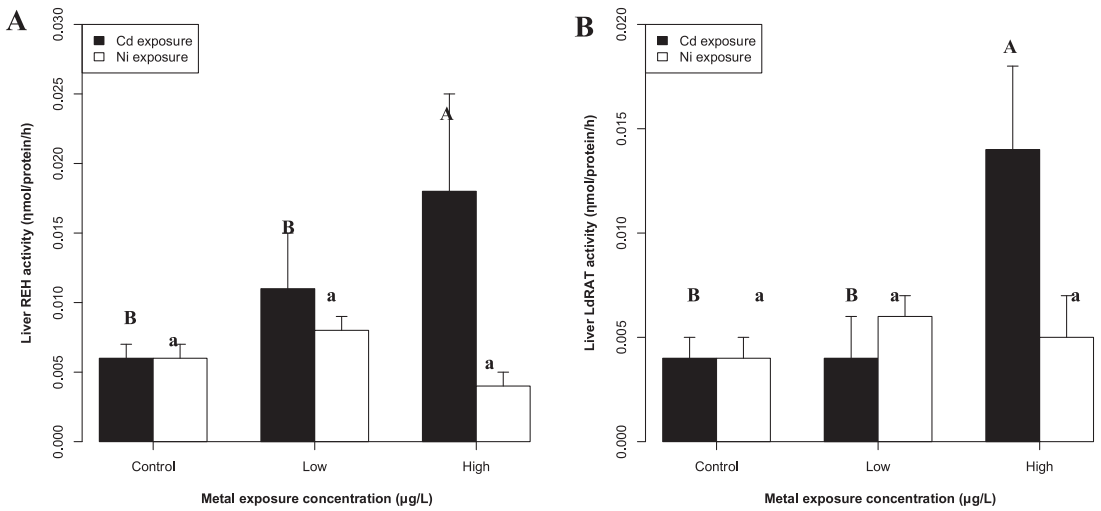

Fig. 3. Liver enzyme activities of REH, retinyl ester hydrolase (A), LdRAT, lecithine dehydroretinol acyl transferase (B) after 6 weeks yellow perch exposure to metal. Low and high categories refer to exposure concentrations (see Section 3.1). Each bar represents the mean ± SE of two separate experiments. Values indicated with different letters within same metal exposure are significantly different (p-value < 0.05). Capital letters represent comparison among Cd exposure and control, and lowercase letters indicate comparison among Ni exposure and control. The same control fish was used to compare exposure to Cd and exposure to Ni.

3.6. *Effects of metals on molecular biomarkers of oxidative stress*

Liver *g6pdh* transcription levels were significantly higher in fish exposed to both metals compared to controls, except for fish exposed to low Cd concentration (Fig. 4B). Muscle *g6pdh* transcription levels increased in fish exposed to low Cd but no change was observed in fish exposed to high Cd compared to controls (Table 3). In contrast, exposure to Ni did not affect the transcription level of muscle *g6pdh* (Table 3). The same increasing trend was observed in *mgst-3* (microsomal glutathione-S-transferase-3) transcription levels (Fig. 4C) and *cat* transcription levels (Fig. 4D) in livers of fish exposed to Cd and Ni compared to the control fish. In contrast to the observation for livers, there were no differences in mean muscle *mgst-3* and *cat* transcription levels among all conditions for both metals (Table 3).

3.7. *Effects of metals on enzyme activities and oxidative stress biomarkers*

Liver SOD activity did not increase significantly as a consequence of metal exposure (Fig. 5A). The activity of liver G6PDH was significantly higher in fish exposed to Cd either at low (2-fold) or high (1.66-fold) concentrations, relative to the control. In contrast, no effect of Ni exposure was detected in the activity of this enzyme when fish were exposed at either Ni concentration (Fig. 5B). Also liver MGST activity did not increase significantly as a consequence of metal exposure (Fig. 5C), but significant differences in liver CAT activity were found in yellow perch exposed at high concentrations of both Cd and Ni. The increase in CAT activity was 1.46- and 1.61-fold, for HCd and HNi groups, respectively, compared to controls (Fig. 5D).

