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How does exposure to nickel and cadmium affect the transcriptome of yellow perch (*Perca flavescens*) – Results from a 1000 candidate-gene microarray☆

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ABSTRACT

The molecular mechanisms underlying nickel (Ni) and cadmium (Cd) toxicity and their specific effects on fish are poorly understood. Documenting gene transcription profiles offers a powerful approach toward identifying the molecular mechanisms affected by these metals and to discover biomarkers of their toxicity. However, confounding environmental factors can complicate the interpretation of the results and the detection of biomarkers for fish captured in their natural environment. In the present study, a 1000 candidate-gene microarray, developed from a previous RNA-seq study on a subset of individual fish from contrasting level of metal contamination, was used to investigate the transcriptional response to metal (Ni and Cd) and non metal (temperature, oxygen, and diet) stressors in yellow perch (*Perca flavescens*). Specifically, we aimed at (1) identifying transcriptional signatures specific to Ni and Cd exposure, (2) investigating the mechanisms of their toxicity, and (3) developing a predictive tool to identify the sublethal effects of Ni and Cd contaminants in fish sampled from natural environments. A total of 475 genes displayed significantly different transcription levels when temperature varied while 287 and 176 genes were differentially transcribed at different concentrations of Ni and Cd, respectively. These metals were found to mainly affect the transcription level of genes involved in iron metabolism, transcriptional and translational processes, vitamin metabolism, blood coagulation, and calcium transport. In addition, a linear discriminant analysis (LDA) made using gene transcription levels yielded 94% correctly reassigned samples regarding their level of metal contamination, which indicates the potential of the microarray to detect perch response to Cd or Ni effects.

1. Introduction

Understanding the mechanisms underlying metal toxicity is crucial to predict the impacts of metals in organisms living in aquatic environments subjected to metal release. Different types of biomarkers, including biometric, physiological, and biochemical indicators, for example condition factor, enzyme activity, or lysosomal parameters, have been proposed to measure and predict the consequences of metal exposure in the wild (Lam, 2009; Handy and Depledge, 1999; Regoli, 1992). However, aquatic environments are characterized by fluctuations of various factors, including water temperature, food availability, and dissolved oxygen concentrations, that also affect organisms. As a result, these environmental factors complicate the use and discovery of biomarkers specific to contamination by metals (Forbes et al., 2006; Lam, 2009).

The last decade has seen an increasing use of transcriptomic tools to identify gene expression biomarkers indicative of the modes of action of metal contamination (Altenburger et al., 2012; Fedorenkova et al., 2010; Van Aggelen et al., 2010). For example, a set of genes showing differential expression under varying contaminant concentrations could be used to identify mechanisms of metal toxicity (Jeffries et al., 2012; Pierron et al., 2011; Poynton et al., 2007, 2008). Such genes could be considered as biomarkers of metal toxicity when it is demonstrated that their transcription levels are associated with evidence of toxicity. The potential to discover biomarkers of exposure to, and effects of, chemical compounds

☆ Microarray data have been deposited with the NCBI/Gene Expression Omnibus (GEO) under accession number: GSE50975.

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using gene expression approaches was recently demonstrated by Garcia-Reyero et al. (2009) in a study that found six genes showing specific expression to chemical exposures (e.g. TNT, DNB and zinc) in *Daphnia magna*. In order to gain further insights into the transcriptomic responses to aqueous metal exposure in wild animals, it is important to study species that occupy a broad geographic range with variable types of habitats and the capacity to tolerate medium to high levels of pollution, such as the yellow perch. However, despite the growing interest in the use of transcriptomic tools for ecotoxicological studies, custom development of low-cost tools is still required, especially for aquatic species where genomic resources or a microarray are not already available.

Indeed, whereas RNA-seq may a priori represent the ideal tool to analyze global transcription patterns, two of the great advantages of microarrays are a low cost per sample and the simplicity of analyzing the data. RNA-seq data, on the other hand, remain quite expensive to produce, thus imposing a limit on the number of individuals being analyzed. They also typically require more computationally intensive analyses, and have more potential biases to correct for (Sîrbu et al., 2012). However, these two methods complement each other (Kogenaru et al., 2012). For example, one strategy to combine the main advantages of both approaches is to first analyze the transcriptome by means of RNA-seq in a pilot study and subsequently construct a large set of differently transcribed genes based on a small subset of individuals from contrasting environments (e.g. clean vs. polluted). Then, sequences from "candidate-genes" that differ in expression are used to develop a low cost custom microarray that can be employed to study the impacts of different experimental conditions, including metal contamination, on the transcriptome.

Because of their high tolerance to contaminated environments and widespread presence in lakes and rivers throughout North America, yellow perch (*Perca flavescens*) are an ideal species to study the impact of metal exposure on the transcriptome. Yellow perch are abundant in metal contaminated lakes of Canada, where they accumulate metals such as cadmium (Cd), zinc (Zn), nickel (Ni), and copper (Cu) found in their environment in their tissues (Giguère et al., 2004). Cadmium and Ni are the two major elements responsible for freshwater metal contamination by atmospheric deposition from mining and smelting activities in eastern Canada. Chronic exposure to high levels of Ni or Cd leads to well documented negative consequences on yellow perch, e.g. oxidative stress and disruption of ion homeostasis, metabolic capacities and the immune system (McGeer et al., 2011; Pyle and Couture, 2011). However, the specific effects and the modes of action of both metals on the molecular mechanisms underlying the more readily observable physiological effects remain largely unknown. The lack of understanding of the specific effects of these two metals in aquatic species is caused by their broad spectrum of action and the fact that some of the effects, for example induction of oxidative stress, are shared by the two metals and also modulated by environmental factors.

