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Exposure to municipal wastewater effluent impacts stress performance in rainbow trout

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ABSTRACT

The objective of the study was to examine the impact of municipal wastewater effluents on the functioning of the cortisol stress axis in rainbow trout (*Oncorhynchus mykiss*). Juvenile rainbow trout were caged upstream (reference) and downstream (100% and 10% effluent) of a tertiary-treated municipal wastewater treatment plant outfall and sampled at 14 d later (0 time samples). A second set of fish were then subjected to a 5 min handling disturbance and sampled at 1 and 24 h post-stressor exposure. Plasma cortisol, glucose and lactate concentrations, liver and brain glucocorticoid receptor (GR) protein levels, head kidney mRNA abundances of corticosteroidogenesis genes, including steroidogenic acute regulatory protein (StAR), cytochrome P450 side chain cleavage (P450scc), 11β-hydroxylase and melanocortin 2 receptor (MC2R), and key liver metabolic enzyme activities, were measured. Exposure to effluent for 14 d significantly elevated plasma cortisol and lactate levels in 100% effluent group compared to the reference and 10% effluent sites. There was a significantly higher StAR mRNA abundance in the effluent groups compared to the upstream control. GR protein levels in the liver, but not the brain, were significantly higher in the 100% effluent group compared to the upstream control group. Chronic exposure to 100% effluent for 14 d significantly lowered liver hexokinase and glucokinase activities, but did not affect glycogen content or the activities of phosphoenolpyruvate carboxykinase, pyruvate kinase, lactate dehydrogenase, aspartate aminotransferase and alanine aminotransferase compared to the other two groups. Subjecting these fish to a secondary acute stressor elicited a physiological stress response, including significant transient elevation in plasma cortisol, glucose and lactate levels at 1 h which dropped to pre-stress levels at 24 h after stressor exposure, in the control and 10% effluent groups, but this conserved stress response was impaired in the 100% effluent group. The 100% effluent group fish also had significantly higher StAR and P450scc mRNA abundances at 1 h post-stress, while transcript abundances of all the major corticosteroidogenesis genes were suppressed at 24 h post-stressor compared to the control and 10% effluent groups. Considered together, exposure to full-strength MWWE for 14 d elicits a chronic stress response in rainbow trout, and perturbs the conserved adaptive response to an acute stressor. Our results reveal that the impact of tertiary-treated MWWE on stress performance in rainbow trout is abolished by 90% effluent dilution.

1. Introduction

The stress response is a highly conserved adaptation to intrinsic or extrinsic stimuli that threaten to disturb an animal's homeostasis. It includes a series of behavioral, physiological and cellular changes, all of which are essential for the animal to cope with stress. A key aspect of the physiological response is the mobilization and re-allocation of energy substrates to meet the enhanced energy demand associated with stress (Mommsen et al., 1999). In teleosts, as in mammals, the metabolic adjustments involves the activation of two major neuroendocrine pathways, the hypothalamic–sympathetic–chromaffin cell axis and the hypothalamic–pituitary–interrenal (HPI) axis, leading to the release of catecholamines and corticosteroids, respectively (Iwama et al., 2006; Vijayan et al., 2010; Wendelaar Bonga, 1997).

Cortisol is the principal corticosteroid in teleosts and circulating levels of this steroid is increased in response to stress. This involves stressor-induced stimulation of hypothalamus, leading to the release of corticotropin-releasing factor (CRF), which stimulates the anterior pituitary to release adrenocorticotropic hormone (ACTH), the primary cortisol secretagogue (Vijayan et al., 2010; Wendelaar Bonga, 1997). ACTH binds to the melanocortin2 receptor (MC2R) on the steroidogenic cells of the interrenal tissue and activates the signaling cascade leading to corticosteroid biosynthesis (Vijayan et al., 2010). The key rate limiting steps in steroid biosynthesis involves the transport of cholesterol from

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the outer mitochondrial membrane to the inner mitochondrial membrane by the steroidogenic acute regulator protein (StAR; Stocco et al., 2005) and the conversion of the cholesterol to pregnenolone by the cytochrome P450 side chain cleavage (P450scc) enzyme (Payne and Hales, 2004). The terminal step in corticosteroid biosynthesis involves the conversion of deoxycortisol to cortisol by 11β-hydroxylase and this transcript abundance is not affected by acute stressor exposure in fish (Aluru and Vijayan, 2006).

Recent studies have clearly established that cortisol stress axis is a key target for endocrine disruptors (Hontela and Vijayan, 2008). However, most of these studies have utilized a single chemical exposure, including PCBs, metals, xenoestrogens and pharmaceuticals, in the laboratory to examine the cortisol response to stress in fish. Very little is known about the cortisol stress axis functioning in feral fish from contaminated sites (see Hontela and Vijayan, 2008; Vijayan et al., 2010). The release of municipal wastewater effluents (MWWEs) into the aquatic environment is a growing concern as it contains a diverse complement of chemicals, including AhR ligands, metals, xenoestrogens and pharmaceutical drugs, all of which are known to impact fish (Corcoran et al., 2010). It was recently shown that fish exposed to tertiary-treated MWWEs had higher plasma cortisol and glucose levels, and the transcript and protein abundances of a number of stress- and metabolism-related genes were upregulated (Ings et al., 2011). This suggests an overall enhancement of the metabolic demand in fish exposed to MWWE as protein synthesis constitutes a major energy demand for fish coping with stress (Mommsen et al., 1999).

