Stress And Metabolic Responses To Municipal Wastewater Effluent Exposure In Rainbow Trout

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Municipal wastewater effluent (MWWE) is an important source of pollution in the aquatic environment impacting fish. MWWE is a complex mixture of chemicals including pharmaceuticals, personal care products, industrial chemicals and pesticides. A link between reproductive endocrine disruption and MWWE exposure has been established in fish, but less is known about the effects of MWWE on non-reproductive endocrine disruption. The overall objective of this thesis was to examine the impacts of MWWE exposure on the stress response and intermediary metabolism in rainbow trout (*Oncorhynchus mykiss*). In fish, the primary adaptive organismal stress response involves the activation of hypothalamic-sympathetic-chromaffin axis to produce catecholamines, predominantly epinephrine, and the hypothalamic-pituitary-interrenal (HPI) axis to produce cortisol. Both of these hormones play a key role in elevating plasma glucose levels that is essential to fuel the increased energy demand associated with stress. Along with the organismal stress response, the cellular stress response, involving the synthesis of a suite of heat shock proteins (hsps), also plays an important role in protecting cellular protein homeostasis in response to stressors, including toxicants.

The impact of MWWE on stress-related pathways were identified using a low-density trout cDNA microarray enriched with genes encoding for proteins involved in endocrine-, stress- and metabolism-related processes. This was further confirmed by assessing plasma hormone and metabolite levels and stress-related targeted genes and proteins expression and enzyme activities in select tissues in rainbow trout. Studies were carried out in controlled field (caging) and laboratory experiments to examine the impacts of MWWE on stress and

tissue-specific metabolic responses in rainbow trout. Further *in vitro* studies using rainbow trout hepatocytes in primary cultures were carried out to investigate the mechanism of action of two pharmaceuticals, atenolol and venlafaxine, found in relatively high concentrations in MWWE in impacting the stress-mediated glucose response.

In caged fish, MWWE exposure significantly elevated plasma cortisol and glucose concentrations, and altered the mRNA abundance of a number of stress-related genes, hormone receptors, glucose transporter 2 and genes related to immune function. When fish were exposed to an acute handling stress following a 14 d exposure to MWWE, the cortisol response was abolished and the glucose response was attenuated. The effects on cortisol did not correlate with changes in the expression of genes involved in cortisol biosynthesis, but were associated with an increase in hepatic glucocorticoid receptor (GR) protein expression.

Upon further investigation in controlled laboratory studies, MWWE exposure elevated constitutive hsp 70 and hsp90 expression after 8 d exposure, which correlated with a decrease in glycogen levels in the liver in fish exposed to a high concentration of MWWE compared to control fish, pointing to a MWWE-induced increase in liver energy demand. By 14 d, glycogen stores were replenished, and this was commensurate with increases in liver gluconeogenic capacity, including increases in the activities of phosphoenolpyruvate carboxykinase (PEPCK) and alanine aminotransferase (AlaAT), along with a decrease in liver GR expression. In the heart, GR protein expression increased in treated fish, and the activity of pyruvate kinase increased, indicating an increase in glycolytic capacity. Subjecting the MWWE exposed fish to a secondary handling disturbance (acute stress) led to an attenuated plasma cortisol and glucose response compared to the control group. This

corresponded with a reduced liver gluconeogenic capacity and a lower liver and heart glycolytic capacities, reflecting a disturbance in the energy substrate repartitioning that is essential to cope with stress.

While it is difficult to establish causative agents from a complex mixture such as MWWE, the two pharmaceutical that were tested impacted glucose production. Specifically, atenolol and venlafaxine disrupted the epinephrine-induced glucose production, but did not modify cortisol-mediated glucose production in trout hepatocytes. The suppression of epinephrine-mediated glucose production by atenolol and venlafaxine was abolished by cAMP analogue ([8bromo] cAMP) or glucagon (a metabolic hormone that increases glucose production). This suggests that both drugs disrupt β-adrenoceptor signaling, while it remains to be determined if the response is receptor isoform-specific.

Altogether MWWE exposure disrupts the organismal and cellular stress responses in trout. Key targets for MWWE impact leading to the impaired cortisol and metabolic responses to stress include liver and heart GR expression, liver gluconeogenic capacity, and liver, heart and gill glycolytic capacities. Most significantly, MWWE impairs the ability to metabolically adjust to a secondary acute stressor, which is an important adaptive process that is integral to successful stress performance. From an environmental stand-point, long-term exposure to MWWE will lead to reduced fitness and will compromise the capacity of fish to cope with additional stressor, including escape from predators.

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1.1 Introduction

There are various definitions of what constitutes a stress in fish (see reviews Barton et al., 2002; Iwama et al., 2006; Vijayan et al., 2010) but generally stress is considered any change that will disturb the homeostasis of an animal leading to increased energy demand. The stress response involves changes at the molecular, biochemical and physiological levels, including alterations in plasma hormone, protein and metabolite levels as well as intracellular changes in protein expression, all of which are essential to reestablish homeostasis. In the short-term this involves mobilization and reallocation of energy resources to meet the increased energy demand. Under a long-term scenario, this drain on energy resources may occur at the expense of other energy demanding pathways, including growth, reproduction and immune function, leading to an overall reduced fitness. However, the various pathways involved and their adaptive value in the stress response process is only starting to emerge. To this end, transcriptomics, the study of expression patterns of the complete compliment of actively expressed genes either in a cell, tissue or whole organism, of model and non-model animals are beginning to shed light on the molecular basis of the stress response. This introduction chapter will focus on the functional genomics of the stress response in fish and its application in ecotoxicology.

1.2 Stress Response

As one can imagine there are various types of stressors that fish encounter and this to a large extent is dependent upon their habitat and environment. For instance, animals confined to hatcheries and aquaculture related environments will be exposed to physical, chemical, nutritional and pathogen stressors (see Barton et al., 2002). Anthropogenic

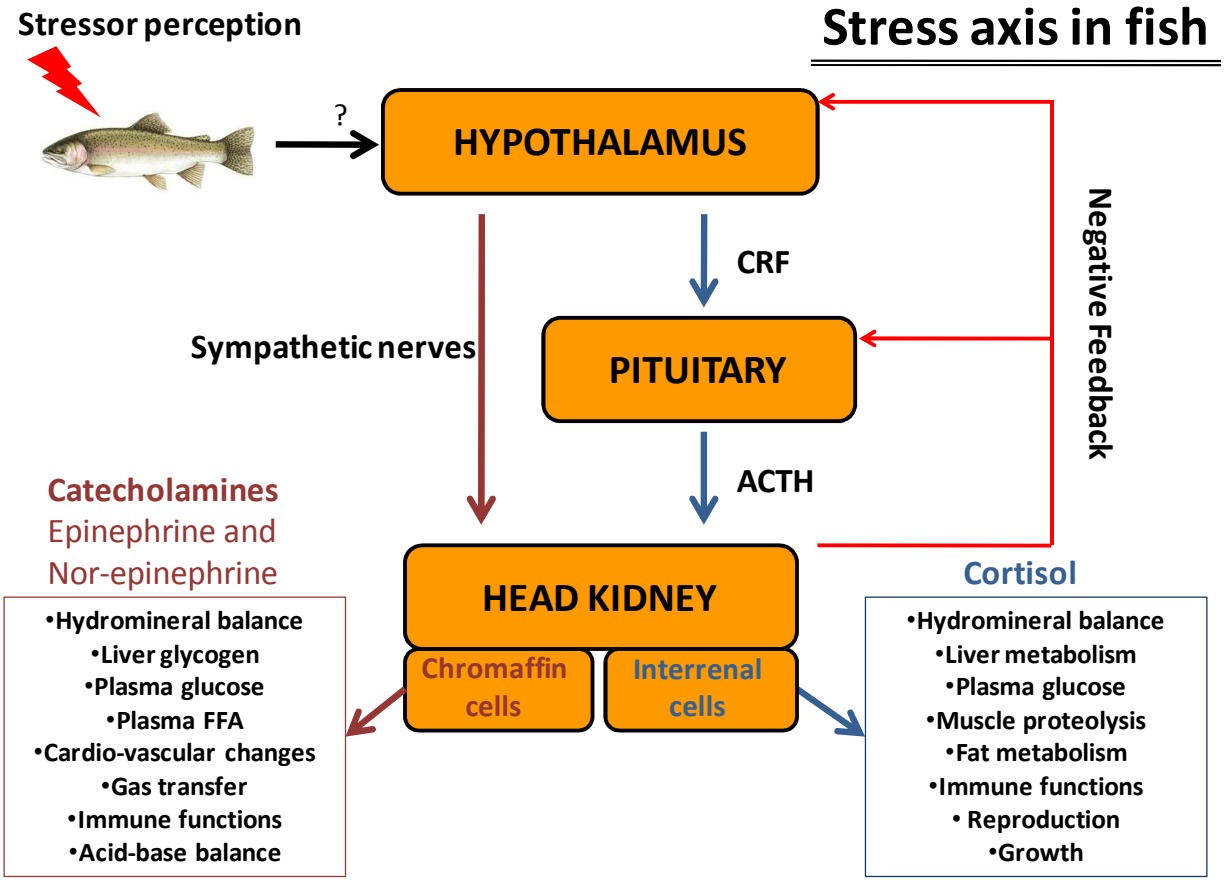
stressors, including toxic chemicals, are present in our water-ways and fish are exposed to these chemicals either singly or as complex mixtures. The toxicants in the aquatic environment impart wide ranging effects on fish health, including endocrine disruption, abnormal acute stress response, impaired ion regulation, altered growth and reproductive dysfunction (Barton et al., 2002; Hontela and Vijayan, 2009).