To identify metal effects at the transcriptomic level in ectothermic organisms, most studies so far have examined the effects of acute exposures (<96 h) at high metal concentrations. Such short exposure times are likely to produce misleading observations about the response of wild fish to metal exposure under environmentally relevant conditions. Others studies have tried to approach the problem with experimental approaches that more closely mimic long-term exposure to metals. For example, recent studies were able to detect transcriptomic changes following chronic exposure to low metal concentrations corresponding to concentrations observed in the environment in order to identify biomarkers of metal effects in yellow perch and other species (Pierron et al., 2011; Reynders et al., 2006).

In the present study, a 1000 candidate-gene microarray, developed from a previous RNA-seq study (Pierron et al., 2011) on a subset of individuals from contrasting level of metal contamination, was used to investigate the transcriptional response to metal (Ni and Cd) and non metal (temperature, oxygen, and diet) stressors in yellow perch. Our main objectives were: (1) to identify transcriptional signatures specific to the exposure to two metals: Ni and Cd; (2) to investigate the mechanisms of toxicity for these two metals; and (3) to develop a predictive tool to identify the sublethal effects of Ni and Cd contaminants in fish sampled from natural environments.

2. Materials and methods

2.1. Specific perch microarray construction

2.1.1. Candidate-gene selection and identification

One thousand oligonucleotide probes (58–62 bp) were designed from a set of transcripts comprising both a yellow perch transcriptome assembled from a previous 454 experiment focusing on metal contaminated and uncontaminated yellow perch (Pierron et al., 2011) and yellow perch transcripts available in online databases. The probes were designed using the command line version of ProDesign (Feng and Tillier, 2007) and synthesized by Sigma®. ProDesign maximizes probe specificity and ensures that each probe cannot be found among the list of interesting genes, as well as among the list of all the unwanted sequences. 6C amine spacers were added to each probe at the 5′ extremity. All probes were resuspended in SSC 3× and stocked in 384-well plates.

In total, 219 gene sequences were chosen from the literature according to their role in key biological pathways, such as lipid and energy metabolism, in fish. The other sequences were chosen from the 454 experiment as follows: 40 genes that presented an interesting SNP and 741 genes from the 1297 unique genes that were annotated from either the *swissprot* or the *nr* protein database (minimal *e*-value < $1.0\,e^{-10}$ using the blastx program from BLAST 2.2.23+, http://blast.ncbi.nlm.nih.gov). Among these 741 genes, 49 genes were manually chosen because of their important role in global metabolism or response to metal exposure, 196 genes were chosen because their expression levels were significantly correlated with hepatic Cd or Cu concentrations (Pierron et al., 2011), and the 496 remaining genes correspond to genes annotated with an *e*-value lower than $1.0\,e^{-30}$ and that were significantly different among lakes but not directly correlated with metal concentration. We added four positive control spots containing a mix of all probes and four negative spots that only contained SSC 3× and 6C spacers but no DNA probe.

2.1.2. Microarray printing

The probes were printed on SuperChip®Poly-L-Lysine slides (Thermo Scientific®) with the Spotarray 24 (PerkinElmer Life Sciences) at the Great Lakes Institute for Environmental Research (GLIER, University of Windsor, On, Canada). Stealth 3 Microarray Printing Pins were used to deposit 0.0125 μL (0.011 μg oligonucleotides) per spot onto the slide. The probes were then crosslinked with UV irradiation. The microarray layout consists of a 4-by-4 metagrid with 9 × 14 spots in the subgrids. Each probe was duplicated side by side and the 1000 probes were deposited in a 4-by-4 metagrid. Finally, the 4-by-4 metagrid is replicated three times on the chip. In total, the 1000 probes are thus replicated six times on each slide, improving the robustness and repeatability of the measurements.

2.2. Experiment: fish samples

2.2.1. Temperature, oxygen and diet effects

In 2010, juvenile yellow perch were obtained from Kinmount Fish Farm (Ontario, Canada). Upon arrival, the perch were kept in 1 m^3 indoor tanks for acclimation in water kept at 18 °C with a 12 d/12 n photoperiod at the Laboratoire régional en sciences aquatiques (LARSA, Université Laval, Québec, QC, Canada). After one month of acclimation, the perch were distributed in aquaria at a density of 160 g of biomass in 40 L of water (25 fish per aquarium), maintained at 100% oxygen saturation and fed a diet of frozen *Artemia salina* representing 3% of the tank biomass daily.

For the temperature experiment, fish were exposed to three different conditions: control (20 °C), low temperature (11 °C), and high temperature (28 °C). Temperature was gradually increased or reduced at a rate of 2 °C per day to reach the experimental temperatures. For the restrictive diet experiment, the aquaria parameters were set as follows: 20 °C, 100% oxygen (9.1 mg O_2/L), and diet representing 0.75% of the tank biomass daily. Finally, for the hypoxia experiment, the aquaria parameters were set as follows: 20 °C, 75% oxygen (6.8 mg O_2/L), and diet representing 3% of the tank biomass daily. In these experiments, lower reduced oxygen concentrations for the hypoxia group were not possible with the setup available at the fish facility. Also, diet representing 0.75% of the tank biomass daily was used because it is slightly above the value used in preliminary experiments where no mortality and no growth were observed over a long-term experiment with yellow perch (Gauthier et al., 2008). The temperatures were chosen to represent a wide range of temperatures to which fish from this species are exposed in the wild.

The experiments began after one week of acclimation at the target temperatures. All conditions were replicated in three aquaria.

