

Combined effects of temperature changes and metal contamination at different levels of biological organization in yellow perch



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ARTICLE INFO

Article history:

Received 15 February 2016

Received in revised form 10 June 2016

Accepted 11 June 2016

Available online 14 June 2016

Keywords:

Fish

Yellow perch

Perca flavescens

Temperature

Metals

Cadmium

Nickel

Metabolic capacities

Gene transcription level

Biometric condition indicators

Oxidative stress

ABSTRACT

In this study, we measured the effects of temperature (9 °C, 20 °C, and 28 °C), metal contamination (cadmium and nickel) and their interaction on yellow perch (*Perca flavescens*) using liver enzymatic and transcriptomic endpoints and biometric indices. Kidney metal concentrations increased with a rise of temperature. The biometric indices analysed (Fulton condition factor, pyloric caeca, hepatosomatic and gonadosomatic indices) generally decreased with an increase of temperature but not with metal contamination. At the enzymatic level, the activity of superoxide dismutase (SOD), involved in antioxidant response, was affected by both temperature and metal contamination, whereas the activity of glucose-6-phosphate dehydrogenase (G6PDH), involved in energy accumulation but also in antioxidant response, was only affected by metal exposure. The response of perch to the stressors at the transcriptional level differed from the metabolic response. In particular, the transcription level of the *cco* and *g6pdh* genes sharply decreased with increasing temperature, while the activities of the corresponding enzymes remained stable. The normal response of the transcription level of the apoptotic gene (*diablo*) to heat stress was also altered in metal-contaminated fish. The combination of metal and temperature stresses also modified the response of antioxidant metabolism induced by these stressors individually. This study contributes to a better understanding of the influences of natural stressors like temperature on biomarkers commonly used in ecotoxicological studies and will facilitate their interpretation in the context of multiple stressors characteristic of field situations.

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1. Introduction

Among the major contaminants of concern in the environment, metals can cause devastating effects to aquatic life because of their propensity for bioaccumulation and toxicity. Metal mining and smelting are historically among the most important economic activities in Canada, but the industry has caused large-scale water contamination and impacts on aquatic habitats, in particular by nickel (Ni) and cadmium (Cd) (Csavina et al., 2012; Iles and Rasmussen, 2005; Klinck et al., 2007; Lemly, 1994).

In addition to metal effects, aquatic organisms from mining areas are exposed to numerous natural stressors in their environment. Among these natural factors, changes in water temperature can have important impacts on aquatic fauna and their environ-

ment (Cochrane et al., 2009; Rosenzweig et al., 2007). In ectotherms including fish, temperature is a major driver of metabolic and locomotor capacities, although other exogenous and endogenous factors such as food availability and reproductive status can also influence these capacities (Guderley, 2004). In this context, yellow perch (*Perca flavescens*) are particularly relevant to examine combined effects of multiple stressors, as they are considered eurythermal and tolerant to a wide range of physico-chemical conditions (Scott and Crossman, 1974) including metal contamination (Couture and Pyle, 2015; Eastwood and Couture, 2002; Pierron et al., 2009).

Yellow perch exposed to changes in water temperature or to metal contamination can use various defence mechanisms to fight against these stressors. In a previous study (Grasset et al., 2014), we reported that an increase in temperature affected biometric indices (Fulton condition factor (FCF) and pyloric caeca index (PCI)), the activity of glucose-6-phosphate dehydrogenase (G6PDH) and the transcription level of genes involved in apoptosis (the *diablo*-

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like protein gene, *diablo*) and in energy metabolism and oxidative stress response (*g6pdh*). Metal contamination is also well known to impact biometric indices, enzymatic activities and gene transcription levels in fish. In yellow perch, Cd induces oxidative stress in gills and liver (Giguère et al., 2004). Exposure to Ni resulted in changes in biomarkers of oxidative stress such as an increase in the transcription of *g6pdh* and *cat* (Defo et al., 2014) and of CCO activity (Pierron et al., 2009). Negative effects of Ni on iron metabolism have been demonstrated in yellow perch (Bougas et al., 2013; Wood et al., 2011).

Clearly, there is compelling evidence that both temperature and metal contamination induce various stress responses in yellow perch. In this context, the objective of this study was to investigate the combined effects of temperature changes and metal contamination (Ni or Cd) on yellow perch using biometric indicators (FCF, PCI, hepatosomatic index (HSI) and gonadosomatic index (GSI)), hepatic metabolic capacities (enzyme activities of cytochrome C oxidase (CCO), an indicator of aerobic capacities, nucleoside diphosphate kinase (NDPK), an indicator of biosynthetic capacities, G6PDH and superoxide dismutase (SOD), indicators of antioxidant capacities) and hepatic gene transcription levels (*cco*, *diablo*, *g6pdh* and *sod*).

2. Materials and methods

2.1. Fish

Young of the year yellow perch (*Perca flavescens*) were bought from a fish farm (Trevor Thomas, Abbey Road Fish Farm, Wainfleet, ON) and brought back to the Laboratoire de Recherches en Sciences Aquatiques (LARSA) at Université Laval (Québec, QC). During the acclimation period (2 weeks), perch were kept in a 1 m³ circular tank at 9 °C, where they were fed daily at 3% of their biomass with frozen brine shrimp. After this period, fish were acclimated in eighteen 40-L glass aquaria for 2 weeks with a gradual increase of temperature up to their experimental temperature. After acclimation, the experimental conditions were initiated.

At the end of the experiment, perch were sacrificed by a blow to the head, immediately measured, weighed and dissected. Pyloric caeca, gonads, liver, kidney and muscle were collected and frozen in liquid nitrogen. All procedures were approved by our institutional animal care committee and followed the guidelines of the Canadian Council on Animal Care.

2.2. Experimental conditions

Each condition, described below, was replicated in two separate aquaria each containing 22 fish, yielding a total of 44 fish per condition. Fish were divided among various projects, and a number were set aside for method optimizations. For this project, in order to examine physiological and transcriptomic endpoints, 16 fish were randomly sampled from each experimental condition: eight yellow perch were used to measure gene expression (for qRT-PCR) and eight were used to measure enzyme activities. After acclimation, yellow perch were exposed to combined stressors for seven weeks. Three metallic conditions were used: control (no metal), cadmium exposure and nickel exposure. Fish from each metal exposure condition were exposed to one of three different temperatures (9 °C, 20 °C and 28 °C), yielding nine conditions. The oxygen level and daily ration were the same for the exposure as they were during the acclimation period. Metal concentrations were chosen based on previous studies in our lab and were similar to concentrations measured in contaminated Canadian lakes (eg. Couture et al., 2008; Defo et al., 2012). They were measured twice a week in each aquarium and adjusted as required using stock solutions of Cd and Ni with certified concentrations of 1000 µg/L in 4% HNO₃ (SCP Science, ICP

Standard item # 140-051-485 for Cd 140-051-285 for Ni). Measured aqueous metal concentrations were statistically identical in aquaria adjusted at different temperatures and did not vary over the seven weeks of exposure. For Cd, measured water concentrations were 3.99 ± 0.21 µg/L (35.5 ± 1.9 nM) (mean ± SEM, n = 84; 3 temperatures, 2 aquaria per temperature, two samplings per week). Values in Ni exposure aquaria were 606.1 ± 13.4 µg Ni/L (10.3 ± 0.2 µM) (n = 84). Concentrations of both metals in control aquaria were consistently below 0.01 µg/L. Aqueous concentrations of Cd and Ni were measured using an inductively coupled plasma mass spectrometer (ICP-MS, Thermo Elemental, Model X-7). The instrument response was calibrated with certified standard solutions (SCP Science, ICP Standard item # 140-051-485 for Cd 140-051-285 for Ni) and possible instrument drift was controlled for by inserting blanks and standards every 10 samples.