Muscle CAT activity increased at the low Cd concentration but no changes were observed at the higher Cd concentration relative to the control. In contrast to Cd exposure, fish exposed to Ni at either low or high concentrations displayed lower muscle CAT activity compared to the control fish (Table 3). Muscle SOD activity of yellow perch exposed to Cd remained unaffected by Cd, but fish exposed to Ni had consistently higher muscle SOD activity compared to the control fish (Table 3). In contrast to muscle SOD activities, muscle total GST activity was lower in high Cd exposed fish, and there were no differences in muscle GST activity in fish exposed to Ni compared to the control fish (Table 3).

4. Discussion

Our experimental conditions induced an accumulation of both cadmium and nickel in the kidney of our test fish. In our experiment, in fish exposed to the higher metal concentrations, renal metal concentrations were comparable to Cd values (Bourret et al., 2008; Kraemer et al., 2006) and Ni values (Giguère et al., 2006; Iles and Rasmussen, 2005) reported in the kidney of wild yellow perch from metal-contaminated lakes. Although in this study we could not measure metal concentrations in the liver (see Section 2.5), kidney and liver Cd and Ni concentrations have been shown to be well correlated in wild yellow perch (Couture et al., 2008a; Kraemer et al., 2005a,b). Also, in yellow perch collected in a metal-impacted lake, total Cd concentrations measured in kidney (525 nmol/g) were similar to values in liver (649 nmol/g) (Kraemer et al., 2005a,b).

4.1. *Effects of metal contamination on tissue retinoid metabolites*

Liver represents the major site of retinoid storage, with REs (esterified retinoids) representing the main stored form. In the present study, hepatic retinoid concentrations decreased after metal exposure, suggesting that waterborne Cd and Ni exposure negatively impacted yellow perch liver retinoid metabolism. This result contrasts with our previous studies where we reported positive correlations between kidney Cd concentrations and liver dehydroretinoids (free and esterified form) in fish inhabiting metal impacted lakes (Defo et al., 2012). The discrepancy between these studies could be due to the interactions of multiple stressors found in the natural environment (for example diet quality and availability) or individual biometric parameters such as fish sex, size and age that probably affected retinoid metabolism (Defo et al., 2014). An alternative hypothesis is that, in contrast to the metal-naïve laboratory fish used here, wild yellow perch may have developed adaptive mechanisms enabling them to live in polluted areas, as recently shown at the genome level (Bélanger-Deschênes et al., 2013).

Low liver dROH concentrations observed in fish exposed to metals could reflect the general circulation levels. The low concentrations of plasma dROH in Cd- and Ni-exposed perch compared to controls suggest that these metals inhibit the synthesis of carrier proteins such as retinol binding protein and/or transthyretin, or interfere with the ability of dROH to bind to RBP (retinol binding protein). By displacing the dROH bound to RBP, metals may