2.2.2. Nickel and cadmium exposures

For the metal exposures, juvenile yellow perch were obtained from Pisculture RG (Trois-Rivières, QC) in 2011. Upon arrival, the perch were kept in 1 m^3 indoor tanks for acclimation in water kept at 18 °C with a 12 d/12 n photoperiod at the LARSA. After one month of acclimation, they were distributed in aquaria with a biomass of 250 g of fish per 40 L tank (15 fish per aquarium). One week before the start of the experiment, water temperature in the aquaria was increase and then maintained at the same level (20 °C) as for the control experiments from 2010. Fish were exposed to different Cd and Ni water concentrations: control (0 μg/L), low Cd concentration (LCd, 0.86 ± 0.09 μg/L), high Cd concentration (HCd, 3.65 ± 0.21 μg/L), low Ni concentration (LNi, 68.5 ± 6.8 μg/L), and high Ni concentration (HNi, 542 ± 16 μg/L). The low Cd and Ni concentrations in water were chosen on the basis of concentrations observed in contaminated lakes from the Rouyn Noranda area for Cd and from the Sudbury area for Ni (Couture et al., 2008). The high Cd and Ni concentrations correspond to low concentrations multiplied roughly by a factor of 10 for Ni and a factor of 5 for Cd, in order to obtain significant metal accumulation in fish after the exposure period. All concentration conditions were replicated twice. Dissolved metal concentrations in the tanks were measured every week and adjusted as necessary. The experiments began after one week of acclimation.

For all the experiments, liver and kidney of fish were sampled after 45 days of exposure and immediately frozen in liquid nitrogen. All samples were stored at −80 °C.

2.3. Experiment: microarray analyses

2.3.1. RNA extraction, labeling, and cDNA hybridization

Total RNA was extracted from liver of the 104 fish (8 individuals per condition: three temperatures, two diet levels, two oxygen concentrations and the three levels for each of Cd and Ni). Total RNA was extracted with the PureLink™ Micro-to-Midi Total RNA Purification System Kit and then treated using Amplification Grade DNase I (3 unit/μL; Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Total RNA was stored in RNAse free water at −80 °C. Quality and integrity of the total RNA were controlled using an Experion Automated Electrophoresis Station and RNA HighSens Chips (Biorad, Hercules, CA). For each sample, 12 μg total RNA was reverse-transcribed and the cDNA samples labeled using the Genisphere 3DNA Array 50 Kit, Invitrogen's Superscript II retro-transcriptase and *Cyanine3* and *Cyanine5* fluorescent dyes (Genisphere), following the procedures described at http://genisphere.com/products/3dna-array-detection/support (Genisphere Array 50 Protocol). Five independent experiments (temperature, diet, oxygen conditions, Cd and Ni concentrations) were conducted and analyzed in loop designs, when there were three conditions, or pair-wise direct comparisons when there were only two conditions, for a total of 88 microarrays that were distributed as follows: 24 microarrays for the three temperature conditions, 8 microarrays for restrictive diet and control, 8 microarrays for hypoxia and control, 24 microarrays for the three Cd concentrations, including the control group, and 24 microarrays for the three Ni concentrations, including the control group. The loop design included pair-wise direct comparisons among samples of the different conditions. Each sample was technically replicated on two bi-colored microarrays and dye-swapped.

2.3.2. Data acquisition, preparation, and statistical analyses

Microarrays were scanned using a ScanArray scanner (PerkinElmer Life Sciences). Spots were localized and quantified with the QuantArray 3.0 software (PerkinElmer Life Sciences), using the histogram quantification method and using the mean value of intensity for each spot. Data from bad replicate spots were excluded from the data set and local background was subtracted from the signal. All of the 1000 genes were used in the analysis. The data were normalized according to the regional lowess procedure, as implemented in the R/MAANOVA package (Kerr et al., 2000), to remove intensity-dependent and region-dependent dye effects on each slide. In order to detect differences in transcription profiles among the conditions, data were analyzed using a mixed model ANOVA (Wolfinger et al., 2001) and the R/MAANOVA package (Kerr et al., 2000, 2002). We tested for the presence of significant treatment effects for the different levels within each experiment (temperature, diet, oxygen, Ni and Cd) with a mixed ANOVA where the Array term was included as random while "Dye" and "Condition type" were fixed terms. A permutation-based *F*-test (Fs, with 1000 sample ID permutations) was then performed and restricted maximum likelihood was used to solve the mixed model equations (Cui et al., 2005). The presence of condition type effects was tested with the ANOVA model. A False Discovery Rate correction (FDR = 0.05) was applied within the R/MAANOVA package to account for multiple testing and the corrected *p*-values were used to determine the significance of differential gene transcription levels among the experimental conditions.

For the temperature and metal experiments, a posteriori contrast tests were conducted with 1000 permutations for the three possible comparisons to generate a list of genes with significant differential expression among the different levels of temperature or water metal concentration (FDR = 0.05). For the Ni and Cd experiments, many more genes were found to be differentially transcribed by the a posteriori contrast tests (FDR = 0.05) than by the ANOVA tests (FDR = 0.05). Since we aimed at characterizing genes that may respond in a specific manner to these two metals, and since these genes are likely candidates, we chose to keep them for our analyses. As explained in the Discussion, subsequent steps

are also planned to further validate these results in the subsequent steps of this research program chapters.

2.3.3. Functional classification

Functional classification and assessment of significant differential representation of functional classes were performed in the Blast2Go software (http://www.blast2go.com/b2ghome) (Conesa et al., 2005) using Gene Ontology annotation (Fisher's exact test, significance threshold: p-value = 0.05). The Blast2Go annotation of all the genes from the microarray is available at http://www.bio.ulaval.ca/louisbernatchez/files/1000_perch_sequences_annotation_blast2go_v1.0.dat or by contacting the authors.