2.3. Sample analysis

2.3.1. Biometric measures

Fish condition was estimated using four biometric measurements. The Fulton condition factor (FCF) was calculated with the following equation: $FCF = (\text{weight (g)} / \text{length (cm)}^3) \times 1000$ (Nash et al., 2006). The pyloric caeca index (PCI) was calculated as described by Gauthier et al. (2008), using the following equation: $W_{corr} = (W_f / W_m)^b \times W_c$, where W_{corr} is the corrected weight of the pyloric caeca, W_m is the mean of the fish weight for the dataset, W_f is the fish weight, b is the slope of the logarithmic relationship between fish weight and caecum weight (0.869 for this dataset) and W_c is the uncorrected weight of pyloric caeca. The pyloric caeca index is a good indicator of recent feeding history of fish and of food availability in their environment (Gauthier et al., 2008). The hepatosomatic index (HSI) and the gonadosomatic index (GSI) were calculated using the equations described by Lloret and Rätz (2000): $HSI = (\text{liver weight (g)} / \text{fish weight (g)}) \times 100$ and $GSI = (\text{gonad weight (g)} / \text{fish weight (g)}) \times 100$.

2.3.2. Metal concentrations

Kidney samples used for metal measurement were lyophilized in acid-washed (15% HNO₃) Eppendorf® tubes. Certified reference material (TORT-2 from the Canadian National Research Council) and blanks were submitted to the same treatment as samples in order to determine analytical accuracy. After lyophilisation, freeze-dried samples were digested in trace metal grade nitric acid at a ratio of 100 µL of HNO₃ for 1 mg of dried sample for 2 days at room temperature and for 24 h in trace metal grade hydrogen peroxide at a ratio of 40 µL for 1 mg of dried sample. The samples were then diluted in ultrapure water (in order to obtain 1 mL/mg of dry weight) and concentrations of Cd and Ni were measured using an ICP-MS (Thermo Elemental, Model X-7) (Bougas et al., 2013).

2.3.3. Enzyme and protein assays

Protein assays followed the Bradford protocol elaborated for microplates by Bio-Rad (Bio-Rad Protein Assay). Enzyme activities were measured in triplicate using a UV/Vis spectrophotometer (Varian Cary 100) on a microplate at room temperature (20 °C). Reactions were recorded over a period of five minutes and a linear portion of at least three minutes was used in order to calculate reaction rates. Activities were expressed as international units (IU)/g wet weight.

All chemicals were bought from Sigma-Aldrich (Canada). Livers were homogenized in ice-cold buffer (pH 7.5, 20 mM HEPES, 1 mM EDTA, 0.1% Triton X-100) for three bursts of 20 s using an Ultra Turrax T25 tissue homogenizer.

Reaction conditions for the enzymes involved in energy production, biosynthetic and anaerobic capacities were as follows:

Cytochrome C oxidase (CCO; EC 1.9.3.1): Phosphate buffer (100 mM, pH 7.0), cytochrome c 0.07 mM, 0.33% potassium ferricyanide (in controls).

Nucleoside-diphosphate kinase (NDPK; EC 2.7.4.6): Imidazole buffer (imidazole 50 mM, MgCl₂ 20 mM, KCl 70 mM), pH 7.5, β-NADH 0.24 mM, adenosine triphosphate (ATP) 2 mM, phospho(enol) pyruvate (PEP) 1.1 mM, LDH and PK in excess, TDP 70 mM (omitted in controls).

Glucose 6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49): Imidazole buffer (imidazole 50 mM, MgCl₂ 25 mM, KCl 70 mM), pH 7.5, β-nicotinamide adenine dinucleotide phosphate (NADP) 0.3 mM, glucose-6-phosphate 200 mM (omitted in controls).

Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured using the Superoxide Dismutase Assay Kit from Cayman Chemical, following the manufacturer's protocol.

2.3.4. RNA extractions

Total RNA was extracted from liver of 72 fish corresponding to 8 individuals per condition. Liver RNA extractions were performed using the PureLink™ RNA Mini Kit from Ambion following the manufacturer's protocol, and a DNase treatment (10U/μL) was made on the entire RNA sample. Sample purity and concentration were verified using a NanoDrop spectrophotometer. A260/A280 ratios were between 1.99 and 2.15. Extracted RNA was stored at –80 °C until used in real-time PCR experiments.

2.3.5. qRT-PCR analysis

One μg of total RNA from eight individuals per condition were reverse-transcribed into cDNA using the High capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies Carlsbad, CA, USA). The four genes (*g6pdh*, *diablo*, *cco* and *sod*) were chosen to be consistent with a related study (Grasset et al., 2014) and are known to be involved in the antioxidant response (*sod*, *g6pdh*), mitochondrial metabolism (*cco*) and in the apoptotic mechanism (*diablo*) (Bougas et al., 2013). Specific primers were determined using PrimerBlast (NCBI software) (Table S1). qRT-PCR reactions were performed in triplicate using Fast SYBR® Green Master Mix (Applied Biosystems, Life Technologies Carlsbad, CA, USA) in a 7500 Fast Real-Time PCR 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 12.5 μL reaction contained 6.25 μL Fast SYBR® Green Master Mix (2X), and the specific primer pairs at final concentration of 15 μM. The transcript abundance for each gene was calculated as relative quantification (RQ) normalized according to the expression of the β-actin gene as a reference gene. Relative quantification of each gene expression level was normalized according to the β-actin gene using the 2^{–ΔCT} method described by Livak and Schmittgen (2001). The output cycle corresponding to the β-actin was investigated. This output was obtained around the same cycle, 20.60 ± 0.07 (Mean ± SE). Consequently, using the β-actin gene as reference gene was relevant under our experimental conditions.

2.4. Statistical analysis

Statistical analyses to determine differences among the nine groups of fish for metal contamination, biometric indices, enzymatic activities and transcript abundances were performed using R software version 2.13.0 (R Team, 2012). Comparisons among fish groups were performed using analysis of variance (ANOVA, p-value < 0.05) after verifying the homoscedasticity and the normality of the data. ANOVAs were followed by Tukey-Kramer post-hoc tests (p-value < 0.05). We considered interactions between temperature and metal contamination, because we hypothesized a non-linear effect of temperature and metal contamination on response variables.

