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Temporal changes in stress and tissue-specific metabolic responses to municipal wastewater effluent exposure in rainbow trout

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ABSTRACT

Sub-chronic exposure to municipal wastewater effluent (MWWE) *in situ* was recently shown to impact the acute response to a secondary stressor in rainbow trout (*Oncorhynchus mykiss*). However, little is known about whether MWWE exposure in itself is stressful to the animal. To address this, we carried out a laboratory study to examine the organismal and cellular stress responses and tissue-specific metabolic capacity in trout exposed to MWWE. Juvenile rainbow trout were exposed to 0, 20 and 90% MWWE (from a tertiary wastewater treatment plant), that was replenished every 2 d, for 14 d. Fish were sampled 2, 8 or 14 d post-exposure. Plasma cortisol, glucose and lactate levels were measured as indicators of organismal stress response, while inducible heat shock protein 70 (hsp70), constitutive heat shock protein 70 (hsc70) and hsp90 expression in the liver were used as markers of cellular stress response. Impact of MWWE on cortisol signaling was ascertained by determining glucocorticoid receptor protein (GR) expression in the liver, brain and, heart, and metabolic capacity was evaluated by measuring liver glycogen content and tissue-specific activities of key enzymes in intermediary metabolism. Plasma glucose and lactate levels were unaffected by exposure to MWWEs, whereas cortisol showed a transient increase in the 20% group at 8 d. Liver hsc70 and hsp90, but not hsp70 expression, were higher in the 90% MWWE group after 8 d. There was a temporal change in GR expression in the liver and heart, but not brain of trout exposed to MWWE. Liver glycogen content and activities liver gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK), and alanine aminotransferase (AlaAT) were significantly affected by MWWE exposure. The glycolytic enzymes pyruvate kinase (PK) and hexokinase (HK) activities were significantly higher temporally by MWWE exposure in the gill and heart, but not in the liver and brain. Overall, a 14 d exposure to MWWE evokes a cellular stress response and perturbs the cortisol stress response in rainbow trout. The tissue-specific temporal changes in the metabolic capacity suggest enhanced energy demand in fish exposed to MWWE, which may eventually lead to reduced fitness.

1. Introduction

Municipal wastewater effluent (MWWE) is a major contributor to endocrine disruption in the aquatic environment (Lange et al., 2009). It is becoming clearer that pathways other than those directly related to reproduction may be important targets of environmental contaminants, including MWWEs. These pathways are critical for physiological performance and survival, and include growth, development, osmoregulation, stress responses, metabolism and immune functions, among others (Filby et al., 2007; Vijayan et al., 2005, 2010), but few studies have addressed these questions to date.

A growing number of studies suggest that the stress response and related physiological processes may be affected by exposure to environmental contaminants commonly found in MWWEs, including polycyclic aromatic hydrocarbons, pharmaceuticals and industrial chemicals (Hontela et al., 1992, 1997; Gravel and Vijayan, 2007; Hontela and Vijayan, 2009; Aluru et al., 2010). The organismal stress response, including elevated plasma cortisol and catecholamine levels in response to stress, is an evolutionarily conserved adaptive response in vertebrates to metabolically adjust the animals to cope with the increased energy demand and to reestablish homeostasis (Vijayan et al., 2010). The cellular stress response involves a highly conserved family of proteins called the heat shock proteins (hsps), and is a protective response that helps combat stress-induced conformational damage to proteins (Hightower, 1991; Iwama et al., 2006).

We showed recently that 14 d *in situ* sub-chronic exposure to MWWE altered the adaptive organismal and cellular stress responses in trout (*Oncorhynchus mykiss*; Ings et al., 2011a,b). Specifically, MWWE exposure disrupted the unstimulated- and secondary stressor-stimulated cortisol and glucose responses. The impact on glucose homeostasis, included suppression of key hepatic glycolytic enzyme activities (Ings et al., 2011b), suggesting a role for MWWE in impacting energy metabolism. This suggests that long-term exposure to MWWE may function both as a chronic stressor, and impair the ability to

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respond to an additional stressor (Ings et al., 2011a,b). However, these studies looked at responses only after 14 d exposure period, so little is known about temporal changes in organismal and cellular stress parameters, and how they relate to secondary metabolic processes.

Stressor-induced elevation of plasma cortisol and its activation of glucocorticoid receptor (GR) is a key signal that maintains elevated plasma glucose levels to fuel energy demanding pathways, including protein synthesis (Mommsen et al., 1999; Aluru and Vijayan, 2007; Vijayan et al., 2010). This is mediated by cortisol stimulation of hepatic gluconeogenesis, including increased transcript abundance and activity of the key rate-limiting enzyme phosphenolpyruvate carboxykinase (PEPCK) (Mommsen et al., 1999; Sathiyaa and Vijayan, 2003; Vijayan et al., 2003). This response is essential for coping with the increased energy demand associated with stressor adaptation, so disruptions in the cortisol response by environmental contaminants, including MWWEs, may present metabolic challenges in exposed fish. Despite this, very few studies have looked directly at tissue metabolic capacities, including glycolytic and gluconeogenic pathways, in response to environmental contaminants (Vijayan et al., 1997; Gravel and Vijayan, 2007; Tintos et al., 2007). We hypothesize that MWWE disrupts the organismal and cellular stress responses, leading to temporal change in tissue-specific intermediary metabolism indicative of perturbation in energy substrate reorganization in juvenile rainbow trout.