Exposure to stressors elicits a specific and/or a non-specific stress response. Selye (1950) classified the stress response as the general adaptation syndrome and categorized the responses temporally into three stages: i) the alarm phase, which includes the perception of the stressor and the physiological recognition of its threat to homeostasis, ii) the resistance phase, involving physiological changes that are essential to cope with the stress and leads to adaptation, and iii) the exhaustion phase, where coping is no longer possible and health effects are inevitable (Barton et al., 2002). While the pathways involved in either adapting or maladapting the animals to stress are not well understood, the role of hypothalamic-sympathetic-chromaffin cell (HSC) axis and hypothalamic-pituitary-interrenal (HPI) axis functioning in stress adaptation is well established (Vijayan et al., 2010). The HSC axis activation leads to the release of norepinephrine from the post-ganglionic neurons (Reid et al., 1998; Vijayan et al., 2010) and epinephrine and norepinephrine into the circulation from the chromaffin cells, located in clusters along with the steroidogenic cells around the post-cardinal vein in the teleost head kidney (Reid et al. 1998). The catecholamines play an important role in cardiovascular and respiratory adjustments, including enhanced blood oxygen transport and cardiac output, which are essential for energy substrate reallocation to cope with the increased energy demand associated with stress (Wendelaar Bonga, 1997;

Vijayan et al., 2010). The HPI axis activation leads to the release of cortisol, the principal corticosteroid in teleosts, into the bloodstream in response to stressor exposure (see Fig. 1). The functioning of HPI axis involves the stressor-mediated stimulation of the hypothalamus causing the release of the peptide neurohormone corticotropin-releasing factor (CRF), which stimulates the anterior pituitary to release adrenocorticotropic hormone (ACTH). Although other neuropeptides and hormones have been shown to stimulate cortisol release from the interrenal tissues, ACTH is the primary cortisol secretagogue (Wendelaar Bonga, 1997; Barton et al., 2002; Iwama et al., 2006). ACTH binds to the melanocortin 2 receptor (MC2R) on steroidogenic cells in the interrenal tissue, leading to the activation of the steroid biosynthetic pathway and the production of cortisol (Aluru and Vijayan, 2008). This is accomplished through a series of enzymatic reactions starting with the transport of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane by steroidogenic acute regulatory protein (StAR; Stocco et al., 2005) and peripheral-type benzodiazepine receptor (PBR; Papadopoulos, 2004), often referred to as the rate limiting step in steroidogenesis. Two key enzymes important for cortisol biosynthesis are cytochrome P450 side chain cleavage (P450scc) and 11β-hydroxylase (Mommsen et al., 1999; Payne and Hales, 2004).

Following a stressor exposure, catecholamines are released within seconds to minutes into the circulation, followed by cortisol release within minutes to hours. This temporal profile is modified by various factors, including the type and intensity of the stressor, life-history types and also depends on the species involved (Iwama et al., 2006; Vijayan et al., 2010). The primary hormonal stress response is important in the regulation of many

Fig. 1: Schematic diagram of the hypothalamic-sympathetic-chromaffin cell (HSC) axis and hypothalamic-pituitary-interrenal cell (HPI) axis function following stressor perception in fish, adapted from Wendelaar Bonga (1997). Following stressor perception, the HSC axis is activated with sympathetic nerves from the hypothalamus stimulating the chromaffin cells in the head kidney to release catecholamines (epinephrine and nor-epinephrine) into the blood stream, stimulating physiological changes required for an alarm, or fight-or-flight response. During HPI axis activation, corticotrophin-releasing factor (CRF) released from the hypothalamus stimulates the anterior pituitary to secrete adrenocorticotropic hormone (ACTH), the primary cortisol secretagogue, into the blood stream, which increases cortisol synthesis and release from the interrenal cells in the head kidney, which promote physiological changes required for regaining and maintaining homeostasis.


Stress axis in fish
Stressor perception
?
HYPOTHALAMUS
CRF
PITUITARY
ACTH
Sympathetic nerves
Negative Feedback
HEAD KIDNEY
Chromaffin cells
Interrenal cells
Catecholamines
Epinephrine and
Nor-epinephrine
•Hydromineral balance
•Liver glycogen
•Plasma glucose
•Plasma FFA
•Cardio-vascular changes
•Gas transfer
•Immune functions
•Acid-base balance
Cortisol
•Hydromineral balance
•Liver metabolism
•Plasma glucose
•Muscle proteolysis
•Fat metabolism
•Immune functions
• Reproduction
•Growth

physiological and cellular processes, including energy substrate mobilization and reallocation, increased cardiac output and blood flow and enhanced oxygen uptake and transport at the gills, all essential for acute stress adaptation (Fig. 1; Mommsen et al., 1999; Iwama et al., 2006; Vijayan et al., 2010). In the short-term, stress responses are geared towards metabolic adjustments and fuel delivery to tissues that have a higher energy demand, while in the long-run this may lead to negative consequences, including impaired immune function, growth and reproduction, due to diversion of energy resources from these growth and health processes in order to reestablish homeostasis.

1.3 Metabolism

The stress response and intermediary metabolism are strongly connected. Studies have used plasma metabolite levels such as glucose and lactate as surrogates for stressor and hormone effects on metabolism (Vijayan et al., 1994; 2003; 2010). A clear link has been made between increased cortisol and increased plasma glucose levels (Mommsen et al., 1999; Vijayan et al., 2010), as the primary role of cortisol is to mobilize energy substrate in order to cope with the increased energy demand associated with stress. This is in part thought to be mediated by cortisol through an increase in the metabolic capacity of the liver, primarily by the stimulation of gluconeogenesis, and to a lesser extent by increasing glycogenolysis, proteolysis and lipolysis (Mommsen et al., 1999). Studies have demonstrated an increase in activity or transcript abundance of key gluconeogenic enzymes, including phosphoenolpyruvate carboxykinase, which catalyzes the conversion of oxaloacetate to phosphoenolpyruvate, in fish exposed to stress (Wiseman et al., 2007) or cortisol (Sathiyaa

and Vijayan, 2003; Vijayan et al., 2003). Amino acid catabolism is also important in providing substrate for hepatic gluconeogenesis and has been estimated in response to contaminant and stress exposure by measuring the activity of aminotransferase enzymes such as alanine aminotransferase and aspartate aminotransferase, which convert their respective amino acids into pyruvate (Vijayan et al., 1994; Gravel and Vijayan, 2007; Tintos et al., 2008) An increase in the activity of hepatic and extra-hepatic glycolytic enzymes, such as hexokinase, pyruvate kinase and lactate dehydrogenase, has also been associated with acute stress and cortisol exposure, likely to provide metabolic fuel required to cope with the increased energy demand associated with stressor adaptation (Vijayan et al., 1994; Gravel and Vijayan, 2007). Very few studies have looked at tissue-specific metabolic adjustments in response to chronic contaminant exposure or how this primary stressor exposure affects the ability of a fish to respond and adjust to subsequent secondary acute stressors, but it is well known that tissues, including heart, gill and brain increase their energy demand during stress (Mommsen et al., 1999).

1.4 Functional Genomics

Gene expression has been shown to be a sensitive indicator of stressor exposure, as well as of animal health and metabolic status, and can provide a plethora of information on how an organism adapts to its environment (Denslow et al., 2007; Gracey and Cossins, 2003). While gene expression may not always equate to protein synthesis, the effector molecules that are essential for adaptation, it does reflect the first line of genetic response elicited by an organism in response to disturbed homeostasis. Therefore, the analysis of global gene expression changes has the potential to identify and develop markers of exposure

and effects that are stressor non-specific and stressor-specific, as well as to identify the mode of action of stressors, including environmental toxicants (Lettieri, 2006). If used in this way, functional genomics provides a sensitive and often stressor-specific platform to understand the consequences of environmentally relevant anthropogenic impacts in fish and other animals. The pathways involved in the short-term and long-term adjustments to stress in animals, and how they can be modulated by exposure to environmental contaminants, are far from clear. Functional genomics may be useful for identifying stressor-specific and stressor-non-specific regulatory pathways and gene networks that are involved in the stress response.

1.4.1 Microarrays

By far, the most commonly applied method for transcriptomics and functional genomics studies is the microarray. DNA microarray technology evolved from the principle that nucleic acid molecules attached to a solid support could be differentiated and studied using complimentary labeled nucleic acids (Gracey, 2007; Lettieri, 2006). Microarrays consist of strands of nucleic acid material adhered to a solid surface, usually a glass slide, in a recognizable pattern. There are many different types of microarrays, each with immense value depending on the research questions. Microarrays can be made using two different probe types: oligonucleotides (Oligo; 25-120 nucleotides), or cDNA (100-3000 base pairs) (Lettieri, 2006). In cDNA microarrays, the probes are made from normalized/subtracted cDNA libraries, or targeted PCR amplification. These microarray platforms produce strong signals and high specificity. The short fragment size of oligonucleotide arrays, the most recent and advanced with oligo synthesis occurring directly on the slide, allow for high density spotting resulting in strong signals with reasonable specificity.