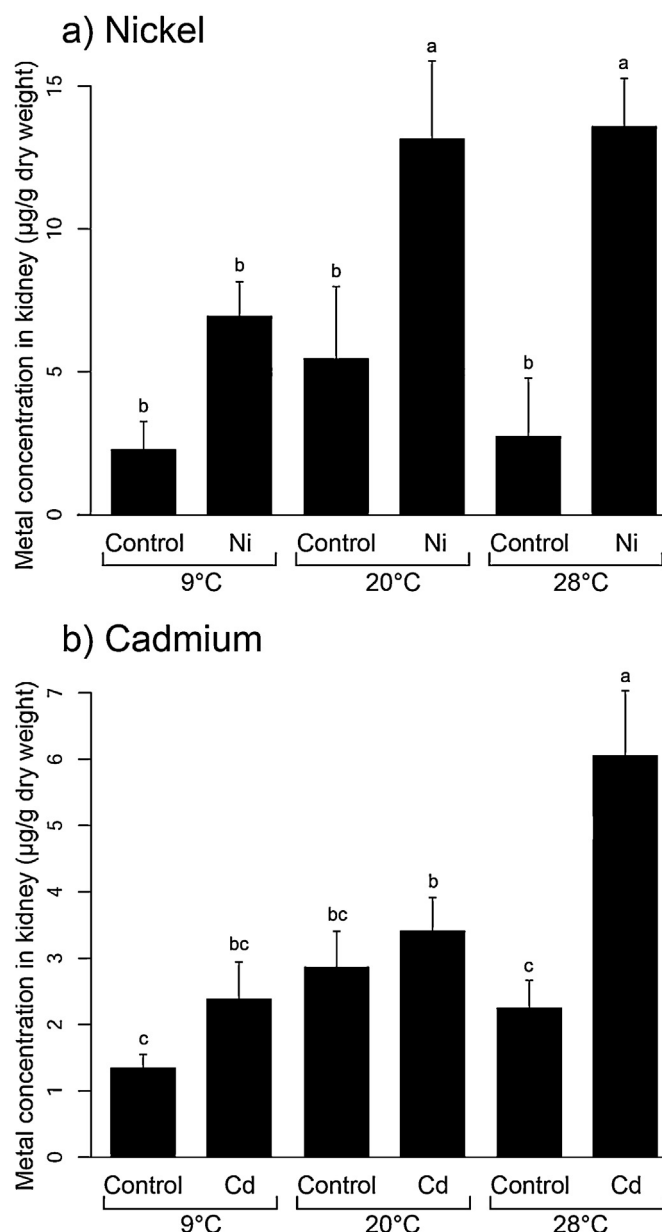


Fig. 1. Kidney concentrations of Ni (a) and Cd (b) for each exposure condition (mean ± se; n=40). Bars with different letters differ significantly (p < 0.05; Tukey post-hoc test).

To examine correlations among kidney metal contamination, biometric condition indicators, transcript abundances and enzymatic activities, Spearman coefficients (p value < 0.05) were calculated using JMP software (version 7.0; SAS Institute Inc.).

3. Results

3.1. Kidney metal contamination

Kidney Ni concentrations showed a significant increase with temperature at 20 °C and 28 °C (Fig. 1a) whereas no difference between the control and the contaminated condition was observed at 9 °C. Fish exposed to Cd showed a higher metal accumulation at 28 °C compared to lower temperatures (Fig. 1b).

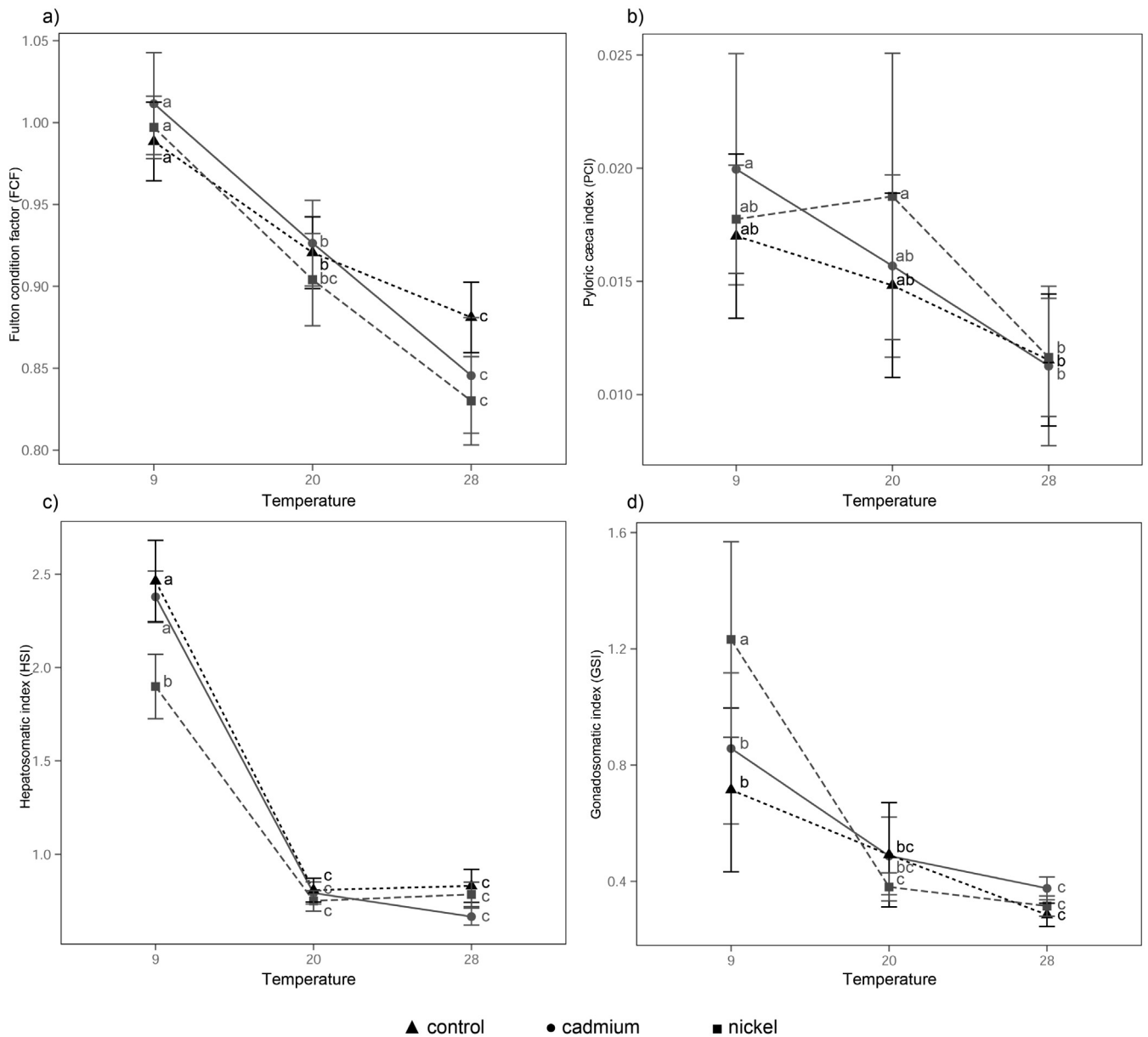


Fig. 2. Biometric indices for each exposure condition: Fulton condition factor (a), pyloric caeca index (b), hepatosomatic index (c) and gonadosomatic index (d) (mean ± se; n = 40 for each condition; control ▲, cadmium ●, nickel ■). Dots with different letters differ significantly (p < 0.05; Tukey post-hoc test).

Table 1

Spearman correlations among the various response parameters for the different conditions examined (* = significant at p-value < 0.05).

