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Transcriptional responses to environmental metal exposure in wild yellow perch (*Perca flavescens*) collected in lakes with differing environmental metal concentrations (Cd, Cu, Ni)

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Abstract To investigate the mechanisms involved in metal stress in wild fish, yellow perch (*Perca flavescens*) were collected in eight lakes of the Rouyn-Noranda and Sudbury regions (Canada). Due to mining and smelting activities, these two regions indeed present a broad contamination gradient in metal concentrations (Cd, Cu, Zn and Ni; water, sediment and prey) and offer a unique research opportunity to investigate relationships between metal bioaccumulation and resulting deleterious effects in indigenous biota chronically exposed to metal mixtures. The expression level of genes encoding for proteins involved in metal detoxification (metallothioneins, *mts*), protein protection (heat shock protein-70, *hsp-70*), growth (insulin-like growth factor-1, *igf-1*), aerobic energy metabolism (cytochrome c oxydase, *cco-1*) and protection against oxidative stress (Cu/Zn superoxide dismutase, *sod-1*) were assessed in fish liver and muscle in association with protein and enzymatic assays for cytochrome c oxidase (CCO). Bioaccumulation of both Cd and Cu increased in response to higher ambient metal concentrations, but the two metals clearly have different modes of action. For Cd, changes in gene expression levels were more marked in the liver than in the dorsal muscle, whereas for Cu the opposite trend was observed. Hepatic Cd accumulation was linked to decreased *cco-1* and *sod-1* gene expression, whereas Cu accumulation was associated with a decrease in CCO enzymatic activity and an increase in total protein concentration and in *cco-1*, *mts* and *hsp-70* gene expression levels. For Ni, no significant correlations were observed at the transcriptional level, but increasing hepatic Ni concentrations were significantly and positively correlated with protein concentrations and CCO activity. By coupling gene expression to biochemical and physiological endpoints, this work provides new insights into the mechanisms involved in metal stress and the adaptive response of fish chronically exposed to metal mixtures.

Keywords Metal contamination · Field-collected fish · *Perca flavescens* · Gene expression · Physiological endpoints

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Introduction

Human activities, such as mining and smelting operations, have greatly increased the global flux of many metals in the Earth's surficial environment, leading to elevated concentrations in aquatic ecosystems. In recent years, considerable progress has been achieved in reducing metal releases to the environment (Mahler et al. 2006); ambient metal concentrations in mining areas are decreasing and the local ecosystems are showing signs of recovery. However, fish living in areas subjected to metal contamination still today show evidence of lower condition and overall health, suggesting that ambient metal concentrations continue to exceed levels that are safe for the environment (Rajotte and Couture 2002; Bervoets and Blust 2003; Maes et al. 2005; Giguère et al. 2005; Hansen et al. 2006).

Yellow perch (*Perca flavescens*) is a fish species that is broadly distributed across North America. Previous studies examining wild yellow perch inhabiting metal-contaminated lakes have reported impairments in cortisol stress response, intermediary metabolism, seasonal storage of energy reserves and aerobic capacities in the more contaminated fish (Levesque et al. 2002; Rajotte and Couture 2002; Couture and Kumar 2003; Gravel et al. 2005; Couture et al. 2008a). These investigations were carried out in two areas on the Canadian Precambrian Shield that have been subjected to metal emissions from smelters for over 80 years, the Rouyn-Noranda (Quebec) and the Sudbury (Ontario) regions. These two regions present a broad contamination gradient in metal concentrations (Cd, Cu, Zn and Ni; water, sediment and prey) and offer a unique research opportunity to investigate relationships between metal bioaccumulation and resulting deleterious effects in indigenous biota chronically exposed to metal mixtures (Couture et al. 2008b).

Yellow perch are a highly relevant species in the context of metal ecotoxicology. First, they are metal-tolerant and as a result are widely distributed and abundant in many mining and smelting areas of Canada. Second, fish are of particular interest for the examination of metal effects in freshwater systems, since in addition to direct effects from aqueous and dietary exposure, they also integrate effects at lower levels such as disturbances in the food web structure resulting, for example, from the elimination of large metal-sensitive benthic invertebrates and their replacement by smaller metal-tolerant taxa (Rasmussen et al. 2008). In the Rouyn-Noranda region, the growth efficiency (i.e., growth increment relative to food consumption rate) was found to be three times lower in yellow perch from the metal-contaminated lakes relative to fish from reference lakes (Sherwood et al. 2000). The authors argued that metal-contaminated perch experience greater total energetic costs, including the cost of metal detoxification and the cost of hunting for smaller and scarcer prey, in the more metal-contaminated lakes.

In the present work, we investigated whether lifelong exposure to metal mixtures can cause changes in gene expression in these perch populations. Indeed, although analysis of gene expression is generating a growing interest in ecotoxicology (Piña et al. 2007; Steinberg et al. 2008), to date almost all studies that have been performed on aquatic organisms exposed to metals have been restricted to model organisms and short-term laboratory or transplantation experiments. Here, we have used this approach to investigate toxicity and adaptation mechanisms developed at the molecular level in fish chronically exposed to metal contamination, and to strengthen our understanding of the link between environmental metal exposure and the effects observed in natural perch populations. Indeed, recent advances tend to show that analyses of gene expression offer the potential not only to provide information about mechanisms and modes of action for classes of chemicals but also to facilitate the differentiation between intoxication and responses to environmental factors (Piña et al. 2007; Steinberg et al. 2008). The expression profiles of five genes, suspected from the literature to respond to metal stress, were determined in liver and white muscle of wild yellow perch directly collected from eight lakes with differing environmental metal concentrations.