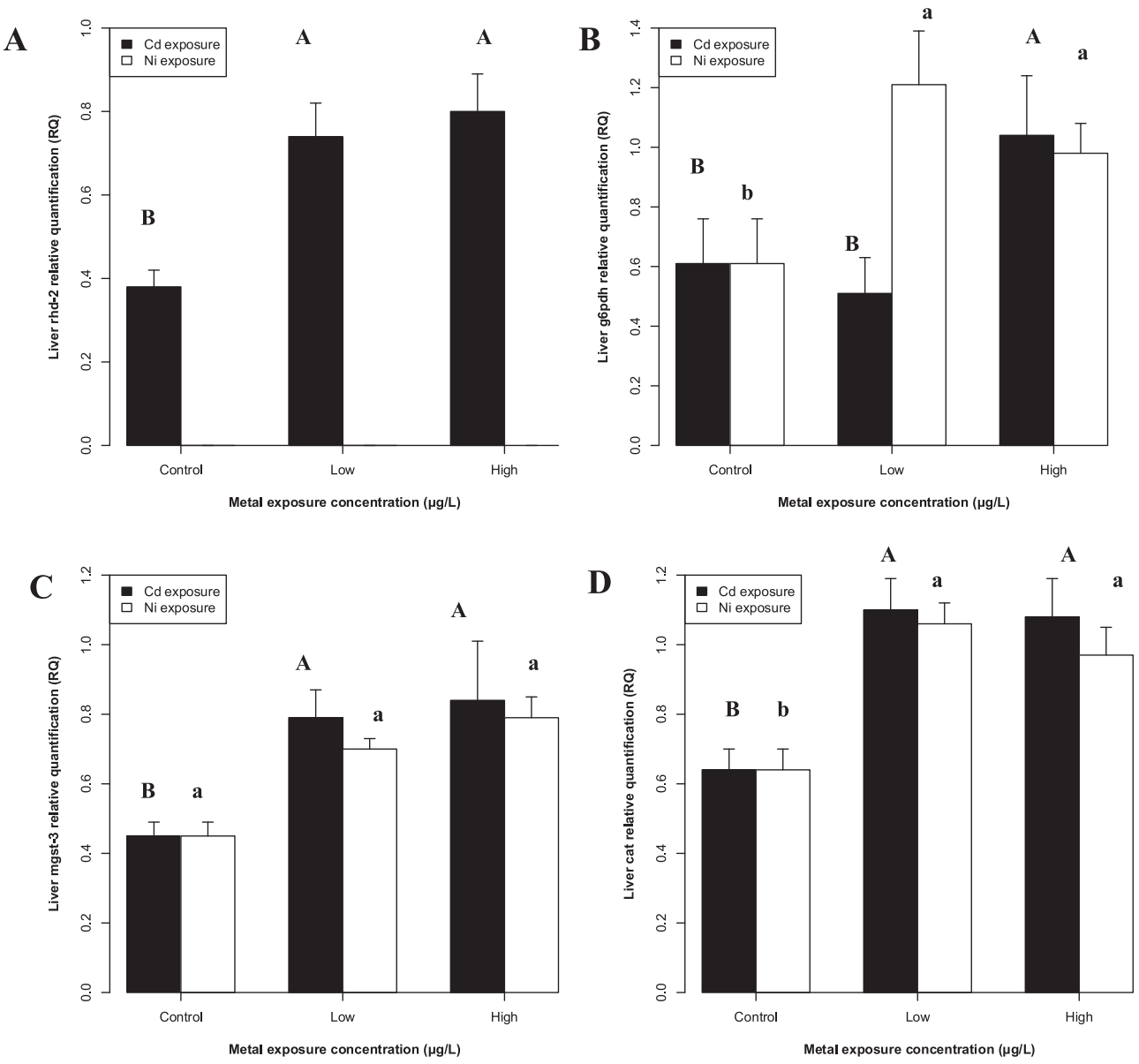

Fig. 4. Liver relative transcription level of *rdh-2* (A), *g6pdh* (B), *mgst-3* (C) and *cat* (D) after 6 weeks yellow perch exposure to metal. Low and High categories refer to exposure concentrations (see Section 3.1). Each bar graph represents the mean ± SE of two separate experiments. Values indicated with different letters within same metal exposure are significantly different (p-value < 0.05). Capital letters represent comparison among Cd exposure and control, and lowercase letters indicate comparison among Ni exposure and control. The same control fish was used to compare exposure to Cd and exposure to Ni.

also inhibit the ability of the dROH–RBP complex to bind to TTR (transthyretin), which protects this complex against degradation during its transfer to extra-hepatic cells (Berry et al., 2012). Interestingly, we have previously reported (Pierron et al., 2011) an increase in the transcription level of the gene encoding for TTR in wild yellow perch populations living in Cd-impacted areas, suggesting a compensatory mechanism aimed at counteracting the effect of Cd in the degradation of dROH–RBP complexes or accelerating the blood clearance rates of dROH. Alternately, the imbalance of yellow perch plasma dROH in metal-impacted environments could occur *via* competitive interactions between trace metals and those carrier proteins. Increasing blood Pb concentrations were also associated with the mobilization (decrease) of plasma retinol in coots (*Fulica atra*) and pochard (*Aythya ferina*) (Martinez-Haro et al., 2011). Taken together, these studies suggest that contaminants affect plasma dROH regulation, but the influences of confounding environmental stressors and adaptation to chronic exposure remain to be clearly identified. Nevertheless, in our experiment, none of the biometric endpoints analyzed (Kn, LSI and corrected pyloric cæca weight) varied among the exposure conditions, implying that nutritional status did not influence the reported differences in tissue vitamin A concentrations. Finally, our study clearly demonstrates that Cd and Ni interfered with tissue retinoid metabolism in yellow perch.