	FCF	PCI	HSI	GSI	<i>cco</i>	<i>diablo</i>	<i>g6pdh</i>	<i>sod</i>	NDPK
PCI	0.51*								
HSI	0.4215*								
<i>cco</i>	0.4023*		0.5291*						
<i>diablo</i>					0.3877*				
<i>g6pdh</i>	0.5276*		0.5793*		0.6133*				
<i>sod</i>						0.3435*	0.2752*		
CCO				0.3462*					
NDPK								0.3598*	
G6PDH	0.3149*								0.7171*
SOD	-0.5247*	-0.3084*	-0.476*		-0.3322*				

3.2. Biometric indices

The FCF showed a significant decrease with increasing temperature, but was not impacted by metal contamination (Fig. 2a). A similar pattern of decrease at warmer temperatures was observed

for the PCI, but the temperature-related trend was not significant in control fish. The decrease was nevertheless significant between 20°C and 28°C for Ni-exposed fish, and between 9°C and 28°C for Cd-exposed fish (Fig. 2b). There was a sharp decline of the HSI between 9°C and 20°C, but the index did not further decrease

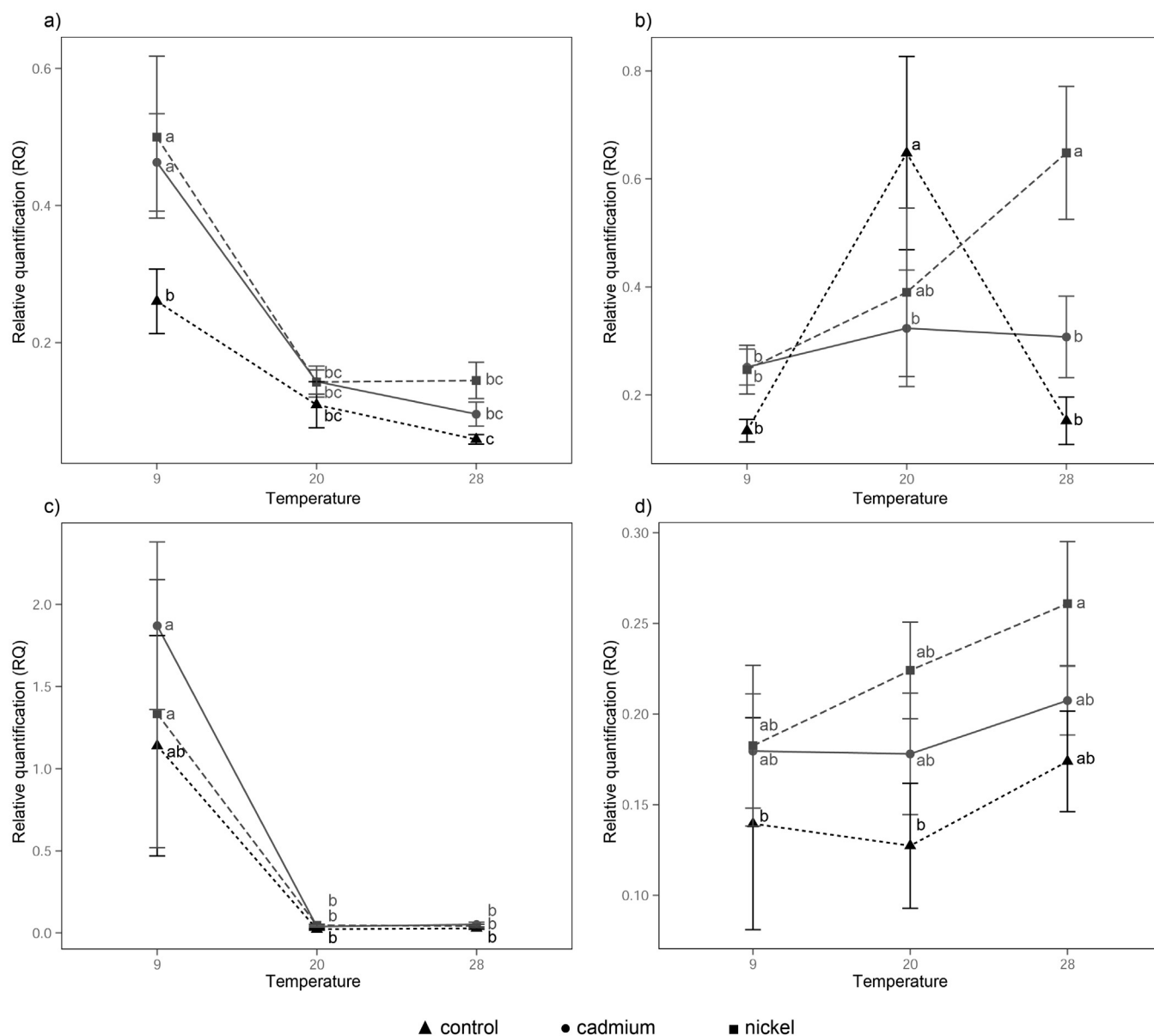


Fig. 3. Liver transcription levels of *cco* (a), *diablo* (b), *g6pdh* (c) and *sod* (d) genes as measured by qRT-PCR for each exposure condition (mean \pm se; n = 10 in each condition; control ▲, cadmium ●, nickel ■). Dots with different letters differ significantly ($p < 0.05$; Tukey post-hoc test).

between 20°C and 28°C (Fig. 2c). Nickel contamination negatively affected the HSI, but only at 9°C. A similar pattern of strong decrease was also observed between 9°C and 20°C for the GSI of Ni-exposed fish (Fig. 2d). Although increasing temperatures also decreased the GSI in control and Cd-exposed fish, the trend was only significant between 9°C and 28°C. The FCF was correlated positively with the PCI and HSI (Table 1), but no relationships were found between the GSI and other biometric indices.

3.3. Transcriptomic analysis

The transcription level of the *cco* gene was stimulated by metal contamination, but only at 9°C (Fig. 3a). This parameter showed a significant decrease with an increase of temperature in metal-contaminated fish between 9°C and 20°C and in control fish between 9°C and 28°C. Finally, the transcription level of the *cco* gene was positively correlated with two biometric indices, the FCF and the HSI, and with the expression of the *diablo* and the *g6pdh*

genes, but it was also negatively correlated with the activity of the SOD enzyme (Table 1).

The transcription level of the apoptotic gene, *diablo*, increased between 9°C and 20°C and decreased at 28°C in control fish (Fig. 3b). This biomarker also increased between 9°C and 28°C in Ni-contaminated fish, but did not vary with temperature in Cd-exposed fish. Transcription of the *diablo* gene was also positively correlated with the transcription of the *cco* and *sod* genes (Table 1).

The *g6pdh* gene showed a sharp decrease of transcription between 9°C and higher temperatures (Fig. 3c) and reached near zero values in all groups, but this decrease was only statistically significant in metal-contaminated fish. The transcription level of *g6pdh* was positively correlated with the biometric indices FCF and HSI, but also with the transcription level of the *cco* and *sod* genes.

Finally, significant increases were observed in the transcription level of the *sod* gene between the control at 9°C and the Ni-contaminated fish at 28°C (Fig. 3d). Although other variations were statistically non-significant, a trend of higher transcription level was observed with an increase of temperature. The transcrip-