Based on previous field studies carried out on this species, and also considering the results from experimental exposures, we chose to measure the expression level of the genes (*mts*) encoding for metallothioneins. Metallothioneins are cysteine-rich proteins involved in maintaining sufficient intracellular supplies of some essential metals (Cu, Zn) and in detoxifying excess intracellular metals (including non-essential metals such as cadmium (Cd); Amiard et al. 2006). However, past studies performed on yellow perch inhabiting the two sampling regions have shown that an appreciable fraction of the total metal accumulated in fish is bound intracellularly to potentially metal-sensitive sites, such as proteins (including enzymes) and organelles, even in the low and moderately contaminated fish (Giguère et al. 2006; Campbell and Hare 2009). In this context, we have also chosen to measure the expression level of the gene (*hsp-70*) encoding for heat shock protein 70, a protein involved in protein folding and protection. Experimental investigations have shown that HSP proteins families are up-regulated not only in response to heat shock but also after exposure to toxic chemicals (Kiang and Tsokos 1998). Since several experimental studies have shown that exposure to metals such as Cu, Cd and Ni can lead to the production of reactive oxygen species (ROS; Stohs and Bagchi 1995; Costa et al. 2002), we also measured the expression level of the gene (*sod-1*) encoding for cytoplasmic superoxide dismutase, an enzyme involved in the fight against oxidative stress (Scandalios 2005). Finally, since a number of studies have reported changes in growth and energy metabolism in fish chronically exposed to metals (Sherwood et al. 2000; Levesque et al. 2002; Rajotte and Couture 2002), we also measured the expression level of the gene (*igf-1*) encoding for the insulin-like growth factor-1, a protein involved in cellular growth and development, as well as the expression level of the gene (*cco-1*) encoding for the cytochrome c oxidase sub unit 1, a mitochondrial enzyme involved in aerobic ATP production. For this latter enzyme, we also measured its in vitro enzymatic activity. With the exception of *igf-1* gene expression, which was only measured at the hepatic level, genetic and enzymatic analyses were performed for both liver and muscle samples.

Genetic and enzymatic analyses were completed by the determination of two general indicators of fish health: total

protein concentrations in liver and muscle tissues and Fulton's condition factor (FCF), an index frequently used in ecology to assess the relative plumpness of fish. Additionally, hepatic Cd, Cu, Ni and Zn concentrations were determined as an integrative measure of recent exposure of fish to metals (Giguère et al. 2006).

Materials and methods

Fish sampling

Four lakes were selected in each mining region on the basis of their known levels of contamination by Cu, Cd and Ni (Giguère et al. 2004; Couture et al. 2008b). In the Rouyn-Noranda region, yellow perch were collected in two clean lakes (Opasatica: 48°04′25N, 78°18′10 and Dasserat: 48°13′04″N, 79°23′27″O) and two metal-contaminated lakes (Dufault: 48°18′23″N, 78°59′58″O and Osisko: 48°14′35″N, 78°59′58″O). In the Sudbury region, clean yellow perch were collected in Geneva (northwest of Sudbury, 46°45′41″N, 81°33′10″O) and James (near North Bay, 46°17′25″, 78°59′27″) Lakes. Metal-contaminated perch were collected in Lakes Hannah (46°26′35″N, 81°02′18″O) and Whitson (46°35′15″N, 80°58′31″O). All fish were collected during August 2006 using a seine net. All yellow perch sampled were size selected to minimize potential allometric bias. The total length (13.6 ± 0.2 cm, mean ± SE, $n = 200$; ≈5–6 years old) and weight (28.2 ± 1.8 g, mean ± SE, $n = 200$) were recorded for each fish in order to estimate the Fulton condition factor (FCF; (weight (g))/(total length (cm))^3 × 1,000). For each sampling region, a total of 25 fish were collected per lake and used for subsequent analyses. Fish were dissected and the liver as well as a sample of white muscle were divided into two similar pieces. One piece was immediately frozen and stored in liquid nitrogen until needed for gene expression analyses. The second piece was stored at −80°C and used for both enzymatic and metal analyses.

Liver metal concentrations

Liver samples for metal analysis were placed in acid-washed (15% HNO_3) Eppendorf® polypropylene tubes and weighed (~15 mg) after lyophilisation. The freeze-dried samples were then digested in 1 ml of trace metal grade nitric acid over 5 days at room temperature. In parallel, certified reference materials from the National Research Council of Canada (TORT2), as well as blanks, were submitted to the same treatment in order to monitor analytical accuracy and recovery. Digests were then diluted with ultrapure MilliQ water and concentrations of Cd, Cu and Zn were measured by inductively coupled plasma atomic emission spectrophotometry (ICP-AES, Varian, Model Vista AX). In the case of Ni, concentrations were measured using inductively coupled plasma mass spectrometry (ICP-MS, Thermo Elemental, Model X-7). Metal recoveries for the reference material averaged 102.5 ± 1.1% for Cd, 101.4 ± 1.2% for Cu, 110.0 ± 2.2% for Zn and 92.7 ± 2.7% for Ni (mean ± SE).

Enzyme and protein assays

Liver and white muscle tissue samples that had been kept at −80°C were thawed on ice, weighed, diluted 50- and 20-fold, respectively with HEPES buffer (HEPES 20 mM, EDTA 1 mM, Triton X-100 0.1%, PMSF 1 mM, pH 7.5) and homogenized mechanically (Ultra Turrax T25 homogenizer; Janke and Kunkel). All enzyme activity determinations were performed within 6 h of homogenization. Preliminary experiments established the optimal substrate concentrations and pH values for measurement of CCO activity. Reactions were performed in potassium phosphate buffer (KH_2PO_4/K_2HPO_4 1 M, pH 7) with reduced cytochrome c (0.07 mM) as substrate. The reduction of cytochrome c was carried out by the addition of sodium dithionite and the solution was bubbled with air to eliminate the excess reducing agent (Couture et al. 2008a). Reactions were run against a control of 70 µM cytochrome c oxidized with 0.33% (w/v) potassium ferricyanide. Cytochrome c oxidase activity was measured at 550 nm by following the oxidation of reduced cytochrome c. For each sample, enzyme activities were measured in duplicate for 5 min using a temperature-controlled spectrophotometer (Varian, Model Cary 100) at 20°C with assay volumes of 1 ml, including 10 µl of tissue homogenate.

Total protein concentration was determined using a bicinchoninic acid assay. Tissue homogenates were first diluted 20-fold with a fresh solution (pH 11.25) containing bicinchoninic acid (29 mM), sodium carbonate (189 mM), sodium tartrate (8.25 mM), sodium hydroxide (100 mM), sodium bicarbonate (113 mM) and copper sulfate (250 mM). Samples were then diluted 5-fold with urea solution (urea 10 M, acetic acid 23.8 mM) and incubated at 37°C for 20 min. Absorbance at 562 nm of each sample was then measured in duplicate within 20 min. Protein concentrations were finally determined with reference to a bovine serum albumin (BSA) standard curve. All chemicals were purchased from Sigma–Aldrich.