2.3.4. Discriminant analyses

Linear discriminant analysis (LDA) was applied on the transcription level data of the 1000 genes for the 104 fish using *R* to test and develop a predictive tool for the presence of metal specific contamination. All the gene transcription data from individuals of the temperature, diet and oxygen experiments were included as control individuals in addition to control individuals from the Ni and Cd experiments. Contamination levels for Cd and Ni were grouped into Cd contamination (both low and high) and Ni contamination (both low and high). We then used the LDA to produce a model that discriminated these three groups: control, Cd contamination, and Ni contamination. Finally, we used the model to reassign the individuals in order to determine its power to discriminate among the three categories. All microarray data were obtained after the rlowess normalization step and were adjusted using the scaling method, as implemented in the 'scale' *R* function.

2.4. Renal metal concentration analysis

Kidney metal concentrations were measured for the 24 samples from the Cd and Ni exposures using inductively coupled plasma-mass spectrometry. Previous studies on yellow perch showed that kidney and liver are both important for metal accumulation and that accumulation of metals in these tissues is correlated to water metal concentrations (Giguère et al., 2004; Couture et al., 2008). In our study, measurement of metal concentrations in kidneys was done to confirm that metal accumulation had occurred rather than to directly correlate the metal concentration in the fish to gene transcriptional levels. For these reasons, we measured the metal concentrations in kidneys and treated these measures as representative of fish contamination.

The samples were first freeze-dried and digested at room temperature for one week in trace-grade nitric acid, followed by hydrogen peroxide, according to the method described in Pierron et al. (2009). In order to monitor analytical accuracy and recovery, a certified reference material (TORT-2, National Research Council, Ottawa, ON, Canada) was submitted to the same protocol.

Normality and homoscedasticity of the Cd and Ni concentration data were verified, respectively, with the Shapiro and Bartlett tests. To detect the potential differences in Cd and Ni concentration, data were analyzed with an ANOVA test in *R*. Differences among the means were tested with either Tukey or Wilcoxon tests, dependent on whether they followed normality or not.

3. Results

3.1. Nickel and cadmium kidney concentration results

Nickel and Cd concentrations in kidneys were significantly different between the high concentration (Ni mean = 15.7 ± 4.2 μg/g dry weight (dw); Cd mean = 7.9 ± 1.4 μg/g dw) and both the control (Ni mean = 1.9 ± 1.4 μg/g dw; Cd mean = 3.6 ± 0.7 μg/g dw) and the low concentration treatments (Ni mean = 2.9 ± 1.0 μg/g dw; Cd mean = 4.7 ± 0.6 μg/g dw) (Fig. A1). However, the renal Cd and Ni concentrations from the low concentration treatment were not significantly different from those of the control group.

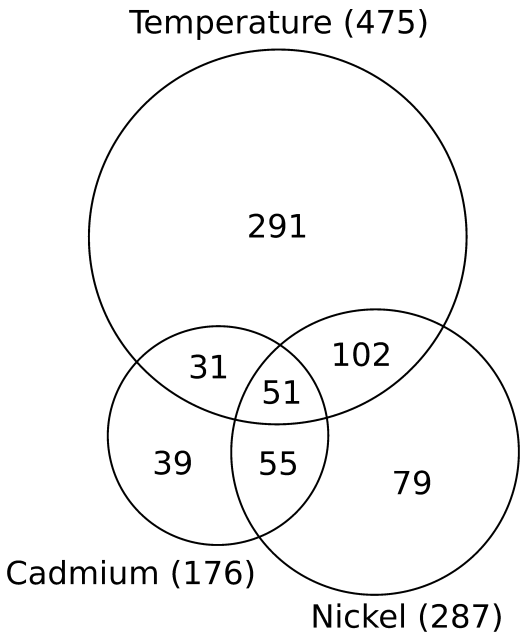

Fig. 1. Venn diagram presenting the number of differentially expressed transcripts among temperature and metal experiments. Intersection regions correspond to significant transcripts that are common among the experiments.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2013.09.009.

3.2. Gene transcription differences

3.2.1. Temperature, diet and oxygen experiments

The comparison between the low (11 °C) and high (28 °C) temperature treatments showed more differentially transcribed genes than the other comparisons; 385 genes were differentially transcribed between 11 °C versus 28 °C, compared to 222 and 163 differentially transcribed genes between control versus 11 °C and control versus 28 °C, respectively (FDR < 0.05, Table A1). Among these, the transcription level of 120 genes increased and that of 145 genes decreased with increasing temperature. For oxygen and diet comparisons, respectively, zero and only three genes showed a significant differential transcription level among the control and test level (FDR < 0.05).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2013.09.009.

3.2.2. Nickel and cadmium experiments

In the Ni experiment, only the comparison between the control and high Ni (HNi) concentration revealed differentially transcribed genes, with 136 over-transcribed and 151 under-transcribed genes in HNi fish relative to control fish (Table A2). Similarly, in the Cd experiment, only the comparison between the control and the high Cd (HCd) concentration showed differentially transcribed genes, with 82 over-transcribed and 94 under-transcribed genes in HCd fish relative to control fish (Table A3).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2013.09.009.

Table 1
Over-represented processes and functional categories in the metal and non metal tests (Fisher exact test, significance threshold: p-value = 0.05; http://www.blast2go.com/b2ghome). *Abbreviations*: OG, over-transcribed gene; met., metabolic; p., process; reg., regulation; endop., endopeptidase; act, activity; a.a, amino acid; cell., cellular; prot., protein; mol., molecular.