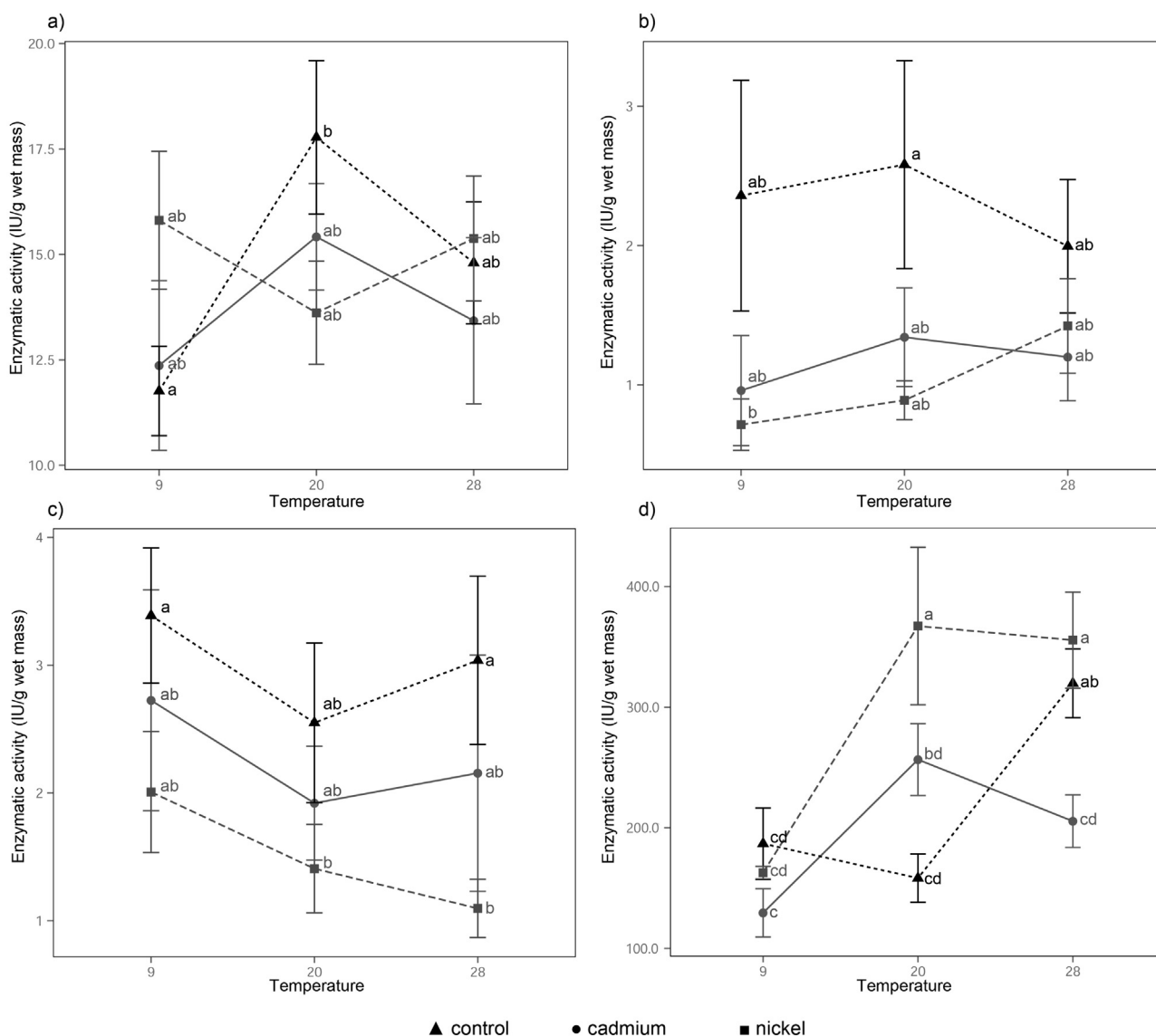


Fig. 4. Liver enzyme activities of CCO (a), NDPK (b), G6PDH (c) and SOD (d) for each exposure condition (mean \pm se; n = 8 in each condition; control ▲, cadmium ●, nickel ■). Dots with different letters differ significantly ($p < 0.05$; Tukey post-hoc test).

tion level of *sod* was positively correlated with the transcription level of *diablo* and *g6pdh* and with NDPK activity (Table 1).

3.4. Enzymatic activities

The activity of CCO showed a significant increase in control fish with increasing temperature, but only between 9 °C and 20 °C (Fig. 4a), and did not vary with temperature in metal-exposed fish. Liver CCO activity was positively correlated with the GSI (Table 1).

The activity of NDPK was higher in control compared to metal-contaminated fish (Fig. 4b) although a high variability precluded significant differences. Liver NDPK activity was also positively correlated with G6PDH activity and with *sod* gene transcription level (Table 1).

Liver G6PDH activity was significantly higher in control fish compared to those exposed to Ni at 28 °C, with intermediate values in Cd-exposed fish (Fig. 4c). Liver G6PDH activity was also positively correlated with NDPK activity and with FCF (Table 1).

The activity of liver SOD increased significantly in Ni- and Cd-exposed fish between 9 °C and 20 °C but not in control fish (Fig. 4d). In contrast, SOD activity increased in control fish between 20 °C and

28 °C, but not in Ni- or Cd-exposed fish. The Spearman coefficient analysis revealed that this enzyme activity was negatively correlated with the three biometric indices FCF, PCI and HSI, as well as with the transcription level of the *cco* gene (Table 1).

4. Discussion

4.1. Temperature effects

In a previous study, we demonstrated that an increase in temperature impacted biomarkers of condition, energy metabolism, oxidative stress and apoptosis (Grasset et al., 2014). In the current study, a temperature of 9 °C appeared to be optimal for perch growth and development, as confirmed by higher values of FCF, PCI, HSI and GSI. The PCI is a biomarker reflecting recent feeding intensity in yellow perch (Gauthier et al., 2008), but has also been reported to be positively correlated to food conversion efficiency and growth (Gauthier et al., 2008; Krogdahl and Bakke-McKellep, 2005). Since in our experiment all fish were fed the same ration, the decrease of the PCI can therefore be explained by an increase of metabolic costs at higher temperatures leading to a decrease

of growth efficiency, in agreement with our earlier conclusions (Grasset et al., 2014), and supported by the strong positive correlation between FCF and PCI, reported here (Table 1) and elsewhere (Gauthier et al., 2008). Among the other biometric biomarkers negatively affected by an increase of temperature, the decrease in HSI and its positive correlation with FCF also clearly indicate that temperatures higher than 9° C lead to a decrease in the accumulation of energy stores in yellow perch.

The transcription levels of the *cco* and *g6pdh* genes were strongly and positively correlated with the FCF and also negatively affected by an increase of temperature. Interestingly, the activities of the corresponding enzymes were not affected by temperature, at least when comparing the two extremes of temperature studied, since CCO activity was strongly increased between 9 and 20° C. Given the involvement of CCO in energy production via the mitochondrial respiratory chain and the role of G6PDH in fatty acid biosynthesis, our results suggest that aerobic capacities and lipid biosynthesis were maintained at higher temperatures, at a lower metabolic cost (lower gene transcription levels). Conversely, at the colder temperature, maintenance of aerobic capacities appears to involve an investment in enzyme synthesis. Given the higher condition of perch in the cold, our study suggests that higher temperatures lead to a decrease in growth efficiency, likely at least partly due to increased metabolic costs related to oxidative stress-induced damage repair mechanisms. Temperature is known to induce oxidative stress as shown in our previous study for perch (Grasset et al., 2014). The absence of a thermal response of liver NADPH activity in control fish however suggests that global investment in protein synthesis does not vary with temperature in liver. The substantial depletion of liver energy stores at higher temperatures, revealed by the HSI, coupled with the maintenance of lipid synthetic capacities in this tissue suggested by the activity of G6PDH, also indicates that metabolic costs are increased in perch exposed to the higher temperatures examined.

Evidence of changes at the mitochondrial level in fish under thermal stress has been reported in the literature. Beck and Fuller (2012) showed that thermal stress induces a decrease in the reserve capacity of the mitochondria (also termed reserve respiratory capacity) in a white bass (*Morone chrysops*) cell line, which may lead to the development of pathophysiological conditions. In gilthead sea bream (*Sparus aurata*), thermal stress leads to perturbations of gene expression related to mitochondrial oxidative capacities including COX genes (Bremer et al., 2014), which may be a regulatory response to the temperature-induced increase of oxidative capacities (Bermejo-Nogales et al., 2014). Our study partly supports these reports, since although we reported a sharp temperature-induced decrease in the expression of the *cco* gene in our perch livers, the activity of the CCO enzyme which increased between 9 and 20° C, supporting an increase in oxidative capacities, decreased at the highest temperature studied. Likely, at 28° C, fish were under thermal stress and no longer displayed the normal thermal response of their aerobic capacities.

