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Effects of cadmium exposure on the gill proteome of *Cottus gobio*: Modulatory effects of prior thermal acclimation

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ABSTRACT

Temperature and trace metals are common environmental stressors, and their importance is increasing due to global climate change and anthropogenic pollution. The aim of the present study was to investigate whether acclimation to elevated temperature affects the response of the European bullhead (*Cottus gobio*) to subsequent cadmium (Cd) exposure by using enzymatic and proteomic approaches. Fish acclimated to 15 (standard temperature), 18 or 21 °C for 28 days were exposed to 1 mg Cd/L for 4 days at the respective acclimation temperature. First, exposure to Cd significantly decreased the activity of the lactate dehydrogenase (LDH) in gills of fish acclimated to 15 or 18 °C. However, an acclimation to 21 °C suppressed the inhibitory effect of Cd. Second, using a proteomic analysis by 2D-DIGE, we observed that thermal acclimation was the first parameter affecting the protein expression profile in gills of *C. gobio*, while subsequent Cd exposure seemed to attenuate this temperature effect. Moreover, our results showed opposite effects of these two environmental stressors at protein expression level. From the 52 protein spots displaying significant interaction effects of temperature and Cd exposure, a total of 28 different proteins were identified using nano LC–MS/MS and the Peptide and Protein Prophet algorithms of Scaffold software. The identified differentially expressed proteins can be categorized into diverse functional classes, related to protein turnover, folding and chaperoning, metabolic process, ion transport, cell signaling and cytoskeleton. Within a same functional class, we further reported that several proteins displayed reverse responses following sequential exposure to heat and Cd. This work provides insights into the molecular pathways potentially involved in heat acclimation process and the interactive effects of temperature and Cd stress in ectothermic vertebrates.

1. Introduction

The current increases in average temperatures and fluctuations in temperature extremes due to global climate change are considered as one of the new and important threats to aquatic ecosystems (Daufresne et al., 2009). Fuller et al. (2010) recognized four possible outcomes for a species under the influence of climate changes. Species may (1) become extinct or extirpated, (2) migrate or shift their current distribution range, (3) adapt to the changes through a change in the genetic composition of the population, or (4) employ phenotypic plasticity. Although one possible way of coping with climate change is migration to suitable new habitats (Parmesan and Yohe, 2003), this task is challenging or even impossible for some species (Fuller et al., 2010). Genetic change and phenotypic plasticity are thus the outcomes that prevent local extinction. Phenotypic plasticity (i.e., the ability of one genotype to adopt different phenotypes) is, however, likely to represent the first response of individual organisms under climate change (Bradshaw and Holzapfel, 2008; Somero, 2010). Such phenotypic plasticity can lead to heat acclimation which is defined as a "within lifetime" phenotypic adaptation involving a suite of physiological and biochemical adjustments that enhance thermotolerance and heat endurance (Horowitz, 2007).

Furthermore many aquatic species are also subjected to polluted environments and increased temperatures may pose additional threats to survival of ectotherms by modulating their susceptibility to other stressors, such as metal contamination. Previous studies have shown that elevated temperatures tend to enhance toxic effects of metals on aquatic organisms that may be partially explained by the higher uptake rates of metals and a higher intrinsic

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sensitivity of organisms (reviewed in Cairns et al., 1975; Heugens et al., 2001; Sokolova and Lannig, 2008). According to Sokolova and Lannig (2008), interference with aerobic metabolism, including energy demand, oxygen supply, and mitochondrial function, forms a physiological basis for interactions between environmental temperature and metal pollution. Within this interaction context it is important to differentiate between combined exposure to temperature and metal stress, and sequential combination of these stressors (Holmstrup et al., 2010). The latter scenario may be more ecologically relevant and attenuates temperature dependence, possibly due to common defense mechanisms against heat and metal stress (Vergauwen et al., 2013). Tedengren et al. (2000), for instance, have found that pretreatment to elevated temperature increased heat shock protein (HSP70) levels in mussels *Mytilus edulis* and conferred greater resistance to subsequent cadmium (Cd) exposure. More recently, Vergauwen et al. (2013) have reported that heat-acclimated zebrafish *Danio rerio* were more able to tolerate a subsequent Cd stress.

Proteomic analysis, the study of the protein complement of the cell, is increasingly used in aquatic toxicology as a powerful tool to examine potentially unforeseen responses to environmental stress (Sanchez et al., 2011a). It is generally admitted that a detailed understanding of the formation of a phenotype requires the study of all the steps during gene regulation and their final products at the proteome level (Feder and Walser, 2005; Karr, 2008). Further, proteomics is close to physiology, gives a functional knowledge of gene expression and is often reported as an important part of the cellular phenotype (Silvestre et al., 2012). Currently, changes of protein expression in aquatic organisms exposed to multiple stressors simultaneously or sequentially have received little attention. Using this approach, Silvestre et al. (2010) investigated the combined effects of heat stress and micro-injected selenium on sturgeon larvae (*Acipenser medirostris* and *A. transmontanus*) until stage D45 (after 8–12 days of exposure). The authors observed that proteins involved in correct protein folding, protein synthesis, protein degradation, ATP supply and structural proteins were predominantly affected by heat and/or selenium.