Partial sequencing of cDNA for cytochrome c oxidase, Cu/Zn superoxide dismutase, heat shock protein 70 and metallothioneins

Total RNA was extracted from 40 mg of liver (wild perch from an unpolluted site) using the PureLink™

Micro-to-Midi™ Total RNA Purification System (Invitrogen), according to the manufacturer's instructions. RNA quality was evaluated by electrophoresis on a 1% agarose gel and RNA concentrations as well as purity were determined by spectrophotometry (GeneQuant RNA/DNA Calculator, Pharmacia). A sample of 5 µg of purified RNA was subjected to DNAse I treatment (2 U μg^{-1}, final volume = 50 µl) for 45 min at ambient temperature in order to remove residual genomic DNA. The reaction was stopped by adding 5 µl of EDTA solution (25 mM) and by heat treatment (10 min at 65°C). First-strand cDNA was then synthesized from 5 µg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions. To obtain a partial coding sequence for cytochrome c oxidase, Cu/Zn superoxide dismutase, metallothioneins and heat shock protein 70, oligonucleotide primers were deduced from alignment of corresponding sequences available in libraries from different fish species (using Clustalw software, EMBL-EBI). These primers, deduced from conserved sequences, were then used in PCR experiments (50 amplification cycles at 95°C for 1 min, 48°C for 1 min and 72°C for 1 min). Both DNA strands of the amplified products of expected size were then sequenced.

Quantitative RT-PCR

For each gene, specific primers and Taqman® probe were determined (Table 1) using the Primer Express® software (Applied Biosystems). Total RNA was extracted from 20 mg of liver and 50 mg of muscle using the PureLink™ Micro-to-Midi™ Total RNA Purification System (Invitrogen), according to the manufacturer's instructions. For muscle, the tissue homogenate was first treated with one volume of Trizol (Invitrogen) prior to RNA extraction in order to eliminate excess collagen fibres and lipids. For each sample, RNA quality and concentration were evaluated as previously described (see above). Purified RNA was subjected to DNAse I treatment (2 U μg^{-1} and 1 U μg^{-1} for liver and muscle tissue, respectively, final volume = 50 µl) as described above. Following the reverse transcriptase reaction (see above), cDNA from liver and muscle was diluted 25- and 20-fold, respectively. Real-time PCR reactions were then performed in triplicate in a ABI PRISM ®7000 Sequence Detection System (Applied Biosystems) following the manufacturer's instructions (50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min). Each 25 µl reaction contained 12.5 µl Taqman Universal PCR master mix (Applied Biosystems), the specific primer pairs at a final concentration of 900 nM each and the specific Taqman® probe at a final concentration of 250 nM. For each primer and probe set, two negative controls were also amplified: non-reverse transcribed total RNA treated with DNAse (as a control for contamination by genomic DNA) and a template negative sample, to control for any contamination of the reagents. Amplification efficiencies for all primer/probe sets were calculated following the manufacturer's instructions; all values proved to be sufficient to allow direct comparison of amplification plots according to the ΔΔCt method (see Livak and Schmittgen 2001). Relative quantification of gene expression was achieved by concurrent amplification of the *β-actin* endogenous control. To this end, total RNAs were quantified and 5 µg used for reverse-transcription. During the subsequent qPCR amplifications, the output cycle corresponding to the *β-actin* was investigated. This output was always obtained around the same cycle for control and contaminated individuals, demonstrating the relevance of the *β-actin* as a reference gene under our conditions.

Data treatment

Comparisons among fish groups were performed by analysis of variance (ANOVA), after checking assumptions of normality and homoscedasticity of the error terms. When the assumptions were not met as deduced graphically and from ad-hoc tests, we used log and box-cox (Peltier et al. 1998) data transformations or the non-parametric Kruskall-Wallis test. If significant effects were detected, the least square deviation test (LSD) and the non parametric *U*-Mann–Whitney test were used to determine whether means between pairs of samples were significantly different from one another. Computations were performed using STATISTICA version 6.1 software (StatSoft, USA). Because fish collected in the two sampled regions were exposed to different metal mixtures and to different ranges in metal concentrations, the relationships between individual metal concentrations in liver and gene expression levels, enzymatic activity or total protein concentrations were investigated separately, per region, using the non-parametric Spearman rank correlation test due to the non-linearity of the data. Numerical results are given as means ± SE (n = 100 per region).

Results and discussion

Hepatic Cd, Cu, Zn and Ni concentrations

Hepatic metal concentrations in yellow perch can be considered as an integrative measure of their prior metal exposure (Giguère et al. 2004). For both Cd and Cu, and for both geographical regions, hepatic concentrations in fish inhabiting the two metal-contaminated lakes were

Table 1 Sequences of specific primer pairs and Taqman® probes used in quantitative PCR analyses

Gene	Function	Specific primers (5′-3′)	Taqman® probe
β-actin	Internal standard	GCCTCTCTGTCCACCTTCCA[a]	CAGATGTGGATCAGCAAG
		GGGCCGGACTCATCGTACT[b]	
cco-1	Aerobic ATP production	GCCCGGCGCTCTCCTA[a]	AGACGACCAGATTTAT
		CGAAGGCATGTGCTGTAACAA[b]	
igf-1	Cell growth	AGTACCGCAGGGCACAAAGT[a]	ACAAGGGCACAGAGC
		CTGGCTGCTGTGCTGTCCTA[b]	
sod-1	Detoxification/oxidative stress	GCATGTAGGAGACTTGGGCAAT[a]	TGACTGCAGGAGGAGATA
		CCGTGATTTCTATCTTGGCAACA[b]	
mts	Metal detoxification	ATCCTGCACTTGCACGAACTG[a]	CACCTGCAAGAAGAG
		ACATCCAGAGGCGCACTTG[b]	
hsp-70	Protein folding and protection	TGTTGGTCGGTGGCTCAA[a]	CCGTATCCCTAAGATC
		TTGAAGAAGTCCTGAAGCAGCTT[b]	

[a] Forward primer

[b] Reverse primer

significantly higher than those determined in fish inhabiting the two clean lakes (Fig. 1). The highest values were observed in the livers of fish inhabiting the Rouyn-Noranda region, namely in fish from Lakes Dufault and Osisko for Cd and Cu, respectively. For Zn, fish inhabiting metal-contaminated lakes showed slightly higher concentrations than those inhabiting clean lakes. However, in each region, no significant differences were observed between fish collected in the two contaminated lakes and between fish caught in the two clean lakes. For this reason, and also because Zn is known to be better regulated at the sub-cellular level in wild yellow perch than Cd, Cu or Ni (Giguère et al. 2004, 2005, 2006), Zn was not considered in the analysis of metal effects on fish. Finally, for Ni, although no significant differences could be observed among fish caught in the four lakes sampled in the Rouyn-Noranda region, fish inhabiting Lakes Whitson and Hannah were significantly more contaminated than those inhabiting James and Geneva, the two clean lakes of the Sudbury region.