Our observation of an increase in antioxidant capacities, indicated by SOD activity and by a similar, although non-significant, trend in *sod* gene transcription level, supports our hypothesis of increased metabolic costs at higher temperatures imposed by heat shock and oxidative stress responses, and is consistent with the negative correlations between SOD and FCF, PCI and HSI. The response of G6PDH, which also plays a role in antioxidant response (Cartañà et al., 1992), is nevertheless intriguing. The positive correlation observed here in the transcription levels of *sod* and *g6pdh* supports a coordinated antioxidant response. However, the activity of the G6PDH enzyme itself did not respond to temperature, hypothetically because of its main role in lipid biosynthesis, which varied in the opposite direction to oxidative stress with temperature, as discussed above.

Finally, renal bioaccumulation of both Cd and Ni was clearly, and positively, affected by water temperature. The influence of temperature on metal bioaccumulation in fish tissues has been reported both in the laboratory and in the field. For example, Köck et al. (1996) reported that liver and kidney concentrations of Cd and Pb in Arctic Char (*Salvelinus alpinus*) were low at the end of the winter and increased during the summer, corresponding to a temperature-induced increase in metabolic rate. In the laboratory, higher temperatures have also been shown to stimulate tissue Cd uptake in Japanese eels (*Anguilla japonica*) (Yang and Chen, 1996), Nile tilapia (*Oreochromis niloticus*) (Abdel-Tawwab and Wafeek, 2014) and in zebrafish (*Danio rerio*) (Vergauwen et al., 2013).

4.2. Effects of metal accumulation

Since our data showed clearly that 9° C was the optimal temperature for perch condition (see Section 4.1), the discussion of metal effects will be limited to biomarker responses to Cd and Ni at 9° C. It should be noted however that at that temperature, mean kidney metal accumulation, although clearly occurring (Fig. 1), did not differ significantly in metal-exposed fish compared to controls. Nevertheless, the trend in metal accumulation corresponded to significant responses for several biomarkers.

Exposure to Ni has been reported to induce various changes in the liver of yellow perch (Eastwood and Couture, 2002; Pierron et al., 2009). In our study, at 9° C Ni contamination negatively affected HSI (Fig. 2c), but stimulated *cco* gene transcription level (Fig. 3a). The decrease of HSI with Ni accumulation suggests that Ni induces an increase of metabolic rate, consistent with the increase in liver *cco* transcription level and the parallel, although non-significant, increase in liver CCO activity, presumably to invest in metal detoxification and damage repair, leading to a reallocation of energy away from cell growth and proliferation (Couture and Pyle, 2015). The stimulation of aerobic capacities by Ni is fully consistent with our previous observations (Couture and Pyle, 2008; Pierron et al., 2009). However, further examination of our data leads us to propose an alternative hypothesis: The higher GSI in Ni-exposed perch suggests that this metal stimulates gonad maturation. A similar result was found for *Daphnia magna* (Vandenbrouck et al., 2011), where an increase in temperature and Ni contamination induced an increase in juvenile production. Investment in gonad development may therefore be the cause of lower HSI values, as reported for European perch (Fontaine et al., 2015). To our knowledge, this is the first study to report such a phenomenon in fish. Whether Ni acts as a micronutrient or as an estrogenic compound remains to be investigated.

Surprisingly, Cd had little effect on the biomarkers studied. As was the case for Ni exposure, Cd-exposed fish expressed a higher transcription level of the *cco* gene, but in contrast to Ni-exposed fish, liver CCO activity was maintained at the same level in Cd-exposed fish as in control fish, clearly suggesting that gene transcription was upregulated to maintain hepatic aerobic capacities. However, the cost of this metabolic readjustment was presumably minor, since the general condition of Cd-exposed fish at 9° C was not different from that of control fish, as indicated by FCF, HSI and GSI.

The discussion above focusing on fish exposed to metals at 9° C, it is not directly comparable to the study of Defo et al. (2014), in which yellow perch were exposed to the same metals under similar conditions but at 18° C, although their highest aqueous Cd concentrations were higher than ours (8 vs. 4 µg/L). Under those conditions, a significant accumulation of Cd and Ni in liver was accompanied by an induction of *g6pdh* gene transcription level but G6PDH activity only increased in Cd-exposed fish. By comparison, in our study, fish exposed to a lower Cd concentration at 20° C accumulated three-fold less Cd in their kidney than in the

Defo et al. study, which likely explains the absence of response of G6PDH in our Cd-exposed fish. Fish exposed to Ni in the Defo et al. (2014) study accumulated Ni in their kidney to reach concentrations similar to what we observed in our fish exposed at 20°C. The liver activity of G6PDH was not affected by Ni exposure in either study. However, although Defo et al. (2014) reported a sharp increase in the transcription level of *g6pdh* in their Ni-exposed fish, which they interpreted as an investment aiming at maintaining G6PDH activity, in the present study at 20°C the transcription level of the gene was strongly suppressed in control and metal-exposed fish, while enzyme activity was maintained. Hence, although the functional response (G6PDH activity) was comparable in both studies, the major difference in the transcription level of the corresponding gene between the two studies cannot be explained within our experimental design, in which the time course of biomarker response was not monitored. Likely, gene transcription levels respond over shorter time scales than enzyme activity in order to maintain physiological function.

4.3. Combined effects of metal contamination and heat stress

Although fish were exposed to the same aqueous metal concentrations in all metal exposure conditions, higher temperatures clearly stimulated renal metal accumulation, reaching a plateau for Ni at 20°C (Fig. 1a), but continuing to increase at 28°C for Cd (Fig. 1b). Although the effects of metal exposure were low to moderate at the colder temperature (see Section 4.2), metal contamination globally exacerbated the effects of heat stress on the biometric, transcriptomic and enzymatic biomarkers examined. Surprisingly, however, the heat- and metal-stress combination did not affect any of the biometric biomarkers examined significantly more than temperature itself.

The most obvious response to metal and temperature multi-stress was observed on biomarkers of apoptosis and oxidative stress. The response of the *diablo* gene, involved in regulating apoptosis, revealed a sharp increase of its transcription level in control fish between 9 and 20°C, which was completely reversed when temperature was further increased to 28°C. This pattern may be considered as a normal response for the species, indicating that at 20°C the cellular machinery was fully engaged in controlling enhanced heat-induced apoptosis, but that these protective mechanisms failed at the higher temperature. In a very similar experiment with yellow perch obtained from a different source (Grasset et al., 2014), the transcription level of the *diablo* gene did not respond at 20°C but peaked at 28°C, highlighting that critical temperature inducing apoptosis may vary according to strain and early life history (Beacham, 1988; Doyle et al., 2011; Jenkins and Hoffmann, 1994). Exposure to both Ni and Cd modified the normal response of the *diablo* gene. At 20°C, the metals inhibited the enhanced transcription of the *diablo* gene (Fig. 3b), possibly leading to a disruption of the normal increase in heat-induced apoptosis. In contrast, at 28°C, both metals prevented the failure of the *diablo* gene observed in control fish, suggesting an enhanced capacity for apoptosis. A stimulation of the *diablo* gene has been reported in hepatocytes of flounder exposed in mesocosms to sediment contaminated by organic and inorganic contaminants (Leaver et al., 2010). The gene therefore appears particularly useful as a biomarker of contaminant exposure, but our study demonstrates that its response to contaminants varies according to acclimation temperature.

Our study reveals a clear synergistic effect of metal exposure and heat stress on both *sod* gene transcription level and enzyme activity. While neither temperature alone or combined with Cd exposure induced an increase in *sod* transcription, the stimulation observed in Ni-exposed fish at 28°C compared to controls at 9°C indicates an active investment in combatting oxidative stress, which was reflected by a sharp increase of the SOD enzyme activity.