Consequently, we tested the hypothesis that chronic exposure to MWWE will disrupt the evolutionarily conserved adaptive stress response in rainbow trout (*Oncorhynchus mykiss*). An in situ caging study was carried out with juvenile rainbow trout exposed to MWWE for 14 d. These fish were then subjected to a secondary acute stressor to determine the effect of MWWE exposure on stress performance. Plasma cortisol, glucose and lactate levels were measured as indicators of stress response, while brain and liver glucocorticoid receptor protein levels were used as markers of target tissue cortisol responsiveness. The liver metabolic capacity in response to MWWE exposure was investigated by measuring glycogen content and the activities of enzymes involved in glycolysis (hexokinase, glucokinase, pyruvate kinase and lactate dehydrogenase), gluconeogenesis (phosphenolpyruvate carboxykinase) and amino acid metabolism (aspartate aminotransferase and alanine aminotransferase). The steroid biosynthetic capacity was assessed by analyzing the head kidney mRNA abundances of key genes encoding proteins critical for corticosteroidogenesis, including MC2R, StAR, P450scc and 11β-hydroxylase.

2. Materials and methods

2.1. Experimental design and sampling

Juvenile rainbow trout (39 ± 7 g) were obtained from Silvercreek Aquaculture (Erin, ON, CAN) and transported to the exposure site and transferred directly to cages. The caging experiment was carried out in the Speed River, upstream and downstream of the municipal wastewater treatment plant, in Guelph, Ontario, Canada, during September and October, 2008. The Guelph municipal wastewater treatment plant serves a population of approximately 118,000 people with an average flow of approximately 54,400 m^3/d. It is a conventional extended activated sludge plant that denitrifies with a solids retention time of 15–28 d. The final effluent is polished with sand filtration and disinfected with sodium hypochlorite (CH2MHill, 2009).

Four sites were selected: two upstream reference sites, and two downstream sites that were chosen to represent approximately 100% and 10% effluent based on conductivity. The control sites were pooled for data analysis as no significant differences existed between the two reference sites. Conductivity at the upstream sites was 1110 μS/cm, while it was 4080 and 1459 μS/cm at the 100% and 10% effluent sites, respectively. The cages were placed near the centre of the river and the downstream distances from the effluent outfall was approximately 10 and 40 m for the 100% and 10% sites, respectively. There were three cages, made from 60 L Rubbermaid™ containers with small holes for water flow, at each site and contained six fish each. A cement block was placed in each cage as a refuge for the fish from the current, and also as a weight to keep the cage in place. They were anchored to fence posts embedded in the substrate in approximately 60 cm of stream water.

The exposure was for 14 d and during this period the fish were not fed. Following the exposure period, one cage from each site was sampled (pre-stress samples). The fish in the remaining two cages at each site were subjected to a handling disturbance for 5 min by shaking the cage up and down in the water, and were sampled 1 and 24 h post-stressor. To minimize sampling stress, fish were anesthetized with buffered MS-222 immediately after removal from the cage in the river, carried to shore and bled using heprinized needles by caudal puncture in quick succession. Fish were killed by spinal severance, weighed and fork length measured. Blood was centrifuged at $3000 \times g$ for 5 min to collect plasma for steroid hormone, glucose and lactate analyses. The fish were all immature and liver weights were recorded. Liver, head kidney and brain were snap frozen in liquid nitrogen and stored at −80 °C for later glycogen determination, RNA extraction, protein analysis, and enzyme activity assays. This experiment was conducted in accordance with animal use protocols approved by the Canadian Council for Animal Care and approved by the University of Waterloo Animal Care Committee.

2.2. Plasma measurements

Plasma levels of cortisol were measured directly from plasma by radioimmunoassay (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 Deigo, CA, USA) and lactate levels (Trinity Biotech, St. Louis, MO, USA).

2.3. SDS–PAGE and Western blotting

Livers and brains were homogenized followed by sonication in 50 mM Tris buffer supplemented with protease inhibitors (Roche, Mannheim, Germany) according to Vijayan et al. (2006). Protein concentration was determined using bicinchoninic acid (BCA) method using bovine serum albumin as a standard. The procedures for SDS–PAGE and Western blotting followed established protocols (Sathiyaa and Vijayan, 2003). Briefly, 40 μg of total protein was separated using an 8% polyacrylamide gel along with a prestained molecular mass ladder (Bio-Rad Precision Plus prestained ladder). The proteins were transferred onto a nitrocellulose membrane using a semi-dry transfer unit (Bio-Rad) at 20 V for 25 min with a transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 10% methanol). Following transfer, the membranes were blocked in 5% skim milk with 0.02% sodium azide in TTBS (20 mM Tris, pH 7.5, 300 mM NaCl, and 0.1% Tween 20) for 1 h followed by incubation in primary antibody (polyclonal rabbit anti-trout GR, 1:1000) overnight at 4 °C. Membranes were washed in TTBS (3×10 min) and incubated in secondary antibody (goat-anti-rabbit IgG coupled