The majority of microarray studies to date have utilized model species. This is advantageous, as the knowledge of genomic sequences for these species and the availability of commercial microarrays allows for more complex and comprehensive research. Zebrafish (*Danio rerio*) in particular has been the work-horse in many areas of genomics research related to environmental toxicology, including studies on the effects of estrogens and estrogenic compounds (Hoffmann et al., 2008; Ruggeri et al., 2008; Baker et al., 2009; De Wit et al., 2010), pharmaceuticals (van der Ven et al., 2006), metals (Craig et al., 2009; Sawle et al., 2010) and persistent organic pollutants (Lyche et al., 2010). However, the question always remains as to whether information gained from zebrafish research can be extrapolated for stressor impact on feral fish species, especially those occupying different habitats and exhibiting different life-history strategies.

Indeed, more effort has been made to develop microarrays for non-model species that are more relevant due to either their natural distribution or commercial importance, for instance aquaculture. There are high-density salmonid microarrays available for use through the Genomics Research on All Salmon Project (GRASP, von Schalburg et al., 2005). Microarray platforms are also available for commercially important fish species such as cod (*Gadus morhua*; Lie et al., 2009) and seabream (*Sparus aurata*; Calduch-Giner et al., 2010) (Table 1). From an ecotoxicological stand-point, apart from salmonids, arrays for several non-model species have been developed, including fathead minnows (*Pimephales promelas;* i.e. Garcia-Reyero et al., 2009a,b,c; Gust et al., 2010), three-spined stickleback (*Gasterosteus aculeatus*; Leder et al., 2009), largemouth bass (*Micropterus salmoides*; Garcia-Reyero et al., 2008), horneyhead turbot (*Pleuronichthys verticalis*; Baker et al.,

Table 1: Recent publications using microarray analysis technology for environmental research in fish.

Species	Array Description	Array Platform	Application	Reference
Zebrafish	Oligo, 22000 genes	Agilent	Microcystin toxin exposure	Rogers et al., 2011
Zebrafish	Oligo, 16327 genes	Custom	DCP, DCA, PCP, cadmium chloride exposure	Sawle et al., 2010
Zebrafish	Oligo, 3479 genes	Custom	17α-ethinylestradiol exposure	De Wit et al., 2010
Zebrafish	Oligo, 16228 genes	Custom	Persistent organic pollutant exposure	Lyche et al., 2010
Zebrafish	Oligo, 16730 genes	Custom	Copper exposure	Craig et al., 2009
Zebrafish	Oligo, 22000 genes	Agilent	Fadrozole exposure	Villeneuve et al., 2009
Zebrafish	Multi-species oligo, DNS	Custom	Estradiol and 4-nonylphenol exposure	Baker et al., 2009
Zebrafish	Oligo, 21000 genes	Agilent	Hypoxia exposure	Martinovic et al., 2009
Zebrafish	Oligo, 16399 genes	Custom	Estradiol and 4-nonylphenol exposure	Ruggeri et al., 2008
Zebrafish	Oligo, 14900 genes	Affymetrix GeneChip®	17α-ethinylestradiol exposure	Hoffmann et al., 2006
Zebrafish	Brain-specific cDNA, 683 genes	Custom	Mianserin exposure	van der Ven et al., 2006
Rainbow Trout	Low density specific cDNA, 147 genes	Custom	Municipal wastewater effluent	Ings et al., 2011
Rainbow Trout	cDNA, 16000 genes	GRASP	Heat stress	Lewis et al., 2010
Rainbow Trout	cDNA, 16000 genes	GRASP	Crude oil exposure	Hook et al., 2010a
Rainbow Trout	cDNA, 16000 genes	GRASP	Hydrocarbon mixture exposure	Hook et al., 2010b
Rainbow Trout	cDNA, 16000 genes	GRASP	Diesel exposure	Mos et al., 2008
Rainbow Trout	Liver-specific cDNA, 21120 genes	Custom	Confinement, handling stress	Cairns et al., 2008
Rainbow Trout	Low density specific cDNA, 147 genes	Custom	Beta-naphthoflavone exposure	Aluru and Vijayan, 2008
Rainbow Trout	Oligo, 1450 genes	Custom	Estrogen exposure	Benninghoff and Williams, 2008
Rainbow Trout	Oligo, 1672 genes	Custom	Acute stress	Momoda et al., 2007
Rainbow Trout	Low density specific cDNA, 147 genes	Custom	Acute stress	Wiseman et al., 2007
Rainbow Trout	cDNA, 16000 genes	GRASP	Model chemicals and mixture	Finne et al., 2007
Trout	Low density specific cDNA, 147 genes	Custom	Contaminated lakes survey	Moran et al., 2007
Rainbow Trout	Low density specific cDNA, 32 genes; cDNA, 16000 genes	Custom, GRASP	17α-ethinylestradiol exposure	Skillman et al., 2006
Fathead minnow	Brain-specific cDNA, 4128 genes	Custom	Cyclotrimethylenetrinitramine exposure	Gust et al., 2010
Fathead minnow	Oligo, 22000 genes	EcoArray	Municipal wastewater effluent exposure	Garcia-Reyero et al., 2011
Fathead minnow	Oligo, 15000 genes	EcoArray	Pulp and paper mill effluent exposure	Popesku et al., 2010
Fathead minnow	Oligo, 22000 genes	EcoArray	Municipal wastewater effluent in situ exposure	Garcia-Reyero et al., 2009a
Fathead minnow	Oligo, 22000 and 2000 genes	EcoArray	Estrogen/antiestrogen exposure, males	Garcia-Reyero et al., 2009b
Fathead minnow	Oligo, 22000 genes	EcoArray	Androgen/antiandrogen exposure, females	Garcia-Reyero et al., 2009c
Fathead minnow	cDNA, 5000 genes	Custom	Lead exposure	Mager et al., 2008
Fathead minnow	Oligo, 22000 genes	EcoArray	17β-trenbolone exposure	Dorts et al., 2008
Fathead minnow	Oligo, 11000 genes	Custom	Fadrazole exposure	Villeneuve et al., 2008
Fathead minnow	Oligo, 15000 genes	EcoArray	Methylmercury exposure	Klaper et al., 2008
Fathead minnow	cDNA, 5000 genes	Custom	2,4-DNT exposure	Wintz et al., 2006
Medaka	cDNA, 3055 genes	Custom	Arochlor 1260 exposure	Yum et al., 2010
Three-spine stickleback	cDNA, 14496 genes	Custom	17α-ethinylestradiol exposure	Katsiadaki et al., 2010
Three-spine stickleback	cDNA, 14496 genes	Custom	Copper exposure	Santos et al., 2010b
Three-spine stickleback	Oligo, 19274 genes	Custom	General	Leder et al., 2009
Three-spine stickleback	cDNA, 4972 genes	Custom	Estradiol, dibenzanthracene exposure	Geoghegan et al., 2008
Atlantic salmon	cDNA, 17000 genes	Custom	Acetaminophen, carbamazepine, atenolol exposure	Hampel et al., 2010
Atlantic cod	cDNA, 744 genes	Custom	Alkylphenol and 17β-estradiol exposure	Lie et al., 2009
Horny-head turbot	Multi-species oligo, DNS	Custom	Coastal region survey	Baker et al., 2009
Goldfish	cDNA, 8664 genes	Custom	Fadrozole exposure	Zhang et al., 2009
Githead seabream	cDNA, 4876 genes	Custom	Confinement exposure	Calduch-Giner et al., 2010
Largemouth bass	Oligo, 16350 genes	Custom	Non-model species microarray development	Garcia-Reyero et al., 2008

2009), killifish (*Fundulus heteroclitus;* Healy et al., 2011) and goldfish (*Carassius auratus*; Zhang et al., 2009) (Table 1). High density arrays are often comprised of numerous gene fragments of unknown identity, especially when constructed from expressed sequence tag (EST) databases where repetitive sequences are common. cDNA microarrays constructed from EST sequences can be limited when it comes to sequence-oriented interpretation of the data (Gracey, 2007). The primary advantage of these arrays is gene discovery and to generate hypothesis to test novel pathways that were not previously associated with stress in animals. In addition to high-density arrays, low-density microarrays are also used for targeted research. Low-density targeted arrays are typically made from cDNA and may contain hundreds up to several thousand genes. These arrays are often targeted to a specific tissue or physiological process and generally are fully annotated with full-length cDNA sequences. These arrays are more common in non-model species where less information is available on their genomic sequences, and are also very powerful tools for hypothesis-driven research where questions surrounding specific physiological pathways exist (Aluru and Vijayan, 2009). For example, Wiseman et al. (2007) developed a targeted low-density rainbow trout specific microarray enriched with genes involved in endocrine, immune, metabolic and housekeeping functions ideal for the study of hormonal actions and their responses in target tissues, such as the actions of cortisol and the stress response. These arrays are extremely valuable in functional genomics studies providing information on pathway-specific mechanisms.

1.4.2 Transcriptomics and the Stress Response

It is well known that increased stress results in perturbations to metabolic, reproductive and immune functions in mammals, and studies suggest the same is true in fish (Mommsen et al., 1999; Aluru and Vijayan, 2009), although the mechanisms are far from clear. Microarrays provide a platform from which to study the mechanistic linkages between various stressors and the physiologically and functionally relevant changes that occur as a result. Although transcript-level changes do not always translate into responses at the whole-organism level, understanding the molecular implications of stressor exposure will help direct studies looking at physiologically relevant responses at higher levels of organization.