Post-transcriptional regulation of this metabolic pathway was also modulated by this multi-stress. While in control fish SOD activity only increased at 28°C, in both Cd- and Ni-exposed fish the activity of this antioxidant enzyme was enhanced at 20°C, and maintained at 28°C for Ni-exposed fish. However, at the higher temperature, the lower SOD activity in Cd-exposed fish compared to controls and Ni-contaminated fish suggests an increased vulnerability of these fish to oxidative damage. The addition of Ni contamination to thermal stress induces an increase of SOD activity (Banni et al., 2014), reflecting an increase of oxidative stress induced by temperature (Bagnyukova et al., 2007) and metal accumulation (Cao et al., 2012; Kubrak et al., 2014).

5. Conclusion

The present study contributes to improving our understanding of the effects of temperature, a common natural environmental stressor, on biomarkers of metal effects commonly used in wild fish. Given the high sensitivity of the activities of G6PDH and NDPK to metal exposure and their apparent lack of thermal sensitivity, we propose that these biomarkers of growth and energy storage be favoured in ecotoxicological studies. In contrast, most biometric indicators appeared to be poor indicators of metal stress under our experimental conditions, but were greatly affected by temperature. However, the Ni sensitivity of HSI and GSI warrants further consideration in field studies. Our data also support a growing body of literature suggesting that the combination of metal and temperature stress exacerbates or alters the normal apoptotic and antioxidant responses that these stressors alone induce. From a toxicological perspective, our study suggests an increased vulnerability of metal-contaminated fish to heat stress, which may result in ecological consequences. For environmental monitoring, however, we suggest that these biomarkers of metal stress may be too temperature-sensitive to be used in field studies unless a tight control of the field temperatures to which fish have been recently exposed is possible.

Acknowledgments

The authors wish to thank the staff of the Laboratoire régional des sciences aquatiques (LARSA) at Laval University for their invaluable help during the experiment. This study was supported by a research grant from the Canadian Natural Science and Engineering Research Council (NSERC Collaborative Research and Development Program) to P. Couture, P.G.C. Campbell and L. Bernatchez with Vale Ltd. as the industrial partner. L. Bernatchez and P.G.C. Campbell are supported by the Canada Research Chair Program.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.aquatox.2016.06.008>.

References

- Abdel-Tawwab, M., Wafeek, M., 2014. Influence of water temperature and waterborne cadmium toxicity on growth performance and metallothionein-cadmium distribution in different organs of Nile tilapia, *Oreochromis niloticus*. J. Therm. Biol. 45, 157–162. <http://dx.doi.org/10.1016/j.jtherbio.2014.09.002>.
- Bagnyukova, T.V., Lushchak, O.V., Storey, K.B., Lushchak, V.I., 2007. Oxidative stress and antioxidant defense responses by goldfish tissues to acute change of temperature from 3 to 23 °C. J. Therm. Biol. 32, 227–234. <http://dx.doi.org/10.1016/j.jtherbio.2007.01.004>.
- Banni, M., Hajer, A., Sforzini, S., Oliveri, C., Boussetta, H., Viarengo, A., 2014. Transcriptional expression levels and biochemical markers of oxidative stress in *Mytilus galloprovincialis* exposed to nickel and heat stress. Comp. Biochem.