Comparison	Processes and functions	GO number	Number of unigenes	% OG temp. (HT/LT)	% OG Cd (HCd/control)	% OG Ni (HNi/control)
Temperature	Cellular aldehyde met. p.	GO:0006081	7	72		
	Glyoxylate met. p.	GO:0046487	5	80		
	Response to nutrient	GO:0007584	6	33		
	Oxidoreductase activity	GO:0016614	13	38		
	Enzyme regulator activity	GO:0030234	18	50		
	NADP or NADPH binding	GO:0050661	6	50		
	GTP binding	GO:0005525	13	54		
Cadmium	Vitamin binding	GO:0019842	5		60	
Nickel	Iron ion homeostasis	GO:0055072	5			20
	Cellular homeostasis	GO:0055080	7			14
	Response to wounding	GO:0042592	6			50
	Gene expression	GO:0010467	25			36
	Translation	GO:0006412	13			46
	Ribosome biogenesis	GO:0042254	9			33
	Macromolecule met. p.	GO:0043170	38			37
	Iron ion binding	GO:0005506	6			34
	Structural constituent of rib.	GO:0003735	7			28
Nickel and cadmium	Blood coagulation	GO:0007596	5		100	100
	Cell adhesion	GO:0007155	6		50	50
	Cellular homeostasis	GO:0019725	6		66	66
	Ribosome biogenesis	GO:0042254	6		50	50
	Endopep. act.	GO:0004175	6		100	100
	RNA binding	GO:0003723	7		42	42
	Structural constituent of rib.	GO:0003735	6		50	50
	Calcium ion binding	GO:0005509	5		40	40
Cadmium and temperature	Heterocycle metabolic p.	GO:0046483	6	33	50	
	Reg. of multicellular organismal p.	GO:0051239	5	40	60	
	Response to chemical stimulus	GO:0042221	7	43	42	
Nickel and temperature	Glucose met. p.	GO:0006006	8	37		75
	Protein localization	GO:0008104	9	89		66
	Ion transport	GO:0006811	10	30		30
	Generation of precursor metabolites and energy	GO:0006091	14	21		42
	Phosphorus met. p.	GO:0006793	12	33		25
	Polysaccharide met. p.	GO:0005976	5	20		80
	Small molecule biosynthetic p.	GO:0044283	14	43		78
	Translational elongation	GO:0006414	6	33		50
Nickel, cadmium and temperature	Immune system p.	GO:0002376	6	0	67	67
	Apoptosis	GO:0006915	6	33	33	33
	Primary met. p.	GO:0044238	34	47	47	47
	Structural molecule activity	GO:0005198	7	28	43	43

Fig. 1 presents the overlap of the significant genes for the Ni, Cd, and temperature experiments. The differentially transcribed genes in the Ni and Cd experiment are distributed as follows: 79 genes were only significantly affected in the Ni experiment, 39 genes were only significantly affected in the Cd experiment, 102 genes were differentially transcribed in both the temperature and Ni experiments, 31 genes were differentially transcribed in both the temperature and Cd experiments, 55 genes were differentially expressed in the both the Ni and Cd experiments and, finally, 51 genes were differentially transcribed in all (temperature, Ni and Cd) experiments.

3.3. Functional categories of differentially transcribed genes

Gene Ontology annotation was used to identify over-represented GO terms in the lists of significant genes that were differentially transcribed in the temperature, Cd and Ni experiments, as well as in the following lists of overlapping genes among the three experiments: Ni and Cd, temperature and Ni, temperature and Cd, as well as temperature, Ni, and Cd (Table 1). In some GO biological processes and molecular functions, more than 70% of the genes represented in a given process were either over-transcribed or under-transcribed. We refer to this pattern as either general over-transcription or general under-transcription. General over-transcription was observed for (i) blood coagulation among the genes that are differentially transcribed in the Ni and Cd experiments; (ii) the immune system process among the genes that are differentially transcribed in the Ni, Cd and temperature experiments; and (iii) in the glucose metabolic process among the genes that are differentially transcribed in the Ni and temperature experiments. However, general under-transcription was observed for (i) iron and cellular homeostasis among the genes that are differentially transcribed in the Ni experiment; (ii) apoptosis among the genes that are differently transcribed in the Ni, Cd, and temperature experiments; (iii) ion transport and phosphorus metabolic process among the genes that are differently transcribed in the Ni and temperature experiments. Table A4 presents the functional categories and the genes represented in each of them.

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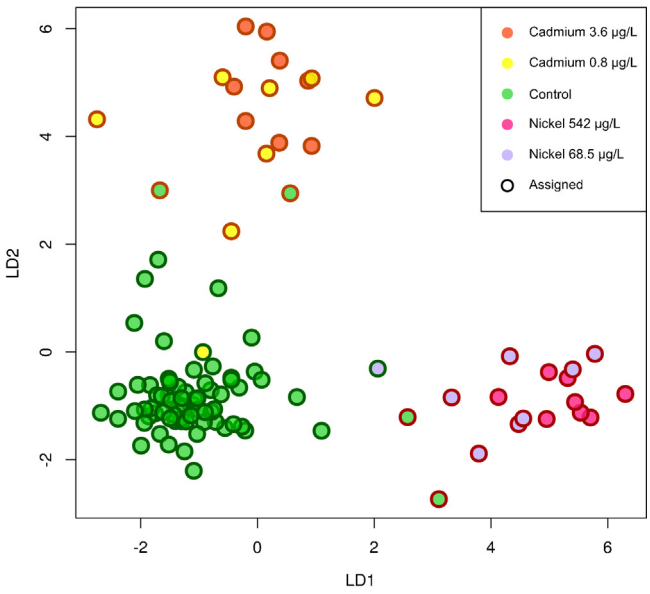

Fig. 2. Linear discriminant analysis (LDA) using the transcription level data and showing fish reassignation according to metal contamination (correct reassignment proportion = 0.94).