Liver retinyl esters are hydrolysed to retinol, which is the form available to be transported to extra-hepatic tissues or target cells by binding to RBP and TTR (Defo et al., 2014). Surprisingly, in our study only dROH-stearate was found as an esterified form of vitamin A in muscle. As was the case for the liver, muscle retinoid (dROH and dROH-stearate) concentrations decreased after high metal exposure. Our data suggest that waterborne Cd and Ni negatively impact yellow perch muscle retinoid metabolism and that dROH-stearate is main storage form of dehydroretinyl esters in this tissue.

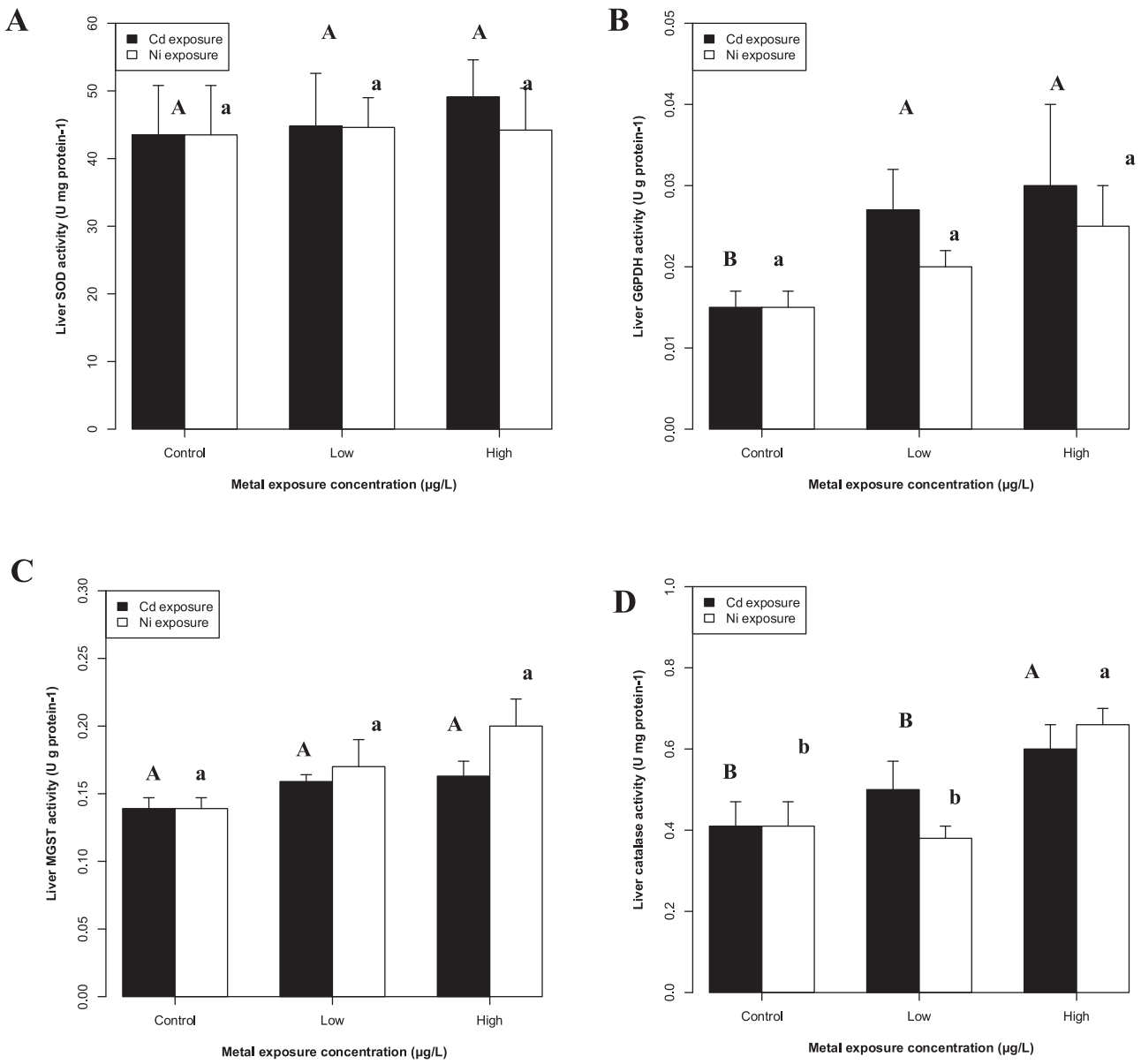

Fig. 5. Liver enzyme activities of SOD (A), G6PDH (B), MGST (C) and CAT (D) after 6 weeks yellow perch exposure to metal. Low and high categories refer to exposure concentrations (see Section 3.1). Each bar represents the mean ± SE of two separate experiments. Values indicated with different letters within same metal exposure are significantly different (p-value < 0.05). Capital letters represent comparison among Cd exposure and control, and lowercase letters indicate comparison among Ni exposure and control. The same control fish was used to compare exposure to Cd and exposure to Ni.

4.2. Effects of Cd and Ni on tissue retinoid metabolism and oxidative stress biomarkers

4.2.1. Liver

The role of retinoids in the protection against metal-induced oxidative stress was recently reviewed (Defo et al., 2014). In the present study, exposure to elevated aqueous Cd concentrations caused an increase in liver REH activity, combined with a higher percentage of free liver ROH and an up-regulation of the transcription level of *rdh-2* gene, encoding for the synthesis of enzymes involved in the oxidation of retinol to retinaldehyde for retinoic acid