- Physiol. C Toxicol. Pharmacol. 160, 23–29, <http://dx.doi.org/10.1016/j.cbpc.2013.11.005>.
- Beacham, T.D., 1988. A genetic analysis of early development in pink (*Oncorhynchus gorbuscha*) and chum salmon (*Oncorhynchus keta*) at three different temperatures. *Genome* 30, 89–96.
- Beck, B.H., Fuller, S.A., 2012. The impact of mitochondrial and thermal stress on the bioenergetics and reserve respiratory capacity of fish cell lines. *J. Aquat. Anim. Health* 24, 244–250, <http://dx.doi.org/10.1080/08997659.2012.720637>.
- Bermejo-Nogales, A., Nederlof, M., Benedito-Palos, L., Ballester-Lozano, G.F., Folkedal, O., Olsen, R.E., Sitjà-Bobadilla, A., Pérez-Sánchez, J., 2014. Metabolic and transcriptional responses of gilthead sea bream (*Sparus aurata* L.) to environmental stress: new insights in fish mitochondrial phenotyping. *Gen. Comp. Endocrinol.* 205, 305–315, <http://dx.doi.org/10.1016/j.ygcen.2014.04.016>.
- Bougas, B., Normandeau, E., Pierron, F., Campbell, P.G.C., Bernatchez, L., Couture, P., 2013. How does exposure to nickel and cadmium affect the transcriptome of yellow perch (*Perca flavescens*) – results from a 1000 candidate-gene microarray. *Aquat. Toxicol.* 142–143, 355–364, <http://dx.doi.org/10.1016/j.aquatox.2013.09.009>.
- Bremer, K., Kocha, K.M., Snider, T., Moyes, C.D., 2014. Energy metabolism and cytochrome oxidase activity: linking metabolism to gene expression. *Can. J. Zool.* 92, 557–568, <http://dx.doi.org/10.1139/cjz-2013-0267>.
- Cao, L., Huang, W., Shan, X., Ye, Z., Dou, S., 2012. Tissue-specific accumulation of cadmium and its effects on antioxidative responses in Japanese flounder juveniles. *Environ. Toxicol. Pharmacol.* 33, 16–25, <http://dx.doi.org/10.1016/j.etap.2011.10.003>.
- Cartañá, J., Romeu, A., Arola, L., 1992. Effects of copper: cadmium and nickel on liver and kidney glutathione redox cycle of rats (*Rattus sp.*). *Comp. Biochem. Physiol. C* 101, 209–213.
- Cochrane, K., De Young, C., Soto, D., Bahri, T., 2009. Climate change implications for fisheries and aquaculture: overview of current scientific knowledge. (No. 530), FAO Fisheries and Aquaculture Technical Paper., FAO Fisheries and Aquaculture Technical Paper. FAO Fisheries and Aquaculture Technical Paper. No. 530, Rome.
- Couture, P., Pyle, G., 2008. Live fast and die young: metal effects on condition and physiology of wild yellow perch from along two metal contamination gradients. *Hum. Ecol. Risk Assess. Int. J.* 14, 73–96, <http://dx.doi.org/10.1080/10807030701790322>.
- Couture, P., Pyle, G.G., 2015. Using *Perca* as biomonitors in ecotoxicological studies. In: Couture, P., Pyle, G.G. (Eds.), *Biology of Perch*. CRC Press.
- Couture, P., Busby, P., Rajotte, J.W., Gauthier, C., Pyle, G.G., 2008. Seasonal and regional variations of metal contamination and condition indicators in yellow perch (*Perca flavescens*) along two polymetallic gradients I. Factors influencing tissue metal concentrations. *Hum. Ecol. Risk Assess.* 14, 97–125.
- Csavina, J., Field, J., Taylor, M.P., Gao, S., Landázuri, A., Betterton, E.A., Sáez, A.E., 2012. A review on the importance of metals and metalloids in atmospheric dust and aerosol from mining operations. *Sci. Total Environ.* 433, 58–73, <http://dx.doi.org/10.1016/j.scitotenv.2012.06.013>.
- Defo, M.A., Pierron, F., Spear, P.A., Bernatchez, L., Campbell, P.G.C., Couture, P., 2012. Evidence for metabolic imbalance of vitamin A2 in wild fish chronically exposed to metals. *Ecotoxicol. Environ. Saf.* 85, 88–95.
- Defo, M.A., Bernatchez, L., Campbell, P.G.C., Couture, P., 2014. Waterborne cadmium and nickel impact oxidative stress responses and retinoid metabolism in yellow perch. *Aquat. Toxicol.* 154, 207–220, <http://dx.doi.org/10.1016/j.aquatox.2014.05.024>.
- Doyle, C.M., Leberg, P.L., Klerks, P.L., 2011. Heritability of heat tolerance in a small livebearing fish, *Heterandria formosa*. *Ecotoxicology* 20, 535–542.
- Eastwood, S., Couture, P., 2002. Seasonal variations in condition and liver metal concentrations of yellow perch (*Perca flavescens*) from a metal-contaminated environment. *Aquat. Toxicol.* 58, 43–56.
- Fontaine, P., Abdulfatah, A., Teletchea, F., 2015. Reproductive biology and environmental determinism of perch reproductive cycle. In: Couture, P., Pyle, G. (Eds.), *Biology of Perch*. CRC Press, Boca Raton, FL, pp. 167–192.
- Gauthier, C., Campbell, P.G.C., Couture, P., 2008. Physiological correlates of growth and condition in the yellow perch (*Perca flavescens*). *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 151, 526–532, <http://dx.doi.org/10.1016/j.cbpa.2008.07.010>.
- Giguère, A., Campbell, P.G., Hare, L., McDonald, D.G., Rasmussen, J.B., 2004. Influence of lake chemistry and fish age on cadmium, copper, and zinc concentrations in various organs of indigenous yellow perch (*Perca flavescens*). *Can. J. Fish. Aquat. Sci.* 61, 1702–1716, <http://dx.doi.org/10.1139/f04-100>.
- Grasset, J., Bougas, B., Campbell, P.G.C., Bernatchez, L., Couture, P., 2014. Temperature, oxygen and diet modulate gene transcription and metabolic capacities in yellow perch. *Can. J. Fish. Aquat. Sci.*, 140708143759004, <http://dx.doi.org/10.1139/cjfas-2014-0059>.
- Guderley, H., 2004. Locomotor performance and muscle metabolic capacities: impact of temperature and energetic status. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 139, 371–382, <http://dx.doi.org/10.1016/j.cbpc.2004.04.001>.
- Iles, A.C., Rasmussen, J.B., 2005. Indirect effects of metal contamination on energetics of yellow perch (*Perca flavescens*) resulting from food web simplification. *Freshw. Biol.* 50, 976–992, <http://dx.doi.org/10.1111/j.1365-2427.2005.01380.x>.
- Jenkins, N.L., Hoffmann, A.A., 1994. Genetic and maternal variation for heat resistance in *Drosophila* from the field. *Genetics* 137, 783–789.
- Köck, G., Triendl, M., Hofer, R., 1996. Seasonal patterns of metal accumulation in Arctic char (*Salvelinus alpinus*) from an oligotrophic Alpine lake related to temperature. *Can. J. Fish. Aquat. Sci.* 53, 780–786, <http://dx.doi.org/10.1139/f95-243>.
- Klinck, J.S., Green, W.W., Mirza, R.S., Nadella, S.R., Chowdhury, M.J., Wood, C.M., Pyle, G.G., 2007. Branchial cadmium and copper binding and intestinal cadmium uptake in wild yellow perch (*Perca flavescens*) from clean and metal-contaminated lakes. *Aquat. Toxicol.* 84, 198–207, <http://dx.doi.org/10.1016/j.aquatox.2007.03.024>.
- Krogdahl, A., Bakke-McKellep, A.M., 2005. Fasting and refeeding cause rapid changes in intestinal tissue mass and digestive enzyme capacities of Atlantic salmon (*Salmo salar* L.). *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 141, 450–460, <http://dx.doi.org/10.1016/j.cbpa.2005.06.002>.
- Kubrak, O.I., Poigner, H., Husak, V.V., Rovenko, B.M., Meyer, S., Abele, D., Lushchak, V.I., 2014. Goldfish brain and heart are well protected from Ni²⁺-induced oxidative stress. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 162, 43–50, <http://dx.doi.org/10.1016/j.cbpc.2014.03.011>.
- Leaver, M.J., Diab, A., Boukouvala, E., Williams, T.D., Chipman, J.K., Moffat, C.F., Robinson, C.D., George, S.G., 2010. Hepatic gene expression in flounder chronically exposed to multiply polluted estuarine sediment: absence of classical exposure 'biomarker' signals and induction of inflammatory, innate immune and apoptotic pathways. *Aquat. Toxicol.* 96, 234–245.
- Lemly, A.D., 1994. Mining in northern Canada: expanding the industry while protecting arctic fishes—a review. *Ecotoxicol. Environ. Saf.* 29, 229–242, [http://dx.doi.org/10.1016/0147-6513\(94\)90022-1](http://dx.doi.org/10.1016/0147-6513(94)90022-1).
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔC_T} method. *Methods* 25, 402–408, <http://dx.doi.org/10.1006/meth.2001.1262>.
- Lloret, J., Rätz, H.-J., 2000. Condition of cod (*Gadus morhua*) off Greenland during 1982–1998. *Fish. Res.* 48, 79–86.
- Nash, R.D.M., Valencia, A.H., Geffen, A.J., 2006. Essay: the origin of Fulton's condition factor – setting the record straight. *Fisheries* 31, 236–238.
- Pierron, F., Bourret, V., St-Cyr, J., Campbell, P.G.C., Bernatchez, L., Couture, P., 2009. Transcriptional responses to environmental metal exposure in wild yellow perch (*Perca flavescens*) collected in lakes with differing environmental metal concentrations (Cd, Cu, Ni). *Ecotoxicology* 18, 620–631, <http://dx.doi.org/10.1007/s10646-009-0320-7>.
- R Team, 2012. A Language and Environment for Statistical Computing [Computer Program]. Foundation for Statistical Computing, Vienna, Austria.
- Rosenzweig, C., Casassa, G., Karoly, D., Imeson, A., Liu, C., Menzel, A., Rawlins, S., Root, T.L., Seguin, B., Tryjanowski, P., 2007. Assessment of observed changes and responses in natural and managed systems. In: *Climate Change*. Cambridge University Press, pp. 79–131.
- Scott, W.B., Crossman, E.J., 1974. *Poissons d'eau douce du Canada*, Ministère de l'environnement, Service des pêches et des Sciences de la mer. Ottawa Bulletin, pp. 184.
- Vandenbrouck, T., Dom, N., Novais, S., Soetaert, A., Ferreira, A.L.G., Loureiro, S., Soares, A.M.V.M., De Coen, W., 2011. Nickel response in function of temperature differences: effects at different levels of biological organization in *Daphnia magna*. *Comp. Biochem. Physiol. Part D Genomics Proteomics* 6, 271–281, <http://dx.doi.org/10.1016/j.cbd.2011.06.001>.
- Vergauwen, L., Hagenaaers, A., Blust, R., Knapen, D., 2013. Temperature dependence of long-term cadmium toxicity in the zebrafish is not explained by liver oxidative stress: evidence from transcript expression to physiology. *Aquat. Toxicol.* 126, 52–62, <http://dx.doi.org/10.1016/j.aquatox.2012.10.004>.
- Homeostasis and toxicology of essential metals Wood, C.M., Farrell, A.P., Brauner, C.J. (Eds.), 2011. *Fish Physiology*, vol. 31 Part 1. Academic Press.
- Yang, H.-N., Chen, H.-C., 1996. Uptake and elimination of cadmium by Japanese eel *Anguilla japonica*, at various temperatures. *Bull. Environ. Contam. Toxicol.* 56, 670–676, <http://dx.doi.org/10.1007/s001289900098>.