3.4. Linear discriminant analysis

The linear discriminant analysis had enough power to clearly separate the control, Cd contaminated, and Ni contaminated groups using the patterns of transcription of the 1000 genes. Using the model to reassign the individual to these three groups, the reassignment probability was 0.94 (Fig. 2), and only six fish were assigned to the wrong group. In detail, two control fish were reassigned to the group of Ni-contaminated fish, two other control fish were reassigned as Cd-contaminated fish, one Cd-contaminated and one Ni-contaminated fish were assigned as controls. None of the fish under a high level of metal contamination were reassigned to the control group and no fish from the control group was assigned as highly contaminated.

4. Discussion

4.1. Effects of natural factors on the perch transcriptome

Comparing the results of the temperature and metal experiments revealed that up to 40% of the genes that are affected by metals are also affected by temperature. This confirms the importance of including natural factors when characterizing the specific effects of single metals in wild fish exposed to both natural stressors and complex metal mixtures. In addition, our results revealed that variations in temperature had more impact on transcription regulation than did changes in oxygen concentration, diet levels, and even metal concentration.

The important impact of temperature variation on transcriptome regulation has been characterized using microarray experiments or RNA-seq in various tissues, including liver, in *Austrofundulus limnaeus* (Podrabsky and Somero, 2004), *Cyprinus carpio* (Gracey et al., 2004), *Ictalurus punctatus* (Ju et al., 2002), *Pomacentrus moluccensis* (Kassahn et al., 2007), *Gillichthys mirabilis* (Logan and Somero, 2010, 2011; Buckley et al., 2006), *Oncorhynchus mykiss* (Vornanen et al., 2005), *Danio rerio* (Long et al., 2012; Vergauwen et al., 2010), *Melanotaenia duboulayi* (Smith et al., 2013). Ectothermic organisms such as fish have developed mechanisms to compensate for temperature fluctuations that can affect the properties and functions of cell components, such as the rate of biochemical reactions or cell membrane permeability and fluidity. Gene Ontology (GO) analysis of the genes affected by temperature variation revealed biological processes and mechanisms used by yellow perch to compensate for the physiological constraints linked to temperature stress. Our results largely agree with previous studies on fish transcriptome responses to temperature acclimation. For example, in carp and zebrafish (Long et al., 2012; Gracey, 2007; Malek et al., 2004), several genes involved in energy metabolism and ion transport were also over-transcribed at lower temperatures. This phenomenon has been explained as a mechanism to compensate for the decline of mitochondrial activity at lower temperatures (Johnston et al., 1994). Energy metabolism and ion transport are also known to be affected by Cd and Ni contamination (McGeer et al., 2011; Pyle and Couture, 2011). Moreover, it has been demonstrated that higher temperatures can lead to an increased accumulation of Cd and Ni in *Daphnia* and *Sparus aurata* (Guinot et al., 2012; Heugens et al., 2003, 2006; Vandenbrouck et al., 2011). It is therefore likely that variations in temperature will modulate the effects that metal contamination has on transcription regulation in yellow perch in the wild, making them more difficult to measure.

Comparing strengths and weaknesses of different types of biomarkers is beyond the scope of the study, but thorough reviews on this topic have previously been published to this end (e.g. Forbes et al., 2006; Lam, 2009; Fedorenkova et al., 2010). Yet, as a few examples, molecular biomarkers of antioxidant capacity and oxidative stress, such as glutathione S transferase, superoxide dismutase, catalase, and cytochrome P450, have all been used or proposed for detection of metal exposure in aquatic organisms. Here, we demonstrate that the transcriptional level of these specific genes is also clearly influenced by temperature variation in addition to being affected by Ni and Cd exposures (see supplementary Tables A1–A3), potentially making them a poor choice for field studies and monitoring, where temperature varies outside the control of the experimenter. Ongoing studies examining the combined effects of temperature and metal contamination on the transcriptome will allow testing this hypothesis.

In contrast with our results on temperature, no pronounced effect of either hypoxia or diet reduction was observed. We argue that this is likely because large differences in temperature used in this experiment (11 °C versus 28 °C) were much more biologically stressful than those applied to both hypoxia and diet reduction. Transcriptional responses to hypoxia have also been well documented in *C. carpio* (Fraser et al., 2006), *Oryzias latipes* (Ju et al., 2007), and *D. rerio* (Ton et al., 2003), also using microarrays. In some of these studies, the level of dissolved oxygen was as low as 5% of normoxia, compared to 75% of normoxia (6.8 mg/L O_2) in our experiment. An oxygen level at 75% of normoxia is likely not a very stressful hypoxic condition for yellow perch and a previous study by Roberts et al. (2011) showed no evident effects on food consumption, growth or RNA:DNA ratios in yellow perch between 5 and 8 mg O_2/L at 11 °C, 20 °C and 28 °C.

Food restriction has also been documented to have measurable impacts on the transcriptome. For example, a total of 229 unique transcripts were found to be differentially transcribed in liver tissues of *O. mykiss* that were deprived of food for three weeks (Salem et al., 2007). However, since our fish were subjected to food restriction (a reduction from 3% to 0.75% of tank biomass per day), but not total food deprivation, important changes in gene transcription levels may not have been necessary to react to the restricted diet regime in our experiment.