biosynthesis (Lee et al., 2009). An up-regulation of this gene coupled with high REH activity indicates a synchronous reaction at the molecular and biochemical levels in order to accelerate retinol synthesis. This reaction suggests a compensatory mechanism aimed at counteracting the direct effects of Cd induced oxidative stress. In contrast, our microarray did not reveal any change in the transcription level of genes involved in retinoid metabolism when yellow perch were exposed to Ni at either low or high concentrations (Bougas et al., 2013). This finding agrees with the absence of effects of this metal on liver REH activity, although the percentage of liver free ROH was higher in fish exposed to the higher Ni concentration. Higher liver REH activity or the higher percentage of ROH suggests a high conversion or hydrolysis of retinyl palmitate to retinol, potentially in order to fight against oxidative stress induced by high metal concentrations (Defo et al., 2012).

LdRAT transforms dehydroretinol into dehydroretinyl esters, and the activity of this enzyme serves as an indicator of the dehydroretinoid metabolic pathway. Studies using ROH as substrate reported negative effects of agricultural practices on LRAT activity in frogs (Boily et al., 2009) and of organic pollutants in fish (Ndayibagira and Spear, 1999). However, to our knowledge, this is the first study using dROH as substrate to examine the effects of

metals on the enzymes involved in retinoid esterification in vertebrates. Our observation that HCd but not HNi exposure increased liver LdRAT activity suggests a higher conversion of dROH to dROH-palmitate, potentially to fight against oxidative stress induced by high metal concentrations, and that the stimulation of LdRAT activity is metal-specific.