It is well established that glucose elevation occurs in response to stress, either short-term through catecholamine-induced glycogenolysis or longer-term through cortisol-induced gluconeogenesis (Mommsen et al., 1999). There are several key enzymes involved in these processes, and recent studies using microarrays have shown the transcripts of these genes to be elevated in the liver following acute stressor exposure (Wiseman et al., 2007; Momoda et al., 2007). These gene expression studies also revealed that peripheral proteolysis and energy substrate mobilization are key aspects of stress adaptation in trout. As most of the stress-related changes in trout were also seen with cortisol treatment, it appears that stressor-induced elevation in plasma cortisol levels may act as a key signal for stress adaptation (Aluru and Vijayan, 2007). These changes also include immune function as seen by the changes in the expression of immune-responsive genes, including those involved in antigen presentation and the inflammatory response (Wiseman et al., 2007; Momoda et al., 2007; Cairns et al., 2008; Aluru and Vijayan, 2007). While in the short-term these changes are

thought to be adaptive, the affected immune pathways are yet to be fully characterized in fish, and also the long-term implications are currently not known. Reproductive impairment is another consequence of stressor exposure observed in mammals, although the mechanisms by which this occurs is still largely unknown. In general, it is thought that stressor exposure may require a reallocation of energy away from somatic growth and reproduction in order to regain homeostasis, and maintain it in the case of chronic stress (Mommsen et al., 1999). In fish, microarray technology has been used with increasing frequency as a tool for understanding the molecular basis of gonadal development and reproduction (von Schalburg et al., 2005; 2006; Bobe et al., 2006), but have yet to be fully exploited to study the linkages between stressor exposure and reproductive performance. Past studies have shown delayed gonadal development, decreased plasma hormone levels and decreased vitellogenin levels in response to cortisol (Pankhurst and Van Der Kraak, 2000; Consten et al., 2001), as well as a down-regulation of hepatic estrogen receptor and vitellogenin mRNA (Lethimonier et al., 2000). Recent microarray studies have identified other estrogen responsive genes such as vitelline envelope protein subunits to be affected by acute stressor exposure, implying that cortisol may have a direct affect on estrogen-dependant gene expression, but this is not yet fully understood (Wiseman et al., 2007; Aluru and Vijayan, 2007).

Overall, the potential for using microarray technology to study the linkages between stressor exposure and the effects on various molecular aspects of performance is promising. Understanding the consequences of stress on reproduction, immune and metabolism related pathways in fish has important implications with respect to evaluating the population-level risk associated with exposure to stressors in the environment. Fish are susceptible to

countless and diverse stressors in the wild, all of which may have the potential to impact growth, survival and population sustainability. As such, there is a need for a greater understanding of the mechanisms by which these stressors exert effects to fully evaluate the long-term consequences and impact. Transcriptomics, by providing a snap-shot of genome wide changes, may provide a basis to identify and catalogue gene patterns that could be used as signatures for toxicant exposure and/or impacts.

1.5 Application of Transcriptomics in Ecotoxicology

The goal of ecotoxicology, and thus environmental risk assessment, is to measure the effects of pollutants in biological organisms, with the intention of predicting whether the observed effects will have an impact on populations, communities or the ecosystem as a whole (Hinton et al., 2005). The majority of research addresses this goal through well-defined and controlled laboratory studies, which have been successful at determining adverse effects of numerous chemicals that are dose- and time-dependant. These studies often focus on single or several sub-cellular biomarkers in an attempt to determine effects of specific chemicals. Although this is useful and valuable information, it is still unclear whether a concrete link can be made between laboratory exposures looking at single compounds and potential effects in the wild where organisms are receiving multiple stressors (Forbes et al., 2006). This research is also limited in that it requires previous knowledge of potential effects to ensure the chosen endpoints are representative of significant effects in the environment, and will sufficiently highlight the relevant impacts. This is particularly difficult when trying to assess the effects of emerging contaminants, such as pharmaceuticals, where very little is known about their effects in non-target organisms (Santos et al., 2010a), or when trying to

evaluate effects of complex mixtures when the spatial and temporal exposure is variable and interaction of contaminants is not clearly understood.

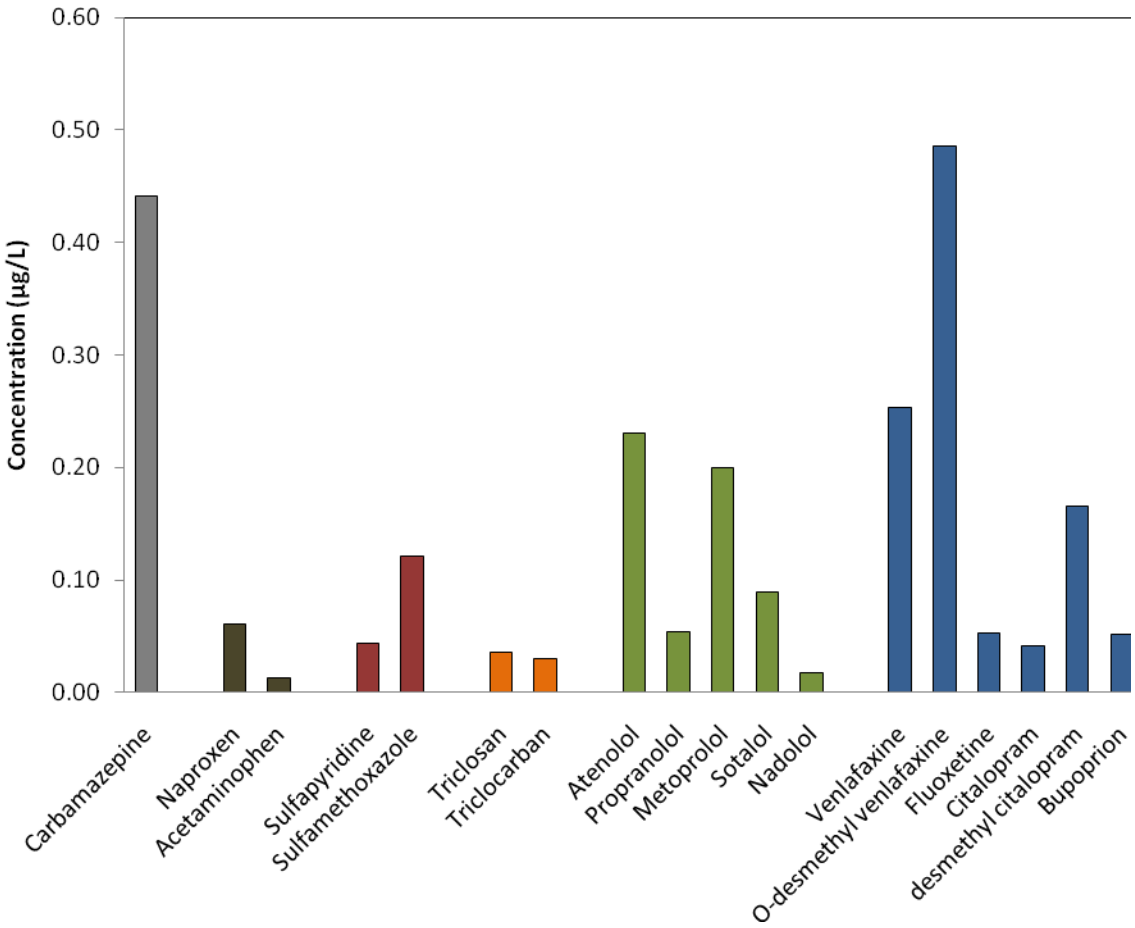
1.5.1 Municipal Wastewater Effluent

MWWEs represent one of the largest point sources of contaminant input into the aquatic environment (Chambers et al. 1997). Concern for the effects of exposure to MWWEs in aquatic organisms is high as treatment plants discharge anthropogenic chemicals from numerous sources directly into the environment. Recently, MWWEs have developed into an important emerging issue for endocrine disruption research. Historically, reproductive endocrine disruption has been the focus of numerous studies on MWWEs, as there is substantial evidence supporting estrogenic effects such as intersex, upregulation of estrogen-dependent proteins such as vitellogenin in male and juvenile fish and reproductive impairment in exposed fish (Jobling et al., 1998). However, it is becoming more apparent that other physiologically relevant pathways such as stress, metabolism, thyroid and immune function and seawater adaptation are also susceptible to disruption by anthropogenic contaminants such as those found in MWWEs (Hontela and Vijayan, 2009; Brar et al., 2010). Numerous classes of compounds have been detected in effluents and in surface waters including natural and synthetic estrogens (Ternes et al. 1999; Servos et al., 2005), non-estrogenic human and veterinary pharmaceuticals (Daughton and Ternes, 1999; Metcalfe et al. 2003; Lishman et al. 2006) and industrial surfactants such as alkylphenolic ethoxylates (Servos et al., 2003; Gross et al., 2004). Although these compounds have been detected at low levels, there remains reason to believe a threat may exist due to their pseudo-persistent nature and because, as therapeutic drugs, they are designed to be bio-active at relatively low

concentrations. The possibility also exists for additive effects if multiple compounds are acting through the same mechanism of action.