Previous studies in fish showed that genes involved in lipid metabolism, energy metabolism, blood functions, immune response, and protein metabolism were differentially transcribed when oxygen or diet levels were reduced. For example, genes coding for ribosomal proteins and those coding for cytochrome

c oxidase and ATP synthase were heavily down-regulated under hypoxia or food deprivation (Salem et al., 2007; Ton et al., 2003). However, since these specific genes and functional categories are well represented on our microarray, it seems plausible that the lack of significant gene transcription differences in our experiment are the consequence of the mildness of the reductions in oxygen and diet used in this experiment.

4.2. Specific transcriptional signatures of nickel and cadmium exposure

Our microarray experiments led to the discovery of genes and biological functions that are affected by aqueous metal exposure. The number of differentially transcribed genes differed between the Ni and Cd experiments, which may be linked to differences in metal concentrations, uptake, and accumulation routes in the organism. For example, only aqueous sources were used for exposure in our experiments and Ni concentrations in the water (600 μg/L) were higher than those of Cd (3.6 μg/L). Moreover, Ni intake in wild fish is more influenced by aqueous sources whereas Cd intake is also influenced by diet contamination (Audet and Couture, 2003; Couture et al., 2008; Kraemer et al., 2006). Renal Cd concentrations in our fish were very low and similar to the concentrations found in kidneys of fish from relatively uncontaminated lakes in the Rouyn-Noranda area in northwestern Quebec (Giguère et al., 2004), whereas kidney Ni concentrations corresponded to those found in the kidneys of fish from highly contaminated lakes in the Sudbury area in northern Ontario (Couture et al., 2008). These factors could all have contributed to the lower number of significant differently transcribed genes found in the Cd experiment compared to Ni. As a result, even though the impact of Cd exposure was less important in terms of the number of affected genes, it would be incorrect to assume that it is less toxic since the concentrations of Cd in the fish were much lower than those of Ni.

Despite the small number of gene transcription differences specific to Cd exposure, the vitamin binding category was over-represented. Cadmium has been reported to affect vitamin metabolism, especially vitamin A, in yellow perch liver (Defo et al., 2012; Pierron et al., 2011). In our study, the transcription level of genes playing a role in folic acid metabolism, *serine hydroxymethyltransferase* ($\log_2$ of fold change: 0.13) and *formimidoyltransferase-cyclodeaminase* ($\log_2$ of fold change: 0.26), and in taurine metabolism, *cysteine sulfinic acid decarboxylase* ($\log_2$ of fold change: 0.21), were also increased at the highest tested Cd concentration. This result suggests a specific cell protection mechanism against Cd-induced oxidative stress since high antioxidant activity has been observed for folic acid in human blood subjected to free radicals (Stocker et al., 2003). In liver, folates are stored by hepatocytes and, when needed, they are metabolized before release into the bile. Folic acid and its derivatives, such as 5-methyltetrahydrofolate, which is synthesized by serine hydroxymethyltransferase are known to interact with nitric oxide (NO) synthase and to increase NO bioavailability. The reaction between NO and superoxide anions releases peroxynitrite which is a reactive nitrogen species being scavenged by 5-methyltetrahydrofolate (Stanger and Wonisch, 2012). From the 5-methyltetrahydrofolate, thymidylate synthase generates the thymidine that is required for DNA synthesis. Since the *thymidylate synthase* gene ($\log_2$ of fold change: −0.03) was under-transcribed, this suggests a tradeoff between protecting the cell against the oxidative damage and DNA synthesis in the context of Cd contamination.

Taurine, a derivative of cysteine, is also known to have an antioxidant action in different fish tissues, such as liver and muscle, and reduces Cd-induced oxidative stress through mechanisms that still remain unclear (Higuchi et al., 2012; Karaytug et al., 2011; Kumar et al., 2009; Manna et al., 2008, 2009). Another hypothesis is that taurine associated with tRNA maintains energy balance and limits the rate of reactive oxygen species production by ensuring the normal functioning of the mitochondrial respiratory chain. This mechanism has been proposed to explain the antioxidant effect of taurine (Schaffer et al., 2009).

The main Ni-specific effect observed in our study suggests that Ni disturbs iron metabolism and could lead to an overall iron deficiency in yellow perch. In the liver, the main organ of iron storage and the site of transferrin synthesis, free iron is transported into the hepatocytes via the divalent metal ion transporter 1 (DMT1). Transferrin is an iron binding transport protein responsible for the transport of iron in blood from absorption and heme degradation sites, i.e. the liver in fish, to storage and utilization sites. Iron can then either be stored as ferritin or released from hepatocytes, where it is bound to transferrins, to be delivered to circulating blood and thence to the rest of the organism (reviewed by Chua et al., 2007). Previous studies on human cells reported that Ni enters cells via DMT1 and interferes with iron homeostasis, leading to decreased cellular iron levels (Chen et al., 2005; Davidson et al., 2005). Kwong and Niyogi (2009) also found that Ni inhibits intestinal iron absorption in *O. mykiss*. Nielsen et al. (1984) showed a synergistic or antagonistic interaction between Ni and Fe, depending on factors that included tissue, Ni intake levels and previous deficiencies of iron. The present study suggests the complexity of interpreting the effects of Ni on iron metabolism. For example, genes playing a role in iron homeostasis, especially those responsible for the transferrin receptor (*transferrin receptor 1a*, $\log_2$ of fold change: −0.04; *transferrin receptor protein 2*, $\log_2$ of fold change: −0.04) and iron storage (*ferritin*, $\log_2$ of fold change: −0.47) were found to be under-transcribed in the liver of Ni-exposed yellow perch. Transferrin receptors are carrier proteins for transferrin involved in the import of circulating iron into the cells. The down-regulation of these genes reflects the disruptive effect of Ni on iron metabolism, especially on iron uptake. The decrease in iron receptors and storage proteins could have induced the over-expression of *transferrin* gene ($\log_2$ of fold change: 0.38) in order to facilitate iron transport into cells. In addition, the *heme oxygenase 1* gene, which encoded for a protein that recycles iron from heme groups, was also under-transcribed, supporting the hypothesis of an overall decrease in iron content in Ni-contaminated yellow perch.