The aim of the present study was to investigate whether physiological acclimation to elevated temperature, in the range predicted by scientists, may modulate the response of ectotherms to subsequent environmental chemical insults. To address this question, the European bullhead (*Cottus gobio*) was chosen as an ecologically relevant organism. Bullhead is a small bottom-dwelling freshwater fish considered endangered in several parts of its distribution area as a result of pollution and habitat destruction and is known for its sensitivity to temperature changes (Utzinger et al., 1998; Dorts et al., 2012a). Fish were acclimated to 15 (standard temperature), 18 or 21 °C for 28 days, and then exposed to Cd at these temperatures for 4 days. The interactive effects of temperature and Cd exposure were investigated on gill tissue by monitoring the response of two enzymes involved in energy metabolism (citrate synthase CS and lactate dehydrogenase LDH). Furthermore, a proteomic approach using the two-dimensional differential in-gel electrophoresis (2D-DIGE) technique (Unlu et al., 1997) was undertaken to reveal molecular responses induced by heat acclimation that could modify the subsequent response to Cd exposure.

2. Material and methods

2.1. Fish capture and maintenance

Investigations and animal care were conducted according to the guidelines for the use and care of laboratory animals and in compliance with Belgian and European regulations on animal welfare. Adult European bullhead of both genders weighing 11.3 ± 2.9 g were caught by electrofishing in the Samson River (Belgium) in May 2009. Fish were acclimatized to laboratory conditions in dechlorinated tap water at 15.3 ± 1.4 °C under a 14:10 h (light/dark) photoperiod for four weeks before the experiment. During the acclimatization period, fish were fed daily to apparent satiation with frozen chironomid (*Chironomus sp.*) larvae.

2.2. Experimental setup

A total of 108 fish were randomly distributed into 9 tanks and acclimated to three temperatures: 15.1 ± 0.5 °C, 18.1 ± 0.2 °C, and 21.1 ± 0.4 °C for 28 days. There were three replicate tanks for each temperature. The temperature was increased by 3 °C per day from 15 to 18 or 21 °C. After heat acclimation, fish were distributed into 18 tanks filled with 12 L dechlorinated tap water and exposed to $CdCl_2$ (Sigma C2544) at nominal concentrations of 0 and 1 mg/L during 4 days at each of the three temperature (15, 18 or 21 °C). The treatments were as follows: (A) 15 °C → 0 mg Cd/L, (B) 15 °C → 1 mg Cd/L, (C) 18 °C → 0 mg Cd/L, (D) 18 °C → 1 mg Cd/L, (E) 21 °C → 0 mg Cd/L and (F) 21 °C → 1 mg Cd/L. The tested treatments were chosen according to our previous studies conducted with *C. gobio* (Dorts et al., 2011a, 2012a, 2012b). Each treatment included three replicate tanks, with 6 fish per tank. After 4 days of exposure, all fish were individually weighed, and gills were collected on ice, directly snap-frozen in liquid nitrogen and stored at −80 °C until homogenization. During the contamination stage, half-water was gently siphoned out and replaced every day. Animals were not fed during contamination while they were fed as described above during heat acclimation. No mortality was observed over the course of the experiment.

Total Cd concentrations in the exposure water were monitored every other day using a Sector Field Inductively Coupled Plasma Mass Spectrometer (Thermo Finnigan Element 2). Actual Cd concentrations in water samples were stable over the course of the experiment; the mean concentration and standard deviation in the 1 mg/L treatment were 0.89 ± 0.07 mg/L.

2.3. Metabolic enzyme activities

Enzymatic activities were assessed in gills from 3 pooled fish per replicate tank. One unit of gill tissue was homogenized with 15 units of ice-cold phosphate buffer (100 mM, pH 7.4) containing Complete-MiniTM Protease inhibitor cocktail (Roche). The homogenates were centrifuged at 1000 × *g* for 10 min at 4 °C, and the supernatants were kept at −80 °C for enzyme activity assays. Protein concentrations were measured according to Bradford (1976) using bovine serum albumin as a standard.

The experimental conditions for the enzymatic activities assays were as described by Dorts et al. (2011a): *Citrate synthase* (CS): 100 mM Tris/HCl, 0.1 mM DTNB, 0.3 mM acetyl CoA, 0.5 mM oxaloacetate, pH 8.1. *Lactate dehydrogenase* (LDH): 100 mM Tris/HCl, 0.3 mM NADH, 10 mM pyruvate, pH 7.4. Reactions were assayed spectrophotometrically following the reduction of DTNB for CS (at 412 nm) and the oxidation of NADH for LDH (340 nm). Millimolar extinction coefficients used were 13.6 for DTNB and 6.22 for NADH. Enzymatic activities were performed in duplicate. They are expressed in milliunit per mg protein. One unit corresponds to the amount of the enzyme that will convert 1 μmol of substrate into product per minute.

Results for the enzymatic activities were expressed as the mean ($n = 3$) ± S.D. All data were logarithm transformed to stabilize the variance and to approximate normal distribution. Differences between groups were analyzed using two-way analysis of variance (ANOVA 2) followed by a multiple comparison Fisher LSD test at a 5% significant level. All tests were performed using the Statistica 5.5 software (StatSoft, Tulsa, OK, USA). Further, a post hoc power