Many classes of human pharmaceuticals have been detected in MWWEs and in surface waters globally (Corcoran et al., 2010; Daughton and Ternes 1999; Fig. 2, unpublished data). Very little research has focused on the effects and mechanisms of action of pharmaceutical compounds in non-target aquatic organisms, and because each class of drug is pharmacologically distinct and it is unclear which compounds are bioactive and present in sufficient levels to elicit a response, it is difficult to predict what pathways will be affected when fish are exposed to MWWE. Despite the fact that compounds are detected in MWWEs at very low concentrations, if multiple compounds are affecting the same pathway there is a potential for additive effects to be observed. There has been some evidence that some compounds such as estrogens are sufficiently high in undiluted or partially treated effluent to cause a response, which implies that after dilution in the environment concentrations might still be approaching a response threshold. Two of the classes of compounds that have been found at particularly high levels in the environment are selective serotonin (and norepinephrine) reuptake inhibitors (SS(N)RIs) and beta-blockers (Metcalfe et al., 2010). Regardless of the drug, reuptake inhibiting drugs work to increase the levels of certain neurotransmitters at the nerve synapses by blocking the transporters on the pre-synaptic membrane responsible for their removal. The majority of work on antidepressants has focused on the SSRI fluoxetine, and has identified a number of impacts on anorexigenic endpoints and weight loss (Mennigan et al., 2009; 2010), glucose metabolism (Mennigan et

Fig. 2. Concentrations of selected pharmaceuticals (μg/L) measured in municipal wastewater effluent collected downstream of a sewage treatment plant in Guelph, Ontario, Canada. This was the study site for caging experiments presented in this thesis, and the source of MWWE for lab studies presented in this thesis. Measured at the Water Quality Centre, Trent University, Peterborough, Ontario (Metcalfe et al., unpublished).


0.60
0.50
0.40
0.30
0.20
0.10
0.00
Concentration (µg/L)
Carbamazepine
Naproxen
Acetaminophen
Sulfapyridine
Sulfamethoxazole
Triclosan
Triclocarban
Atenolol
Propranolol
Metoprolol
Sotalol
Nadolol
Venlafaxine
O-desmethyl venlafaxine
Fluoxetine
Citalopram
desmethyl citalopram
Bupoprion

al., 2010) and fecundity and ovarian steroid hormone levels and gene expression (Lister et al., 2009). Venlafaxine has been receiving recent attention due to its increased prescription rate and relatively high concentrations in the environment (Melcalfe et al., 2010). Beta-blockers have a well established pharmacological mode of action of competitive antagonism of the β-adrenergic receptors, of which epinephrine is the primary endogenous ligand (Owens et al., 2007). One of the most commonly prescribed beta-blocker is the drug atenolol, which blocks the β1-adrenergic receptor (Rang et al., 2003). Relatively few studies have looked at the impact of exposure to atenolol in non-target organisms such as fish, and minimal effects using traditional toxicological endpoints have been reported to date (Winter et al., 2008).

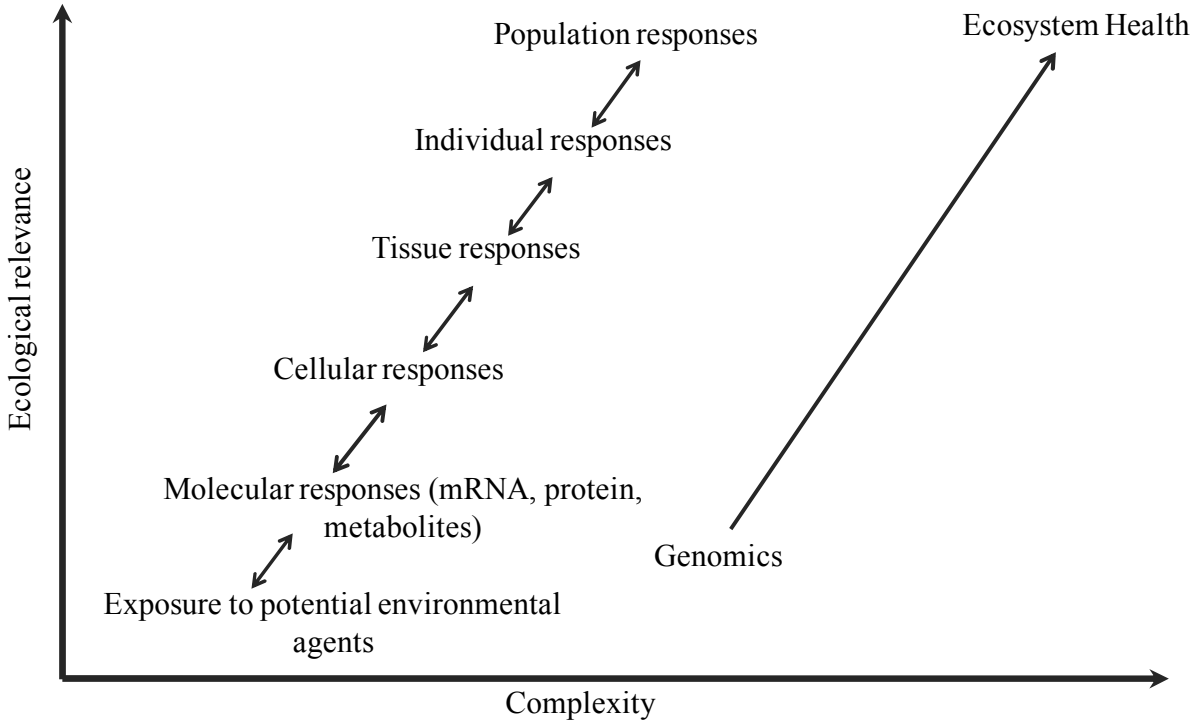
Several factors will modify the concentrations of pharmaceuticals in MWWEs including the physical and chemical properties, sources of influent, differences in the treatment processes at each plant, temperature, season and hydrology of the receiving environment (Clara et al., 2005; Vieno et al., 2005; Lishman et al., 2006; Loraine and Pettigrove, 2006). Despite the overwhelming evidence for the presence of these compounds in the environment, very little research has investigated the effects of these compounds in exposed aquatic organisms. It is difficult to predict what sort of effect could be expected in aquatic organisms as little is known about how human derived drugs will interact in non-target animals such as fish. In addition MWWE are complex mixtures of contaminants and it remains unclear how the numerous compounds present may interact with each other to cause effects.

1.5.2 Transcriptomics and Environmental Risk Assessment

Toxicogenomics is defined as the study of how an organism's genetic material responds to a toxicant stressor, and as such, ecotoxicogenomics is the adaptation of this tool to ecologically relevant species and questions, with the overall goal of predicting the deleterious effects of contaminants and other stressors on an ecosystem (Lettieri, 2006). Ecotoxicogenomics studies focus on the abundance of mRNA transcripts in tissue from organisms subjected to different experimental conditions, usually a control group versus one or more groups treated with a contaminant, mixture of contaminants or another stressor.

Ecotoxicogenomics has the ability to be integrated into current ecotoxicological methodologies as a powerful tool for risk assessment, yet it is important to understand that the technology cannot be used in isolation. A multi-tiered systems-biology approach must be used, which takes into account molecular (transcripts), protein and metabolite profiles at the cellular, tissue, individual and population levels, in order to determine environmental risk (Fig. 3). It is important for changes at lower levels of organization, such as what is discovered using transcriptomics, to be supported by phenotypic changes at the whole organism level which can be associated with impairments of population-relevant endpoints such as reproduction, growth, disease susceptibility and mortality. To this end, field studies that make these critical linkages are limited. A recent study, using a targeted low-density array, demonstrated a differential gene expression pattern in feral trout residing in high altitude lakes receiving different contaminant inputs (Moran et al., 2008). Interestingly, the differences in gene expression pattern between two lakes in that study reflected the differences in contaminant loading. The gene expression patterns seem to suggest

Fig. 3: The relationship between different levels of organization from increasing complexity to increasing ecological relevance in a multi-tiered approach to risk assessment. As research moves from detection of chemicals in the environment, through molecular responses to cellular responses to tissue responses to individual organism responses to population responses, increased complexity occurs, but also increased ecological relevance. The goal of ecotoxicogenomics is identify responses at lower levels of complexity that can help predict highly relevant effects at the ecosystem level.


Ecological relevance
Population responses
Individual responses
Tissue responses
Cellular responses
Molecular responses (mRNA, protein, metabolites)
Exposure to potential environmental agents
Ecosystem Health
Genomics
Complexity

that elevated mercury levels may disrupt metabolic and reproductive function in exposed fish (Moran et al., 2008). While field-based studies are unable to establish direct cause and effect, the gene expression patterns certainly provide a starting point to narrow down the problem areas for further detailed analyses. Therefore, transcriptomics has the potential to be a useful tool for environmental risk assessment. It has an advantage over biomarker-based ecotoxicological studies in that multiple, interconnected pathways can be examined at one time, which is often a necessity when trying to understand the implications of how gene expression changes relate to whole organism, population, community or ecosystem level effects (Van Aggelen et al., 2009; Schirmer et al., 2010). An increasing number of studies are utilizing this technology in ecotoxicology, often with the intention of providing information for risk assessment of contaminants and other stressors. Table 1 provides current examples of transcriptomic research using the different microarray tools available.