Consistent with our hypothesis that higher enzyme (REH and LdRAT) activities and up-regulation of the *rdh-2* gene afforded an increased protection against metal-induced oxidative stress, we observed an increase in the activities of enzymes and in gene transcription levels of a set of toxicologically-relevant biomarkers traditionally involved in liver oxidative stress response. Probably, our Cd exposure conditions were beginning to exceed the capacities of antioxidants involving retinoid metabolites, since free and esterified forms of retinoids decreased along with an increase of other biomarkers of oxidative stress (G6PDH, GST, CAT, SOD). Specifically, yellow perch treated with Cd and Ni displayed high transcription levels of liver *g6pdh* and this up-regulation translated into an increase in liver G6PDH activity, presumably in order to bring the level of ROS generated by metal-exposed fish down to that of unexposed fish. G6PDH is an enzyme involved in lipid and pentose phosphate pathways, which has been used as an oxidative stress biomarker in studies examining the effects of numerous environmental stressors including metals (Cartañá et al., 1992; Regoli et al., 2011). This enzyme regulates the response of glutathione to chemical exposure by influencing the biosynthesis of NADPH, which in turn serves as a reducing equivalent in glutathione reductase activity (Giguère et al., 2005; Regoli et al., 2011). Our findings agree with those of Kubrak et al. (2013), who reported an increase in G6PDH activity in gills of goldfish (*Carassius auratus*), after laboratory exposure to different Ni concentrations. Previous reports in which the European eel (*Anguilla anguilla*) was chronically exposed to a cocktail of pollutants, including Cd and Ni at different concentrations, indicated an increase in liver and gill *g6pdh* transcription level when fish were exposed to moderately polluted sediment (Regoli et al., 2011). However, a down-regulation of this gene was observed in the liver of fish exposed to highly polluted sediment (Regoli et al., 2011).

Gene transcription for liver *mgst-3* was upregulated in Cd-exposed fish. However, the increase that we observed in mGST activity was not significant. It is known that GST contributes to cell detoxification by favoring excretion of ROS-containing reactive electrophilic centres (Elia et al., 2006). Our data suggest that at least at the transcription level, an increased protection is achieved at the metal concentrations used in this study. This protection also occurs *via* the glutathione metabolism (Kowara et al., 2005). A recent study reported an increase in gill GST activity after Ni exposure in goldfish (Kubrak et al., 2013). Regoli et al. (2011) also reported an increase in liver GST activity when eels were exposed to either moderately or highly polluted sediments compared to the control group. However, this increase in enzyme activity was preceded by an increase of liver *gst pi* transcription level, a gene encoding for one of the multiple *gst* isoforms (Espinoza et al., 2012), only in fish exposed to the moderately contaminated sediment (Regoli et al., 2011). In a laboratory experiment, (Hoarau et al., 2006) showed that Cd concentration induced *gst pi* transcription level whereas it did not affect the GST activity in the digestive gland of mussels (*Mytilus gallaprovincialis*). We are not aware of a toxicological study other than ours where microsomal GST activity was measured.

In our study, we observed a high transcriptional level of liver *cat* in fish exposed to metals, and this up-regulation was translated into an increase in liver CAT activity in fish exposed to high Cd and Ni. These findings are not surprising since earlier studies have shown that catalase belongs to the first line of oxidative stress defence (Serpil Mişe Yonar, 2013). High levels of this antioxidant contribute to the neutralization of ROS generated by metal exposure (Hsu et al., 2013; Uren Webster et al., 2013). Our results are also in line with those of Kwong et al. (2011) who reported an increase in the transcriptional level of antioxidant genes after fish were exposed to dietary Cd, and with those of Regoli et al. (2011) who reported an increase in liver and gill *cat* transcription levels when European eels were exposed to highly polluted sediment. However, in contrast with our study, they respectively observed either a decrease or no change in liver and gill CAT activities (Regoli et al., 2011).

4.2.2. Muscle

A significant increase in the percentage of muscle dROH was observed in fish exposed to the lower Cd concentration, while a decrease was observed in fish exposed to the higher Ni concentration. These results suggest that the enzymes (LdRAT for Cd exposure and dREH (dehydroretinyl ester hydrolase) for Ni exposure) involved in muscle vitamin A_2 homeostasis were inhibited by the presence of Cd and Ni respectively and indicate that these two metals might have different modes of action on muscle dehydroretinoid metabolism. Recently, we proposed a mechanism of metal toxicity involving liver retinoid metabolic pathways in vertebrates, and resulting in alteration of retinoid homeostasis by metals occurring *via* the breakdown of hepatic enzymes involved in the hydrolysis of retinyl esters or in the esterification of retinol (Defo et al., 2012, 2014). The finding presented here also supports this hypothesis in yellow perch muscle.