Morphometric, gene expression and proteic measurements

Rouyn-Noranda area—liver tissue

In the Rouyn-Noranda region, due to the absence of significant differences in Ni bioaccumulation among fish collected in the different lakes retained for our investigations, possible impacts of this metal on fish were not investigated. Significant relationships between Cd or Cu concentrations and gene expression levels (*cco-1*, *igf-1*, *sod-1*, *hsp-70* and *mts*), FCF, CCO activity or total protein concentration were observed in both liver and muscle tissue (Fig. 2). At the hepatic level, correlations between gene expression or protein measurements and hepatic Cd concentrations were more important than those for hepatic Cu concentrations: whereas the expression of only two genes (*sod-1* and *mts*) was significantly correlated with Cu concentrations, five of the seven variables considered (*cco-1*, *sod-1*, *hsp-70*, *mts* and [protein]) were significantly correlated with Cd concentrations. The strongest correlations with Cd concentrations were observed with *cco-1* and *sod-1*, the expression of these genes being negatively correlated with hepatic Cd concentrations.

Superoxide dismutase is responsible, in the cytoplasm, for the breakdown of the radical anion superoxide into hydrogen peroxide (H_2O_2). It is now well-established that expression of this gene is controlled by the oxidative status of the cell, as its expression level increases when reactive oxygen species (ROS) production increases (Scandalios 2005). Our results thus suggest that hepatic ROS production decreases in fish most impacted by Cd. This result is counter-intuitive, since numerous experimental investigations have shown that Cd can indirectly promote oxidative stress in cells (Wang et al. 2004; Shi et al. 2005). Analogously, exposure to Cd by intraperitoneal injection (2 mg kg^{-1}) was found to enhance hepatic *sod-1* gene expression levels in the European flounder (Sheader et al. 2006). However, the present results are consistent with previous work carried out on native yellow perch inhabiting the Rouyn-Noranda region. Along the metal contamination gradient, hepatic concentrations of malondialdehyde, an end-product of lipid peroxidation, as well as the in vitro activity of glutathione reductase, an enzyme involved in protection against oxidative stress, were also found to decrease with increasing hepatic Cd concentrations (Giguère et al. 2005). These authors hypothesized that metallothioneins, initially

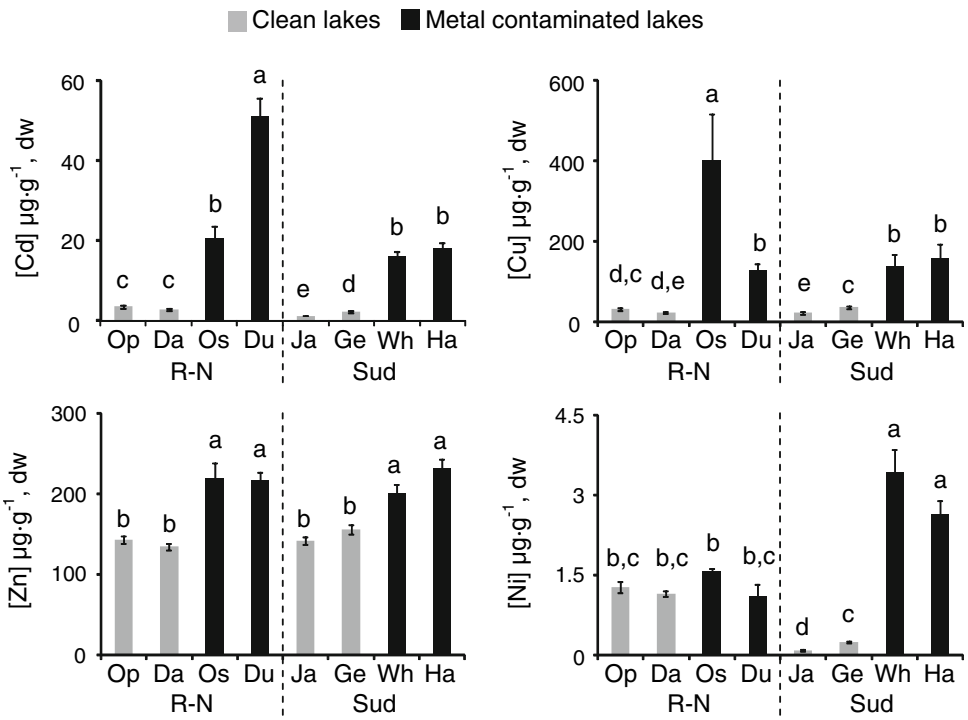

Fig. 1 Hepatic Cd, Cu, Zn and Ni concentrations (mean ± SE, $n = 25$) of fish sampled in the Rouyn-Noranda (*R-N*; Lakes Opasatica, *Op*; Dasserat, *Da*; Osisko, *Os* and Dufault, *Du*) and Sudbury (*Sud*; Lakes James, *Ja*; Geneva, *Ge*; Whitson, *Wh* and Hannah, *Ha*) regions. Means designated with *different letters* (*a, b, c, d, e*) are significantly different (LSD and *U*-Mann Whitney test, $P < 0.05$)