There are several applications of transcriptomics that will likely deem it valuable in the field of ecotoxicology and environmental risk assessment. First, through gene expression profiling researchers are able to identify genetic signatures for contaminants and other stressors and use it as a screening tool (Lettieri, 2006). Ideally it would be possible to take samples from wild fish receiving unknown stressors and, based on the expression profile determined by microarray analysis, identify causative agents present in the environment. Second, microarrays will help in identifying the toxic modes of action of contaminants. By allowing the visualization of genome-wide changes following an exposure, new target pathways can be identified, leading to the discovery of mechanisms by which contaminants exert their effects through directed, hypothesis-based research. This will allow a better

understanding of how contaminants affect an exposed organism, and the potential risks associated with exposure (Miracle and Ankley, 2005; Denslow et al., 2007). A third useful application of microarrays is the identification of molecular biomarkers. This will be important and necessary if wide-scale screening is to be carried out. It will not likely be feasible to use microarrays as a widespread environmental screening tool; their benefit will likely come from their use in biomarker discovery. Microarrays will support the identification of new effects-based and relevant biomarkers that could ideally be used to characterize risk on a site by site manner in the field (Benninghoff and Williams, 2008). A good molecular biomarker would allow the early detection of a contaminant or stressor, with an inferred mechanism of action that would relate back to a phenotypic change that could help predict effects on the growth, reproduction or survival of the fish (Forbes et al., 2006). To this end, risk assessment protocols for identifying impacts of new chemicals, or at new locations, could adapt a quick, cost-effective yet informative screening system based on toxicant-specific effects-based biomarkers.

1.6 Thesis Objectives

The overall objective was to examine the impact of municipal wastewater effluent (MWWE) and selected pharmaceuticals (atenolol and venlafaxine), present in MWWE, on stress response and metabolism in juvenile rainbow trout (*Oncorhynchus mykiss*). This was accomplished through a series of whole animal exposures to MWWE in the field (caging exposures) and controlled laboratory studies, as well as *in vitro* experiments looking at specific impacts of pharmaceutical on glucose production. Specific objectives were to assess if:

1. MWWE exposure affects liver transcriptomics and protein expression in rainbow trout (Chapter 2);
2. Exposure to MWWE impacts the hormonal and metabolite responses to a secondary acute stressor in rainbow trout Chapter 3);
3. MWWE exposure induces temporal changes in stress and tissue-specific metabolic responses in rainbow trout (Chapter 4);
4. Exposure to MWWE impacts tissue-specific metabolic changes in response to an acute secondary stressor in juvenile rainbow trout (Chapter 5);
5. Specific pharmaceuticals (venlafaxine and atenolol) present in MWWE will affect acute stressor-induced glucose production in rainbow trout liver (Chapter 6).

Chapter 2

Hepatic transcriptomics and protein expression in rainbow trout exposed to municipal wastewater effluent

2.1 Overview

Municipal wastewater effluents (MWWEs) represent one of the largest point sources of contamination, but few studies have addressed the impact on fish populations. We tested the hypothesis that MWWEs disrupt multiple stress-related pathways by examining expression of genes and proteins in rainbow trout (*Oncorhynchus mykiss*). A caging study was undertaken by placing juvenile trout for 14 d either at an upstream control or 100%, 50% and 10% MWWE sites. A custom made low-density rainbow trout cDNA microarray was utilized for transcriptomics, and select gene expression was confirmed with quantitative real-time PCR. MWWE exposure significantly elevated plasma cortisol, glucose and vitellogenin levels, and altered the expression of a number of hepatic genes. Notably, expression of stress-related genes, hormone receptors, glucose transporter 2 and genes related to immune function were altered. The gene and protein expression of glucocorticoid receptor, heat shock proteins 70 and 90 and cytochrome P4501A1 were also impacted by MWWE exposure. Our results demonstrate that tertiary-treated MWWEs elicit an organismal and cellular stress response in trout and may lead to an enhanced energy demand in the exposed fish. The disruption in multiple stress-related pathways suggests that tertiary-treated MWWE exposure may reduce fish performance to subsequent stressors.

2.2 Introduction

Municipal wastewater effluents (MWWEs) represent one of the largest point sources of contamination in water resources today. As current municipal wastewater treatment is not designed to completely remove chemical contaminants, many of these potentially hazardous chemicals are released into aquatic environments (*1*). Recent advances in analytical

capabilities has led to a large number of chemicals being detected in the effluents, including industrial chemicals, pesticides, hormones and pharmaceuticals (*1- 4*). Concern over the effects of exposure of MWWEs to aquatic organisms is high as they contain complex mixtures of compounds that have potential to negatively impact aquatic organisms including fish (*1*).

Several factors will modify the concentrations of compounds in MWWEs including the compound's physical and chemical properties, sources of influent, differences in the treatment processes at each plant, temperature, season and hydrology of the receiving environment (*4-6*). Despite the overwhelming evidence for the presence of a large number of chemicals in the aquatic environment, less research has investigated the effects of these complex mixtures in fish. The vast majority of research to date has focused on effects associated with xenoestrogen exposure, including vitellogenin production in male and juvenile fish (*7*) and the presence of oocytes in the testes of male fish (*7*). Very little information, including a lack of endpoints that could be used as bioindicators of effects, exists for other classes of emerging chemicals of concern.

A number of recent studies have focused on individual emerging chemicals and their effects on established endpoints in fish and other organisms (*8-11*). One drawback to this approach is that there are many compounds found in MWWEs, so it is difficult to interpret and extrapolate studies looking at chemicals in isolation. One technique that is becoming increasingly popular is the use of cDNA microarrays for identifying pathways and targets that are impacted by contaminants (*12*). This approach allows the identification of broad-scale gene expression changes, which may help to ascertain specific molecular responses as

endpoints of effects and can lead to the generation of hypotheses into the mechanisms of action of some of these bioactive chemicals in complex mixtures. Few studies have examined the effects associated with exposure to MWWEs, or chemicals detected in these effluents, on gene and protein expression in fish (*13-16*). While the gene expression changes are the first line of response to contaminants, the resultant protein expression changes may be functionally more relevant. The objective of this study was to use a 14 d *in situ* caging study to investigate whether exposure to MWWEs impacts gene and protein expression in rainbow trout (*Oncorhynchus mykiss*) liver. A well established low density trout cDNA microarray (*17-19*) was utilized for gene expression profiling, while select genes and protein expression were confirmed using quantitative real-time PCR and western blotting, respectively.

2.3 Materials and Methods

2.3.1 Animals

Trout (average body mass of 35 ± 8 g) were obtained from Silvercreek Aquaculture (Erin, ON, CAN) transported to the exposure site and transferred directly to cages. Fish were not fed for the duration of the experiment.

2.3.2 Experimental Design and Sampling

The caging experiment was carried out in the Speed River, upstream and downstream of the municipal wastewater treatment plant, in Guelph, Ontario, Canada in September to October, 2007. The Guelph sewage treatment plant is a tertiary treatment plant with denitrification and dechlorination (see Supporting Information - Table 1). Five sites were selected: two upstream controls, and three downstream sites that were chosen to represent

approximate 100, 50 and 10% effluent based on conductivity (see supporting information). The control sites were pooled for data analysis as no significant differences existed between them. Conductivity at the upstream sites was 1110 µS, while it was 4080, 2855 and 1459 µS at the 100%, 50% 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, 20 and 40 m for the 100%, 50% and 10% sites, respectively. Water chemistry, including temperature, dissolved oxygen and pH were measured at all sites on four days during the exposure. No significant differences were observed between the sites (see supporting information - Table 2). Ammonia was also measured in the effluent, but was below the detection limit. There were four cages, made from 60 L Rubbermaid™ containers with small holes for water flow, at each site, each containing four fish. 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 water.

The exposure was for 14 d after which fish were anesthetized with buffered MS-222 and killed by spinal severance, weighed and fork length measured. Blood was collected with heparinized needles by caudal puncture and then centrifuged at 3000 x g for 5 minutes to collect plasma for steroid hormone and vitellogenin (males only) analyses. The fish were sexed, and gonad and liver weights were recorded. All fish were determined to be immature. Livers were snap frozen in liquid nitrogen and stored at -70^{0}C for glycogen determination, RNA extraction and protein analysis later. This experiment was conducted in accordance with animal use protocols approved by the University of Waterloo Animal Care Committee.

2.3.3 Plasma Measurements and Liver Glycogen Determination

Plasma levels of 17β-estradiol (E2), testosterone (T), and cortisol were measured by radioimmunoassay (RIA) using previously established methods (*20*). E2 and T were extracted from plasma using ethyl ether, while cortisol was measured directly from plasma. All antibodies were obtained from MP Biomedicals (Solon, OH, USA), and radiolabeled steroids were obtained from GE Healthcare (Waukesha, WI, USA). Plasma levels of vitellogenin (VTG) in male fish were measured using a competitive enzyme-linked immunosorbent assay (ELISA) with purified rainbow trout vitellogenin and an in-house polyclonal rabbit anti-rainbow trout VTG antibody. Plasma glucose was measured using a commercial kit (Raichem, San Deigo, CA, USA), while glycogen content was determined by measuring glucose content before and after amyloglucosidase hydrolysis according to Vijayan et al. (*21*). The glycogen content is shown as micromoles glucosyl units per milligram protein.

2.3.4 Microarray Analysis

A custom-made low-density trout cDNA microarray containing approximately 150 rainbow trout genes enriched with endocrine, stress and metabolic and immune pathways was utilized for transcriptomics exactly as previously reported (*17-19*). These data have been deposited into the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo, platform GLP3713, series GSE23613).