In contrast to the responses of the liver, muscle CAT and GST activities decreased or remained unchanged with metal exposure, except at low cadmium concentrations, where the activities increased. This result is not counterintuitive as the literature suggests that a decrease in CAT activity is often compensated by enhancements in other antioxidant enzyme activities such as GPx (glutathione peroxidase), as an alternative mechanism for ROS removal (Regoli et al., 2011). A decrease in CAT activity was reported in sea bass (*Dicentrarchus labrax*) kidney after Cd exposure (Romeo et al., 2000). Such a decrease in CAT and GST activities also corroborates the findings of Ahmad et al. (2006), who observed the same response after exposure of European eel (*Anguilla. anguilla*) to a cocktail of environmental pollutants including metals. Although Cd and Ni promote oxidative stress (Hsu et al., 2013; Kubrak et al., 2013), these results suggest that different mechanisms predominate for the clean-up of ROS generated by metals in muscle compared to other tissues. MTs (metallothioneins) are non-enzymatic and cysteine-rich proteins known to have high affinity for ROS and to contribute in the cellular detoxification of metals (Amiard et al., 2006; Viarengo et al., 2000). MTs could thus participate in the cellular antioxidant defence system (Uren Webster et al., 2013). Studies also suggest that the action of MTs depends on the tissue analyzed (De Smet et al., 2001). In support of this hypothesis, we found a slight up-regulation in muscle *mts-1* of fish exposed to metals, although the differences were not statistically significant compared to the control (data not shown).

Another plausible explanation for the decrease or absence of changes in muscle biomarkers for metal-induced oxidative stress is that after their uptake, metals are largely directly transported to organs with high detoxification capacities, such as the liver and kidney. The low accumulation of metals in muscle compared to other tissues, reported previously for yellow perch (Rajotte and Couture, 2002), suggests that muscle may be less susceptible to oxidative stress or may have a reduced need for antioxidant response systems. In agreement with this, we found no evidence of effects in the muscles of metal-contaminated fish, such as impairment of muscle aerobic capacity (*cco-1*, cytochrome c oxidase, transcription level, and CCO activity) or anaerobic capacity (lactate dehydrogenase activity; data not shown). Again, our results differ from studies of wild yellow perch where metal contamination has repeatedly

been reported to impair muscle metabolic capacities (Couture and Kumar, 2003).

In contrast to the liver, which is known to be the first organ to counteract oxidative challenge caused by chemical stressors in fish (Benedetti et al., 2007), responses of muscle to oxidative stressors are more variable. Here, muscle CAT activity decreased with both high and low Ni exposure and increased under conditions of low Cd exposure, while the *cat* gene transcription level remained unchanged. On the other hand, muscle GST activities decreased with Cd exposure but did not vary with Ni exposure, whereas gene transcription (*mgst-3*) levels remained unchanged when fish were exposed to either metal. Increases in antioxidant enzyme activity are the typical response in the presence of ROS in the cell. However a decrease in these activities with metal exposure illustrates the fact that the activities of such enzymes are altered as a consequence of metal exposure (Benedetti et al., 2009). The responses obtained in yellow perch muscle are not unprecedented because in oxidative stress pathways a variety of different effects of pollutants on gene transcription levels and subsequent effects at the biochemical level have been observed, such as concomitant reduction or enhancement or variations in opposite directions. Each situation is time and concentration of exposure dependent (Canesi et al., 2008) and also depends on the pre-existing pool of proteins.