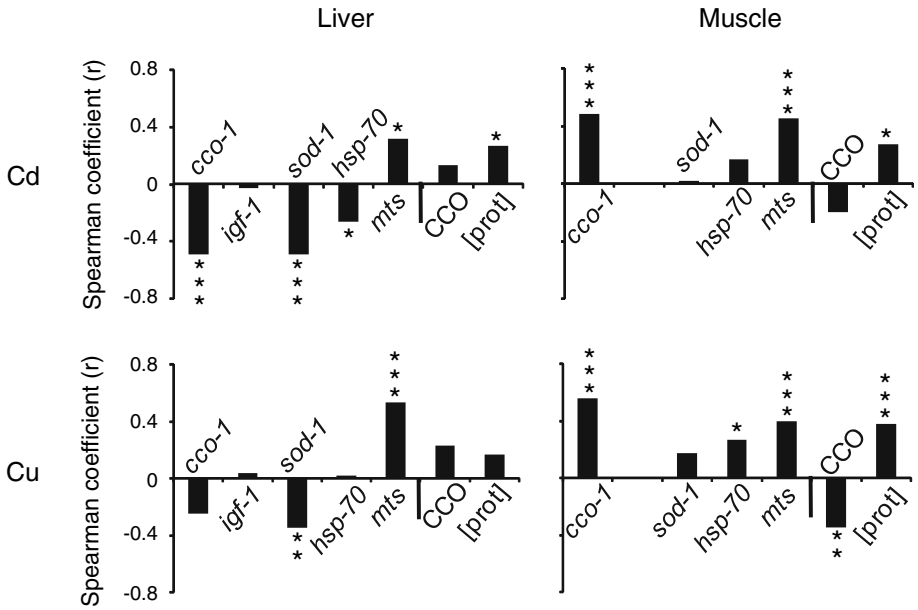

Fig. 2 Spearman correlation coefficients (r) between hepatic Cd (*top panels*) or Cu (*bottom panels*) concentrations and (i) gene expression levels of *cco-1, igf-1, sod-1, hsp-70* and *mts* (see Table 1 for more details), (ii) cytochrome c oxydase enzymatic activity (CCO) or (iii) total protein concentrations ([prot]) of liver (*left panels*) and muscle (*right panels*) from fish sampled in the Rouyn-Noranda region ($n = 100$). * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$

produced to sequester metals, could also scavenge ROS generated by metal exposure. Indeed, MTs are characterized by an important proportion of cysteinyl residues (~30%), which serve as ligands for metal chelation but can also trap ROS efficiently (Viarengo et al. 2000). Additionally, recent reports have demonstrated that *mts* gene expression levels are up-regulated not only by metal exposure but also by ROS generation (Viarengo et al. 2000). In agreement with this hypothesis, the gene expression level of *mts* was found to increase with increasing hepatic Cd concentrations (Fig. 2).

Alternatively, as the mitochondrion is, in absence of any contamination, the main source of ROS in the cell (Wang et al. 2004), another possible explanation of our present results would be that chronically accumulated Cd causes an overall decrease in mitochondrial metabolism. Such an explanation is consistent with the fact that the decrease in *sod-1* gene expression level in Cd-contaminated fish was highly correlated with a decrease in *cco-1* gene expression level ($r = 0.68$, $P < 0.00001$). Such a decrease in *cco-1* gene expression level was first described for the liver of

carp experimentally exposed to low concentrations of a mixture of waterborne and dietary Cd (Reynders et al. 2006). Since this decrease was coupled to an increase in glucokinase and malic enzyme gene expression levels, the authors suggested that Cd exposure triggers a stimulation of anaerobic metabolism and a decreased energy production in the citric acid cycle. In support of this hypothesis, a decrease in *cco-1*, *sod-2* (mitochondrial isoform) and catalase (*cat*), a cytoplasmic enzyme that converts hydrogen peroxide (the product of SOD) into O_2 and water, was, analogous to that found here, also described in gills of glass eels (*Anguilla anguilla*) experimentally exposed not only to waterborne Cd but also to hypoxia. In the case of hypoxia, the decrease in the expression level of genes involved in the mitochondrial respiratory chain or in protection against oxidative stress was attributed to the fact that adaptation of organisms to low oxygen levels proceeds notably, among other pathways, by reducing the activity of the major cellular consumer of oxygen, the mitochondria (Pierron et al. 2007).

Rouyn-Noranda area—muscle tissue

In the muscle tissue of fish collected in the Rouyn-Noranda region, changes in gene expression levels, in CCO activity or in total protein concentration were, unlike the results for liver, mainly correlated not with Cd but rather with accumulated Cu (Fig. 2). Whereas only three variables (*cco-1*, *mts* and [protein]) were significantly correlated with Cd concentrations, five of the six variables analyzed were significantly correlated with Cu concentrations. These significant correlations were observed at both the genetic and protein levels. At the genetic level, Cu concentrations were positively correlated with *cco-1, hsp-70* and *mts* gene expression levels. Although no significant correlation was observed between *sod-1* gene expression level and hepatic Cu concentrations, the mean expression level of *sod-1* was significantly higher in fish collected in Lake Osisko, i.e., in the lake where the fish presented the highest Cu concentrations (Fig. 1), than in the other lakes (Table 2). Such a transcriptional pattern is consistent with a previous work performed on yeast. By means of a DNA microarray, Yasokawa et al. (2008) reported an up-regulation of genes encoding for Cu/Zn superoxide dismutase, metallothioneins, heat shock proteins and key components of the respiratory chain in response to Cu exposure. Concerning the results obtained at the proteic level, increasing Cu concentrations were associated with a significant decrease in CCO activity and, in contrast, with an increase in total protein concentration. Both genetic and protein measurements suggest that chronic Cu contamination could lead to a degradation and/or inhibition of muscle enzymes, particularly CCO. In agreement with this hypothesis, an inhibitory effect of Cu on CCO was first reported in liver of rats (*Rattus norvegicus*) fed long-term (8 weeks) with Cu-enriched food (Sokol et al. 1993). In this context, the increases in *hsp-70* gene expression level, in total protein concentration and especially in the *cco-1* gene expression level suggest a compensatory mechanism. However, this potentially adaptive response appeared to be insufficient to curb the deleterious effect of Cu on muscle in fish inhabiting Lake Osisko. Indeed, whereas no significant differences in muscle CCO enzymatic activity were observed among fish collected in Lakes Opasatica, Dasserat and Dufault, the CCO activity in fish caught in Lake Osisko was significantly lower (Table 2). These results agree with previous research that reported impairment in muscle aerobic capacities of wild Cu-contaminated yellow perch. The activity of citrate synthase (CS), an enzyme involved in the mitochondrial Krebs cycle, was found to decrease in muscle tissue of wild yellow perch with increasing Cu exposure (Rajotte and Couture 2002; Audet and Couture 2003; Couture and Kumar 2003). In contrast, CS activity in the liver was unaffected by increases in Cu exposure (Couture and Kumar 2003). These results suggest that muscle tissue of wild yellow perch is particularly sensitive to Cu contamination. This could be, in part, linked to the sub-cellular partitioning of Cu in fish muscle fibres. Jenkins et al. (1982) collected white croakers (*Genyonemus lineatus*) from two stations in southern California (one more contaminated than the other) and used liquid chromatography to determine the cytosolic metal distribution in liver and muscle tissues. Although most of the hepatic Cu was associated with the metallothionein peak ($\sim$83%), the Cu bioaccumulated in muscle tissue was found to be entirely associated with the high molecular weight pool, i.e., potential sites of toxicity such as the CCO enzyme.