Sample processing. Total RNA was extracted from liver tissue using the RNeasy extraction kit (Qiagen, Mississauga, ON, CAN) and treated with DNase. The RNA integrity was checked by gel electrophoresis on a 1.2% FA gel containing SYBRsafe gel stain (Roche

Diagnostics, Toronto, ON, Canada), and the concentration of RNA was determined spectrophotometrically at 260/280 nm using a NanoDrop™ spectrophotometer (Thermo Scientific, Nepean, ON, CAN). As fish were immature, samples were pooled from each cage to create a sample size of four independent samples per treatment. A reference pool was created by adding an equal amount of total RNA from each sample used in the analysis. The reference pool sample was co-hybridized with every treatment sample and used as an internal standard. The procedure for cDNA synthesis, labeling, hybridization, post-processing and image analysis was exactly as reported before (*18*). A detailed description is available in Supporting Information.

2.3.5 Quantitative real-time RT-PCR

Samples were quantified using a SYBR green fluorescent dye master mix in an iCycler real-time PCR detection system (Bio-Rad, Hercules, CA, USA) exactly as described before (*18*) and also detailed in the Supporting Information. The sequences, amplification sizes, annealing temperatures and accession numbers for each primer pair is found in Supporting Information – Table 3. Genes of interest were normalized to β-actin, as no differences were seen in its transcript abundance between treatments (Supporting information, Fig. 3A).

2.3.6 SDS-PAGE and Western Blotting

The procedures for SDS-PAGE and western blotting followed established protocols (*21*) and the details are provided in the Supporting Information. Briefly, 40 µg of total protein were separated using an 8% polyacrylamide gel and the proteins were transferred onto a nitrocellulose membrane using a semi-dry transfer unit (Bio-Rad). The primary

antibodies used were cytochrome P450 IA1 (CYPIA1; monoclonal mouse anti-fish, 1:3000, Cedarlane, Burlington, ON, CAN), heat shock protein 70, constitutive (HSC70; polyclonal rabbit anti-trout, 1:3000, (*22*)); heat shock protein 70, inducible (HSP70; polyclonal rabbit anti-trout, 1:5000, StressMarq, Victoria, BC, CAN); heat shock protein 90 (HSP90; polyclonal rabbit anti-fish, 1:5000, StressMarq, Victoria, BC, CAN); glucocorticoid receptor (GR; polyclonal rabbit anti-trout, 1:1000, (*23*)). All bands were quantified with Chemi-imager using AlphaEase software (Alpha Innotech, Santa Clara CA, USA). Equal loading of samples was confirmed by probing the blots with β-actin [Cy3-coupled monoclonal primary antibody from mouse, 1:1000 (Sigma, St. Louis, MO, USA)]. No differences were seen in β-actin protein abundance between treatments (Supporting information, Fig. 3B).

2.3.7 Statistical Analysis

Significant differences between treatments for individual genes in microarray analysis, qPCR analysis, western analysis, glycogen content and plasma measurements were carried out with one-way analysis of variance (ANOVA) using SPSS statistical software, followed by the Tukey's post-hoc test. Data were log transformed, if necessary, to meet the assumptions of parametric statistics including equal variance and normal distribution. If log transformation did not satisfy the requirements for ANOVA, data were analyzed non-parametrically, using a Kruskal-Wallis test, followed by a Mann-Whitney U-test to determine where the differences were. A significance level of $\alpha=0.05$ was used.

2.4 Results

2.4.1 Plasma measurements and glycogen determination

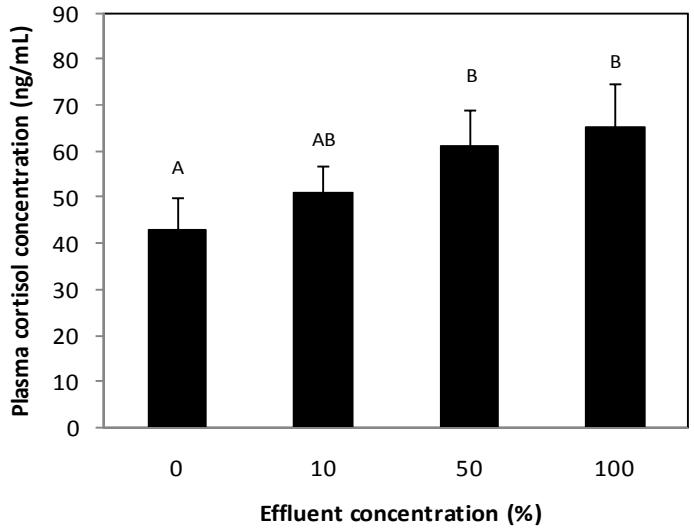
Cortisol levels increased with increasing concentrations of effluent, with significant changes seen in the 50% and 100% treatments (p=0.004; Fig. 1A). Plasma glucose increased significantly in all treatments compared to the upstream control (p=0.050; Fig. 1B). Plasma vitellogenin (VTG) levels in the male fish were significantly higher in the 10 and 50% effluent groups, but not in 100% effluent (p=0.011; Fig. 1C). There was no significant differences in plasma E2 levels in the MWWE groups compared to the upstream control (p=0.828; Supporting Information, Fig. 1A). Plasma T levels were significantly depressed at the 10% effluent concentration (p=0.016), but not at any other effluent concentrations compared to the control group (Supporting Information, Fig. 1B). There were no changes in liver glycogen content between any treatments (p=0.250; Supporting Information, Fig. 1C).

2.4.2 Microarray

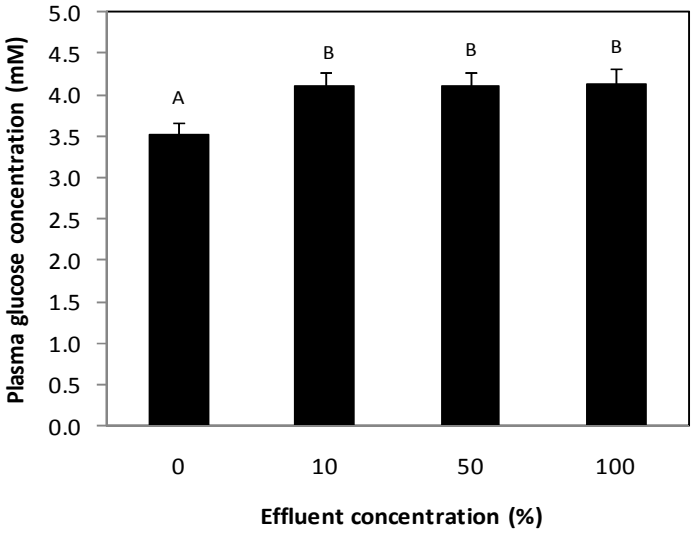
A total of 27 genes significantly changed in our targeted array with exposure to MWWE, representing a variety of functional groups (Table 1 and Supporting Information Fig. 2). A number of genes important for metabolism, energy homeostasis, growth and endocrine stress adaptation were significantly altered by MWWEs. Glucocorticoid receptor (GR), insulin receptor c, growth hormone, and thyroid hormone receptor β, heat shock protein 70 (constitutive and inducible) and heat shock protein 90 transcripts were significantly increased with exposure to MWWEs, while glucose transporter 2 transcripts were significantly decreased (Table 1). Generally, the transcripts of genes involved in

Fig. 1. Effects of municipal wastewater effluent (MWWE) exposure on plasma cortisol (n=9-16 fish; A), glucose (n=8 fish; B) and vitellogenin (VTG: only in male fish; 0%: n=9, 10%: n=4, 50%: n= 6, 100%: n=6 fish; C) concentrations in rainbow trout. Bars represent mean +/- SEM. Different letters indicate significant differences ($P<0.05$; ANOVA).

A.


Plasma cortisol concentration (ng/mL)
90
80
70
60
50
40
30
20
10
0
A
AB
B
B
0
10
50
100
Effluent concentration (%)

B.


Plasma glucose concentration (mM)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
A
B
B
B
0
10
50
100
Effluent concentration (%)

C.

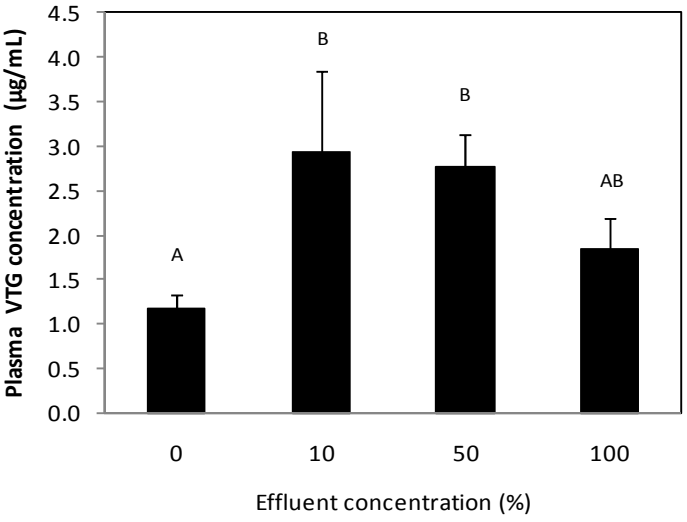

Plasma VTG concentration (µg/mL)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
A
B
B
AB
0
10
50
100
Effluent concentration (%)

Table 1. Hepatic transcripts significantly up or down regulated following exposure to municipal wastewater effluent (MWWE). Values represent the mean as a fold change difference from the upstream control +/- SEM (n=4 independent samples). Different letters indicate significant changes, while light and dark shades of grey reveal significant down- and up-regulation, respectively ($P<0.05$, ANOVA). Rows in bold indicate genes selected for further analysis with quantitative real-time RT-PCR and/or SDS-PAGE and western blotting.