Interestingly, our results also showed that the majority of genes involved in regulation, translation and ribosome biogenesis processes were under-transcribed in the Ni-contaminated group. Previous studies found that genes involved in these processes are also inhibited in L-929 mouse cells treated with Ni (Lü et al., 2010) and in *Daphnia* exposed to Ni (Vandenbrouck et al., 2009). However, previous studies on wild yellow perch from metal contaminated lakes or clean lakes found diverging results concerning biosynthesis indicators. For example, Audet and Couture (2003) found lower biosynthesis in liver of fish from Ni contaminated lakes sampled in the Spring and highlighted the seasonal variations of these results when they found the opposite results in the Fall, whereas studies from Rajotte and Couture (2002), as well as Pierron et al. (2009), showed increased biosynthetic capacities using measurements of protein concentration levels or the activity of nucleoside diphosphate kinase in liver of wild yellow perch. However, these two latter studies used wild yellow perch that were sampled in late summer or early fall. Since the present experiment used chronic, but relatively short, metal exposures when compared to life-time exposures, the observed impact of Ni contamination on protein synthesis and gene expression regulation in livers of yellow perch can be expected to differ from field results. A tentative explanation for the activation of protein biosynthesis observed in wild perch from metal-contaminated lakes is that it represents an adaptive response to compensate for the negative impact of Ni on protein metabolism levels. Indeed, a recent population genomics study on yellow perch

revealed rapid evolutionary change at the gene level in response to metal contamination (Bélanger-Déchênes et al., 2013).

4.3. Common effects of Ni and Cd

Many of the effects of Ni and Cd contamination documented in other organisms and studies were confirmed by our microarray experiments. Our study reveals that there is overlap among the responses to Ni and Cd contamination, as well as temperature variation. More specifically, Cd and Ni both affected genes involved in the blood coagulation cascade, calcium ion binding and apoptosis. A general induction of genes associated with blood coagulation by both metals was observed in our study, supporting the results of earlier studies on Cd contamination in carp liver, *Daphnia*, and wild yellow perch (Pierron et al., 2011; Reynders et al., 2006; Soetaert et al., 2007). The clotting process is an important part of the immune response. It uses proinflammatory signaling to fight invading pathogens and works, in conjunction with the activation of the complement system, as a sentinel for the immune response (Amara et al., 2008; Choi et al., 2006). Over-transcription of the clotting genes could thus be the result of the activation of the complement system genes. Cadmium, Ni and other pollutants are known to suppress the immune response in fish (Krasnov et al., 2007; Kubrak et al., 2012; Pierron et al., 2011; Reynders et al., 2006), but the immune response can also be activated at high temperatures or inhibited when temperature is outside the appropriate thermal range for an immune response (Bowden, 2008; Dios et al., 2010; Dominguez et al., 2004; Podrabsky and Somero, 2004). This complicates the interpretation of the impact of metals on immune response genes.

Nickel and Cd uptake both use the calcium transport pathway. Exposure to these metals has been shown to reduce calcium uptake in zebrafish (Alsop and Wood, 2011). Nickel and Cd can also bind to and activate calmodulin, an important protein in calcium-mediated signal transduction, which is crucial in many processes such as immune response, cell cycle regulation, and regulation of gene expression (Braun and Schulman, 1995; Raos and Kasprzak, 1989; Suzuki et al., 1985). The presence of Cd or Ni could lead to a prolonged activation of calmodulin, which is normally regulated by Ca^{2+}, and consequently to an over-activation of different processes, such as cell proliferation. In our study, the over-transcription of endoplasmic protein genes responsible for calcium storage (*endoplasmin* and *protein disulfide isomerase 3*) and the under-transcription of calcium mediated signal transduction genes (*calmodulin* and *spectrin alpha chain*) after laboratory exposure to Ni and Cd suggest a mitigation mechanism to limit Ni and Cd effects on the calcium pathway.

In conclusion, and in response to objectives (1) and (2) of the study, the 1000 candidate-gene microarray developed in this study allowed identification of differentially transcribed genes specific to aqueous Cd and Ni exposure in yellow perch, which point to different mechanisms of metal toxicity. For instance, Ni was found to affect iron ion homeostasis, while Cd affected folic acid and taurine metabolism and both affected blood coagulation genes. This study also supports the potential of this 1000 candidate-gene microarray as a tool for the detection of metal-induced effects in wild yellow perch exposed to complex metal mixtures, as proposed in objective (3) of the study. These transcriptional results are currently being refined by ongoing experiments examining the combined effects of temperature and metal contamination, as well as metal mixtures, on gene expression. Finally, findings from these laboratory exposures will be compared to data from yellow perch sampled in the field in lakes representing a gradient of metal contamination. In addition, the observed patterns of Ni and Cd effects on fish metabolism, as well as the use of specific genes as potential biomarkers, will be further explored by ongoing studies using qPCR, enzyme assays and determination of concentrations of various gene products. These future studies will attempt to link specific gene expression patterns to the physiological condition of yellow perch exposed to these metals and will investigate the mechanisms of metal toxicity in wild fish.

Conflict of interest

The authors declare no conflict of interest.

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