Sudbury area—liver tissue

In the Sudbury region (Fig. 3), correlations among hepatic metal concentrations, protein and genetic measurements were fewer than those determined for fish collected in the Rouyn-Noranda region. Only the two protein variables analyzed, i.e., CCO enzymatic activity and total protein concentration, were significantly and positively correlated with hepatic Cd, Cu and Ni concentrations.

Past studies analysing the sub-cellular distribution of Cd, Cu and Ni in the liver of yellow perch have demonstrated that Ni, contrary to Cd and Cu, is mainly bioaccumulated in the heat-denaturable proteins fraction (i.e., in proteins other than MT-like proteins), suggesting that Ni could be the main metal responsible for sub-lethal effects in indigenous yellow perch inhabiting the Sudbury area (Giguère et al. 2006). Moreover, a positive correlation between Ni contamination and the enzymatic activities of

Table 2 Means of Fulton condition factor (FCF), basal gene expression level of *cco-1, igf-1, sod-1, hsp-70* and *mts* (see Table 1 for more details), in vitro cytochrome c oxidase activity (CCO) and total protein concentration ([prot]) in liver and muscle tissue of fish sampled in 8 lakes located in Rouyn-Noranda and Sudbury regions. For each region, lakes above the dashed line are considered clean, whereas lakes below the dashed line are metal-contaminated

		Liver						
		Genetic analyses†					Proteic analyses	
	FCF	*cco-1*	*igf-1*	*sod-1*	*hsp-70*	*mts*	CCO#	[prot]*
Rouyn-Noranda								
Op	$1.04^{a,b} \pm 0.02$	$0.86^{b,c} \pm 0.05$	$0.073^{b,c} \pm 0.006$	$0.030^{a} \pm 0.003$	$0.24^{a,b} \pm 0.01$	$0.73^{c} \pm 0.09$	$10.84^{c} \pm 0.26$	$240.6^{a,b} \pm 9.8$
Da	$1.15^{a} \pm 0.02$	$0.87^{b,c} \pm 0.05$	$0.075^{b,c} \pm 0.005$	$0.031^{a} \pm 0.002$	$0.22^{b} \pm 0.01$	$0.50^{d} \pm 0.08$	$11.45^{c} \pm 0.55$	$162.9^{d} \pm 15.5$
Os	$0.89^{c,d} \pm 0.02$	$0.80^{c} \pm 0.03$	$0.072^{c} \pm 0.004$	$0.023^{b} \pm 0.002$	$0.24^{b} \pm 0.01$	$1.81^{a} \pm 0.33$	$14.49^{a,b} \pm 0.55$	$196.2^{c,d} \pm 4.2$
Du	$0.96^{b,c} \pm 0.02$	$0.59^{d} \pm 0.05$	$0.070^{c,d} \pm 0.004$	$0.016^{c} \pm 0.002$	$0.18^{c} \pm 0.01$	$0.83^{b,c} \pm 0.10$	$10.92^{c} \pm 0.56$	$222.0^{b,c} \pm 6.2$
Sudbury								
Ja	$0.87^{d} \pm 0.01$	$0.99^{a,b} \pm 0.07$	$0.050^{e} \pm 0.003$	$0.017^{c} \pm 0.002$	$0.15^{c} \pm 0.01$	$1.56^{a} \pm 0.17$	$13.06^{b,c} \pm 0.59$	$198.3^{c,d} \pm 8.3$
Ge	$1.02^{a,b} \pm 0.01$	$1.06^{a} \pm 0.09$	$0.084^{b} \pm 0.006$	$0.036^{a} \pm 0.002$	$0.26^{a} \pm 0.02$	$1.00^{b} \pm .07$	$14.02^{a,b} \pm 0.41$	$200.6^{b,c,d} \pm 5.7$
Wh	$0.97^{b,c} \pm 0.01$	$0.75^{c} \pm 0.05$	$0.057^{d,e} \pm 0.004$	$0.018^{b,c} \pm 0.001$	$0.16^{c} \pm 0.01$	$0.95^{b,c} \pm 0.15$	$14.54^{a,b} \pm 0.34$	$261.5^{a} \pm 5.6$
Ha	$0.96^{b,c,d} \pm 0.01$	$1.05^{a} \pm 0.06$	$0.099^{a} \pm 0.006$	$0.035^{a} \pm 0.002$	$0.23^{b} \pm 0.01$	$1.82^{a} \pm 0.18$	$16.79^{a} \pm 0.36$	$234.6^{a,b} \pm 6.0$

		Muscle					
		Genetic analyses†				Proteic analyses	
	FCF	*cco-1*	*sod-1*	*hsp-70*	*mts*	CCO#	[prot]*
Rouyn-Noranda							
Op	$1.04^{a,b} \pm 0.02$	$1.50^{d} \pm 0.18$	$0.014^{e} \pm 0.002$	$0.18^{b} \pm 0.01$	$0.020^{e} \pm 0.002$	$3.65^{b,c} \pm 0.10$	$125.0^{d} \pm 7.2$
Da	$1.15^{a} \pm 0.02$	$2.01^{c} \pm 0.19$	$0.033^{b,c} \pm 0.005$	$0.16^{b} \pm 0.02$	$0.042^{d} \pm 0.003$	$3.13^{c} \pm 0.14$	$149.4^{c} \pm 5.0$
Os	$0.89^{c,d} \pm 0.02$	$5.03^{a} \pm 0.30$	$0.048^{a} \pm 0.004$	$0.25^{a} \pm 0.02$	$0.059^{b,c} \pm 0.006$	$1.40^{d} \pm 0.14$	$206.4^{a} \pm 9.8$
Du	$0.96^{b,c} \pm 0.02$	$2.87^{b} \pm 0.19$	$0.017^{d,e} \pm 0.002$	$0.17^{b} \pm 0.02$	$0.059^{b} \pm 0.004$	$3.51^{b,c} \pm 0.18$	$157.4^{b,c} \pm 9.1$
Sudbury							
Ja	$0.87^{d} \pm 0.01$	$3.15^{b} \pm 0.25$	$0.013^{e} \pm 0.001$	$0.12^{c} \pm 0.01$	$0.037^{d} \pm 0.003$	$4.80^{a,b} \pm 0.30$	$171.2^{b} \pm 8.3$
Ge	$1.02^{a,b} \pm 0.01$	$5.15^{a} \pm 0.39$	$0.045^{a} \pm 0.006$	$0.28^{a} \pm 0.02$	$0.094^{a} \pm 0.011$	$5.88^{a} \pm 0.24$	$213.8^{a} \pm 11.7$
Wh	$0.97^{b,c} \pm 0.01$	$4.36^{a} \pm 0.26$	$0.020^{c,d} \pm 0.003$	$0.18^{b} \pm 0.01$	$0.072^{b} \pm 0.008$	$5.84^{a} \pm 0.23$	$167.7^{b,c} \pm 7.6$
Ha	$0.96^{b,c,d} \pm 0.01$	$2.28^{c} \pm 0.17$	$0.027^{c} \pm 0.002$	$0.15^{b,c} \pm 0.01$	$0.047^{c,d} \pm 0.004$	$4.52^{a,b,c} \pm 0.21$	$199.4^{a} \pm 9.0$