2. Materials and methods

2.1. Animals

Juvenile rainbow trout (*O. mykiss*; average body mass of 23 ± 6 g) were obtained from Silvercreek Aquaculture (Erin, ON, Canada) and transported to the University of Guelph Hagen Aqualab (Guelph, ON, Canada). Fish were held in a holding tank (flow-through well water) for one week, and fed to satiety every second day. Fish were then transferred to the experimental tanks (static system) for a one week acclimation period before the start of the experiment. A light: dark regiment of 16:8 h was used. Water was changed completely every 2 d and to minimize handling disturbance during this step, fish were transferred to an identical set of tanks pre-filled with the same treatments and at the same water temperature. Fish were not fed for the duration of the experiment.

2.2. Experimental design

Fish were exposed to either well water (0%) or MWWE diluted with well water to 20% or 90% effluent. Sampling was carried out at 2, 8 and 14 d post-exposure. These time-points were chosen to coincide with water changes, so that fish had 2 d to recover from the handling disturbance. The 14 d duration and the concentrations were chosen based on previous experiments in the field (Ings et al., 2011a,b). Each sampling time-point consisted of triplicate tanks for treatments and two fish were removed from each tank for a total of 6 fish per treatment. A companion study was carried out looking at the chemical composition of the effluent using solid-phase extraction (SPE) and solid-phase microextraction (SPME) methods and various pharmaceuticals (carbamazepine, naproxen, diclofenac, gemfibrozil, fluoxetine, ibuprofen), bisphenol A, and atrazine were detected (Wang et al., 2011).

Fish were anesthetized with buffered MS-222 and killed by spinal severance, weighed and total length measured. Blood was withdrawn with needles and syringes coated in EDTA by caudal puncture and centrifuged at 3000 $\times g$ for 5 min to collect plasma, which was stored at −80 °C for steroid hormone, glucose and lactate analyses. Liver, brain, gill and heart were snap frozen in liquid nitrogen and stored at −80 °C for measuring glycogen content, protein expression and enzyme activities. This experiment was conducted in accordance with animal use protocols approved by the University of Waterloo Animal Care Committee, in accordance with the Canadian Council for Animal Care.

2.3. Plasma measurements

Plasma cortisol levels were measured by radioimmunoassy (RIA) using previously established methods (McMaster et al., 1995). Cortisol antibody was obtained from MP Biomedicals (Solon, OH, USA), and radiolabeled cortisol was obtained from GE Healthcare (Waukesha, WI, USA). Commercial kits were used to measure plasma glucose (Raichem, San Diego, CA, USA) and lactate levels (Trinity Biotech, St. Louis, MO, USA).

2.4. SDS-PAGE and western blotting

Liver, brain, heart, and gill were homogenized followed by sonication in 50 mM Tris buffer supplemented with protease inhibitors (Roche, Mannheim, Germany). Protein concentration was determined using bicinchoninic acid (BCA) method using bovine serum albumin (BSA) as the standard. Samples were diluted to a concentration of 2 mg/mL in Laemmli's buffer (Laemmli, 1970). The procedures for SDS-PAGE and western blotting followed established protocols (Sathiyaa and Vijayan, 2003). Briefly, 40 μg of total protein were separated using an 8% polyacrylamide gel along with a pre-stained molecular mass ladder (Bio-Rad Precision Plus prestained marker). The proteins were transferred onto a nitrocellulose membrane using a semi-dry transfer unit (Bio-Rad). Following transfer the membranes were blocked in 5% skim milk with 0.02% sodium azide in tris-buffered saline (TBS) with tween (TTBS) for 1 h followed by incubation in primary antibody [constitutive (hsc70; polyclonal rabbit anti-trout, 1:3000, (Boone and Vijayan, 2002), inducible heat shock protein 70 (hsp70; polyclonal rabbit anti-trout, 1:5000, StressMarq, Victoria, BC, Canada), heat shock protein 90 (hsp90; polyclonal rabbit anti-fish, 1:5000, StressMarq) or glucocorticoid receptor (polyclonal rabbit anti-trout GR, 1:1000, (Sathiyaa and Vijayan, 2003) for another hour. Membranes were then washed in TTBS (3×10 min) and incubated in secondary antibody (BioRad) coupled to horseradish peroxidase for 1 h. Membranes were again washed in TTBS (3×10 min) and then washed a final time in TBS for 10 min. Band detection was carried out using an ECL-Plus western blotting detection system (Amersham Biosciences, Piscataway, NJ, USA) and scanned by Typhoon imager using Cy2 blue laser. All bands were quantified with Chemi-imager using AlphaEase software (Alpha Innotech, Santa Clara CA, USA). Equal loading of sampling was confirmed by probing the blots with β-actin [Cy3-coupled monoclonal primary antibody from mouse, 1:1000, (Sigma, St. Louis, MO, USA)]. Hsps were measured in the liver, while GR was measured in all tissues. GR was below detection in the gill.

2.5. Liver glycogen and enzyme activities

Glycogen content was determined in the liver homogenate by measuring glucose content before and after amyloglucosidase hydrolysis according to Vijayan et al. (2003). The glycogen content is shown as micromoles glucosyl units per mg protein. The tissue homogenate (liver, brain, heart, gill) for enzyme activity determination was stored in a 50% glycerol buffer (50% glycerol, 21 mM Na2HPO4, 0.5 mM EDTA-Na, 0.2% BSA, 5 mM β-mercaptoethanol, pH 7.5). The enzyme activities were measured in 50 mM imidazole-buffered enzyme reagent (pH 7.4) at 22 °C by continuous spectrophotometry at 340 nm using a microplate reader (VersaMax; Molecular Devices Corp., Palo Alto, CA, USA) exactly as described previously (Vijayan et al., 2006). The following assay conditions were used:

- Hexokinase (HK: EC 2.7.1.1): 1 mM glucose, 5 mM $MgCl_2$, 10 mM KCl, 0.25 mM NADH, 2.5 mM phosphoenolpyruvate (PEP), 5 U/mL