4.3. Implications of transcriptional changes on functional responses

4.3.1. Liver

For liver retinoid metabolism, there was good agreement between transcriptional and functional responses in fish exposed to the higher Cd concentration, e.g. increase *in rdh-2* transcription level was followed by higher REH activity. However, at the lower Cd concentration, REH and LdRAT activities did not increase although there was an up-regulation of the *rdh-2* gene. Translational responses typically follow transcriptional responses (Nikinmaa and Rytkönen, 2011). However, since both processes are under the control of multiple regulation steps, this is not always the case (Regoli et al., 2011). Furthermore, the relationship between both responses depends on several parameters including tissue, mode of exposure, concentration of stress factor, transcription factor activation, gene specificity and substrate relationship (Nikinmaa and Rytkönen, 2011; Regoli et al., 2011). We are not aware of any study that has reported simultaneous effects of metals on vitamin A metabolism at both the transcriptional and translational levels in fish. Although the genes analyzed in this study are not directly involved in the synthesis of the enzymes we examined, they belong to the same metabolic pathways. Thus our findings suggest that at high Cd concentrations, regulation is likely to occur at both levels, but at low Cd concentration regulation of transcription is sufficient to maintain enzyme activity. Since no measurable effect on enzyme activities was observed at low Cd exposure, our results also suggest that the intensity of exposure is likely to influence the regulation of enzyme activity (Regoli et al., 2011). This result contrasts with our previous field studies where we observed a down-regulation of *rdh-2* gene in yellow perch chronically exposed to Cd (Pierron et al., 2011) and a decrease in the percentage of liver free dROH (Defo et al., 2012). Furthermore, confounding effects of environmental stressors to which wild fish are subjected can influence the transcriptional and translational responses (Nikinmaa et al., 2013).

Inconsistent relationships between gene transcription level and biochemical endpoint responses for traditional oxidative stress biomarkers have frequently been observed in studies carried out with different fish species exposed to different classes of pollutants (Bilbao et al., 2010; Craig et al., 2007; Jin et al., 2010) and these responses vary according to the tissue analyzed (Regoli et al., 2011). In relation to other oxidative stress biomarkers, our results generally show good agreement between gene transcription levels and corresponding catalytic activity. For example, gene transcription for liver catalase was up-regulated and followed by an increase in enzyme activity after 6 weeks of exposure to both metals at least for high metal exposure. Optimal protection against oxidative stress is achieved by an appropriate balance among antioxidant defence enzymes, which work cooperatively or synergistically (Bagnyukova et al., 2006; Michiels et al., 1994). Also, in the present study, gene transcription for liver *mgst-3* and *g6pdh* were up-regulated, and associated with an absence and increase in the activities of mGST and G6PDH respectively in fish exposed to Cd. Unlike in prokaryotic cells where transcription is coupled to translation, there is a different temporal relationship between transcription and translation in eukaryotic cells; therefore it is not surprising that gene transcription levels of some antioxidants increase while no change is observed at the biochemical level (Giuliani et al., 2013).

4.3.2. Muscle

Cd induced changes in the transcriptional level of muscle *rdh-2* gene. However at the biochemical level, variation trends in opposite directions in the percentage of dROH were noticed. For example, muscle *rdh-2* was markedly down-regulated after Cd exposure, whereas an increase in the percentage of dROH, at least for LCd, was observed. To our knowledge this is the first study that reports metal impacts on fish muscle dehydroretinoid metabolism. Our results suggest that *rdh-2* transcription level was down-regulated probably in order to activate the inhibition of dehydroretinoic acid synthesis, leading to muscle accumulation of dROH. This result also suggests a delay between muscle transcription and translation (Regoli et al., 2011).

5. Conclusions

Our results clearly indicate that Cd and Ni exposure affected indicators of retinoid metabolism and oxidative stress. Waterborne Cd or Ni exposure led to increased kidney metal concentrations and a decrease in plasma, liver and muscle vitamin A metabolites. Liver antioxidant defence biomarkers were elevated in Cd-impacted fish, indicating a synchronous defence response against Cd-induced oxidative stress. However, no consistent pattern in antioxidant defence responses was observed between molecular and biochemical biomarkers when fish were exposed to Ni, suggesting a non-synchronous response of antioxidant defence in fish exposed to waterborne Ni. In contrast to what we observed in the liver, changes in gene transcription levels in muscle in response to metal exposure were not closely matched by the activities of corresponding enzymes, suggesting a lower susceptibility of this tissue to oxidative stress.

Data collected in this study provide insights into mechanisms used by yellow perch to fight against oxidative stress induced by metal accumulation. These mechanisms involve both well-established responses to oxidative stress and novel responses involving retinoid metabolism. Our approach combining examination of both transcriptomic and biochemical endpoints highlights the benefits of multi-level approaches in the identification of strategies used by fish to fight metal-induced oxidative stress in environmental risk assessment. While liver biochemical responses provide functional information, transcriptional change appears to be more sensitive to metal contamination, supporting their use as additional and complementary biomarkers of effects and exposure, respectively.

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