All data are expressed as means ± SE ($n = 25$). For each parameter, means designated with different letters (a, b, c, d, e) are significantly different (LSD and *U*-Mann Whitney tests, $P < 0.05$)
Units: † expressed as arbitrary units, # expressed as μmol min^{-1} g^{-1}, * expressed as mg g^{-1}

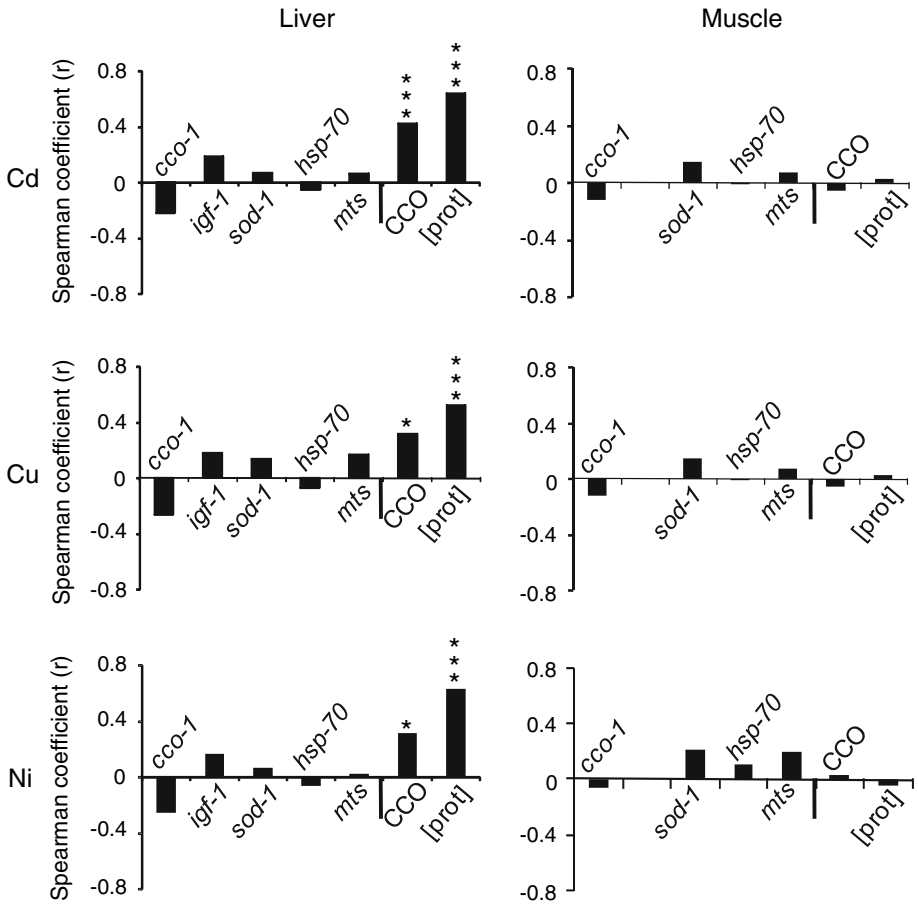

Fig. 3 Spearman correlation coefficients (r) between hepatic Cd (*top panels*), Cu (*middle panels*) or Ni (*bottom panels*) concentrations and (i) gene expression levels of *cco-1, igf-1, sod-1, hsp-70* and *mts* (see Table 1 for more details), (ii) cytochrome c oxydase enzymatic activity (CCO) or (iii) total protein concentrations ([prot]) of liver (*left panels*) and muscle (*right panels*) from fish sampled in the Sudbury region ($n = 100$). * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$

CCO and CS in yellow perch was first reported by Couture et al. (2008a). Thus, we hypothesize that the positive correlations between hepatic metal concentrations and CCO activity in the Sudbury yellow perch are mainly due to Ni contamination (cf. Borgmann et al. 2001) and that the apparent relationships with Cd and Cu are the result of the three metals being spatially correlated among themselves ($r_{Cd–Cu} = 0.79$, $r_{Cd–Ni} = 0.82$ and $r_{Cu–Ni} = 0.76$; $P < 0.000001$ in all cases). Nickel contamination seems to increase, in general, the post-transcriptional activity of the liver, leading to higher protein concentrations and higher CCO activities. Whether this trend reflects a compensation for the direct effects of Ni on proteins (Giguère et al. 2006), or a response to an increase in energy demand in order to curb the stress triggered by Ni bioaccumulation, remains to be determined.

Another interesting observation from the Sudbury fish is the absence of significant correlations among hepatic metal concentrations and expression of any gene. This contrast with the Rouyn-Noranda fish presumably reflects the lower hepatic Cd concentrations observed in the Sudbury fish. For example, if we consider mean expression levels, although no significant differences in liver *cco-1* gene expression levels were observed among fish collected in Lakes Opasatica, Dasserat and Osiko, its expression level in fish caught in Lake Dufault was significantly lower (Table 2). Cadmium contamination levels determined in fish sampled in the two contaminated lakes of the Sudbury region were similar to those defined in fish sampled in Lake Osisko and were significantly lower than those determined in fish sampled in Lake Dufault. Thus, we suggest that correlations were more significant in Rouyn-Noranda fish because the lakes from which the perch were collected represented a greater range of environmental Cd concentrations than did the Sudbury lakes.

Table 3 Spearman correlation coefficients of the relationship between the Fulton condition factor and hepatic Cd, Cu or Ni concentrations in fish sampled in the Rouyn-Noranda and Sudbury regions ($n = 100$ per region)

Region	Hepatic metal concentrations		
	Cd	Cu	Ni
Rouyn-Noranda	−0.59*	−0.52*	ND
Sudbury	0.13	0.16	0.19

ND = not determined

* Denotes significant relationship, $P < 0.0001$

Similar results were obtained at the whole organism level. Whereas the condition factors of fish collected in the Rouyn-Noranda region were negatively and significantly correlated with hepatic metal concentrations, such relationships were not observed in the case of fish sampled in the Sudbury region (Table 3). In the case of Sudbury fish, this latter result reflects the fact that both the lowest and highest values of the FCF were observed for fish from the clean lakes (Table 2), i.e., Lake James (low condition factor) and Lake Geneva (the only condition factor >1).

The foregoing observations suggest that environmental factors other than direct metal exposure have strongly affected the fish inhabiting the four Sudbury lakes that we sampled. In support of this argument, the expression level of the gene encoding for IGF-1 was similar in all fish collected in the Rouyn-Noranda region, whereas its expression level differed significantly among fish caught in the four lakes sampled in the Sudbury region (Table 2). The lowest values were observed in fish collected in Lakes James and Whitson, i.e., a clean and a metal-contaminated lake. As the production of IGF-1 is firmly under the influence of the growth hormone and since the secretion of this hormone is itself partly influenced by the food consumption rate of fish (Lindsey et al. 2007), a decrease in its expression level could represent a decrease in the amount of food available for fish in Lakes James and Whitson.

Sudbury area—muscle tissue

Unlike the results for the Rouyn-Noranda fish, where Cu concentrations and muscle CCO activity were negatively correlated, no significant correlations with metals were observed at the muscle level in fish collected in the Sudbury lakes (Fig. 3). The relationship in the Rouyn-Noranda lakes is influenced by the results from Lake Osisko, where the highest muscle Cu concentrations were observed (Fig. 1); no significant differences in muscle CCO enzymatic activity were observed among fish collected in Lakes Opasatica, Dasserat and Dufault, but its activity in fish caught in Lake Osisko was significantly lower (Table 2). Copper contamination levels determined in fish sampled in the two contaminated lakes of the Sudbury region were similar to those determined in fish sampled in Lake Dufault but significantly lower than those determined in fish sampled in Lake Osisko. Thus the absence of effects in the Sudbury fish likely reflects their lower Cu concentrations.

In general, the highest expression levels, for all genes, were measured in fish caught in Lake Geneva and the lowest were determined for fish caught in the other clean lake, i.e. Lake James. Fish collected in Lakes Whitson and Hannah, i.e., the two metal-contaminated lakes, presented intermediate values, even for *mts* gene expression levels (Table 2). This result is quite surprising and raises several questions. For example, are the elevated values of *mts* gene expression from fish collected in Lake Geneva, compared to those collected in lakes Hannah and Whitson, due to environmental factors other than metals? Several environmental factors other than metal contamination, such as parasitism, reproductive status, hypoxia and food availability, are known to affect *mts* gene expression levels (Murphy et al. 1999; Baudrimont et al. 2006; Hashemi et al. 2008). Clearly, the impact of such factors on the expression of genes examined in this study remains to be investigated in more detail.

Plastic regulation and structural genomic changes

Although plastic/transcriptional regulation seems more probable than genetic evolution of organisms, several studies have highlighted the potential importance of structural genomic changes (e.g., allelic changes, gene duplication, mutations, variations in the promoter region) in the expression of genes encoding for proteins involved in resistance to environmental stresses, such as *mts* and *hsp-70* (see Hoffmann and Willi 2008 for review). In yellow perch, Bourret et al. (2008), using neutral microsatellite DNA markers, demonstrated a negative relationship between levels of genetic diversity within populations and the level of metal contamination. They concluded that 80 years of metal contamination had significantly impacted patterns of genetic diversity among populations of wild yellow perch. However, Ni could not be taken into account in their analysis. Bourret et al. (2008) also demonstrated that Rouyn-Noranda and Sudbury yellow perch populations are genetically very distinct and could have distinct refugial origins. Moreover, genetic diversity was found to be more important among perch populations in Sudbury lakes than in fish inhabiting the Rouyn-Noranda region. These differences could contribute to the divergences we observed in patterns of gene expression between the two regions. However, since analyses by Bourret et al. (2008) were performed using neutral loci, further investigations with functional coding genes, such as those encoding for metallothioneins and heat shock proteins are needed to test such effects.

Conclusions

This study demonstrates that lifelong exposure to elevated but environmentally relevant metal concentrations is linked to changes in gene expression in yellow perch. Our results suggest that measurements of gene expression show promise for field investigations in ecotoxicology. The most important changes were observed in fish inhabiting the Rouyn-Noranda region, particularly in the most

Cd-contaminated perch populations. In these fish, Cd contamination was mainly associated with a decrease in both hepatic *cco-1* and *sod-1* gene expression levels. In contrast, in the same region, Cu contamination was mainly linked to increased *mts* and *cco-1* gene expression levels in muscle tissue, coupled with a marked decrease in CCO enzyme activity. These results strongly suggest that chronic metal exposure to Cd and Cu, at the gene expression and proteic levels, respectively, impairs mitochondrial metabolism. The two metals clearly have different modes of action, the demonstration of which in wild fish would not have been possible without the complementary use of a transcriptomic approach and protein measurements. These specific effects of Cd and Cu on yellow perch were not observed in fish collected in the Sudbury region, presumably because Cd and Cu contamination levels in Sudbury fish were lower than those in Rouyn-Noranda fish. In this context, i.e., lower stress imposed by metal contamination, environmental factors other than metals (e.g., food availability) may well affect gene expression in native yellow perch. However, the fact that bioaccumulated Ni concentrations were significantly associated with an increase in both hepatic CCO activity and total protein concentrations suggests that Ni contamination adversely affects Sudbury fish. In this perspective, a genome-wide investigation of gene transcription profiles by means of cDNA microarray specific to yellow perch would be particularly relevant. Such a study could identify biological functions that are specifically affected by chronic Ni contamination.

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