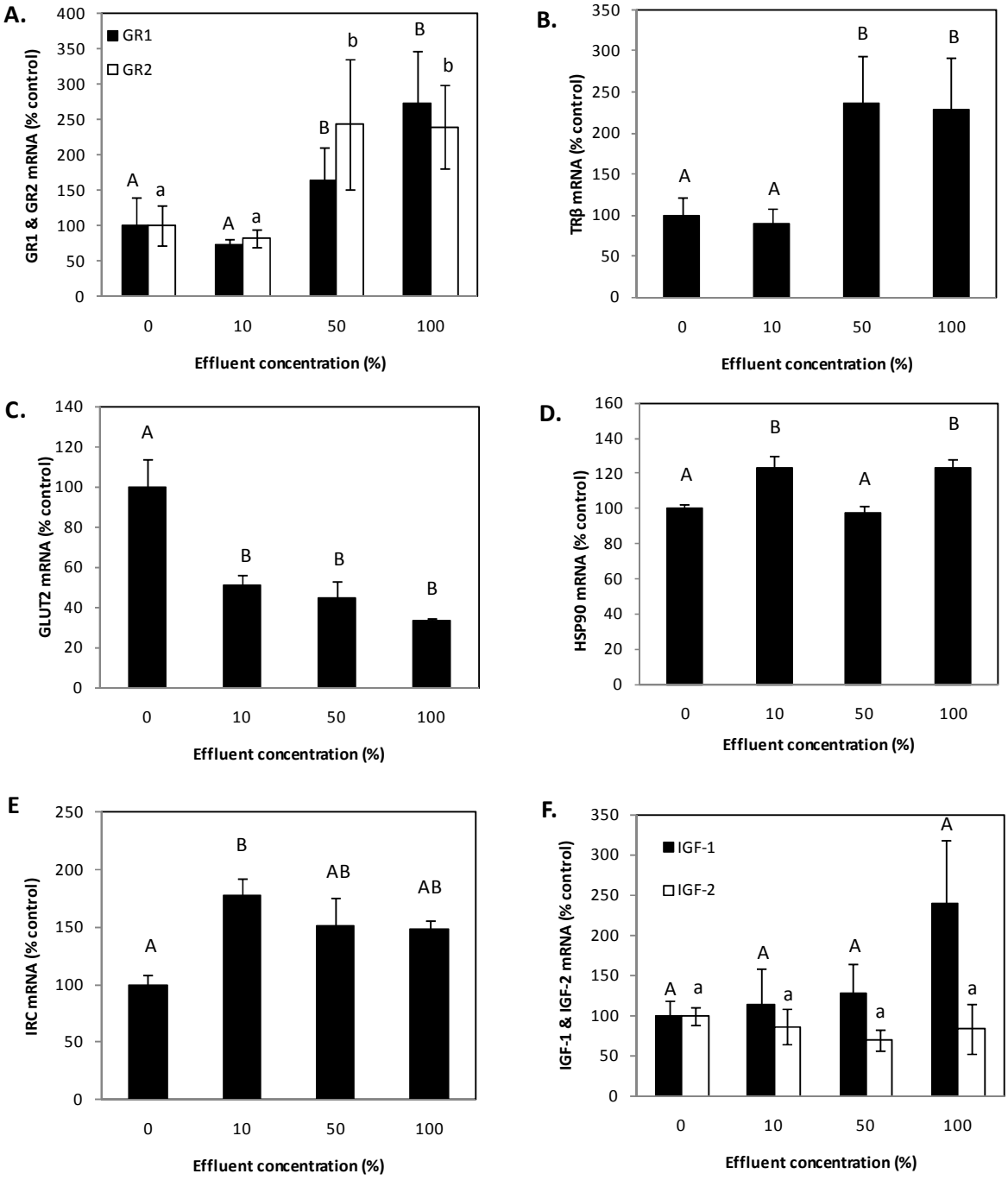
GENE	Functional Group	Accession #	p-value	UPSTREAM	10% EFFLUENT	50% EFFLUENT	100% EFFLUENT
apolipoprotein A-I-2	Binding/Metabolism	AF042219	0.05	1.00 ± 1.81^{A}	2.11 ± 1.29^{AC}	-3.11 ± 1.47^{B}	2.92 ± 1.34^{C}
aryl hydrocarbon receptor a+b	Receptor/Metabolism	AF065138	0.05	1.00 ± 2.65^{A}	-6.25 ± 3.95^{B}	-2.65 ± 1.22^{AB}	-6.68 ± 5.84^{B}
creatinekinase	Metabolism	X53859	0.034	1.00 ± 0.29^{A}	-3.18 ± 0.21^{B}	1.13 ± 1.33^{A}	-3.12 ± 2.07^{B}
Cytochrome p450 1A1	**Metabolism**	**AF059711**	**0.037**	**1.00 ± 0.35^{A}**	**8.64 ± 1.98^{B}**	**0.66 ± 2.55^{A}**	**-0.76 ± 1.89^{A}**
cytochrome p450 monooxygenase 2k1v2	Metabolism	L11528	0.03	1.00 ± 0.47^{A}	-0.30 ± 0.67^{AB}	-1.01 ± 0.51^{B}	1.61 ± 0.69^{AB}
glutathione peroxidase	Metabolism	AF281338	0.047	1.00 ± 0.06^{A}	-0.15 ± 0.18^{AB}	-0.47 ± 0.51^{AB}	-0.62 ± 0.36^{B}
NADH-dehydrogenase (subunit 6)	Metabolism	AF125047	0.031	1.00 ± 0.29^{AB}	2.63 ± 0.29^{A}	0.27 ± 1.11^{B}	1.16 ± 0.52^{AB}
ubiquitin	Protein catabolism	AB036430	0.001	1.00 ± 0.78^{A}	3.76 ± 0.44^{B}	0.38 ± 0.22^{A}	2.19 ± 0.15^{AB}
cholecystokinin	Endocrine	AJ011846	0.004	1.00 ± 0.02^{A}	-2.16 ± 0.04^{B}	-0.48 ± 0.21^{AB}	-0.49 ± 0.53^{A}
glucocorticoid receptor	**Receptor/Endocrine**	**Z54210**	**0.045**	**1.00 ± 3.29^{AB}**	**-1.35 ± 3.4^{A}**	**1.54 ± 5.22^{AB}**	**6.34 ± 2.57^{B}**
growth hormone 1	Endocrine	M24683	0.02	1.00 ± 0.05^{A}	2.45 ± 1.47^{AB}	4.43 ± 0.76^{B}	4.03 ± 0.69^{B}
insulin receptor c	**Receptor/Endocrine**	**AF062498**	**0.023**	**1.00 ± 1.00^{A}**	**13.97 ± 9.9^{B}**	**45.93 ± 25.62^{B}**	**18.87 ± 26.30^{AB}**
proopiomelanocortine-B	Endocrine	X69808	0.023	1.00 ± 0.53^{A}	0.70 ± 0.46^{AB}	-0.18 ± 0.47^{AB}	-1.93 ± 0.66^{B}
thyroid hormone receptor beta	**Receptor/Endocrine**	**AF302246**	**0.036**	**1.00 ± 0.84^{A}**	**10.15 ± 2.19^{B}**	**13.09 ± 5.17^{B}**	**15.33 ± 5.82^{B}**
betaglobin2	Immune	D82926	0.018	1.00 ± 53.75^{A}	12.87 ± 6.65^{B}	-72.10 ± 26.86^{A}	-272.71 ± 239.85^{A}
low molecular mass protein 2	Immune	AF115541	0.002	1.00 ± 0.01^{A}	-0.62 ± 0.13^{B}	-0.18 ± 0.01^{B}	-0.75 ± 0.35^{B}
major histocompatability complex 1	Immune	AF296359	0.04	1.00 ± 0.56^{A}	-2.59 ± 0.98^{B}	-3.01 ± 0.17^{B}	-1.65 ± 0.50^{AB}
Mx2	Immune	U47945	0.05	1.00 ± 1.06^{A}	-2.82 ± 0.91^{B}	-2.16 ± 0.81^{B}	-0.27 ± 0.23^{AB}
Sox24	Immune	AB010741	0.038	1.00 ± 0.08^{A}	-3.31 ± 0.265^{B}	-1.57 ± 0.12^{AB}	-1.51 ± 1.00^{AB}
glucose transporter 2	**Transport**	**AF247728**	**0.018**	**1.00 ± .055^{A}**	**1.01 ± 0.44^{A}**	**1.38 ± 0.23^{A}**	**-0.89 ± 0.36^{B}**
retinol binding protein	Transport	AF503212	0.027	1.00 ± 0.54^{A}	-1.21 ± 0.54^{B}	-0.20 ± 0.05^{AB}	-0.65 ± 0.26^{AB}
hsp70 induced	**Cell stress/Molecular chaperone**	**K02549**	**0.02**	**1.00 ± 1.05^{A}**	**1.92 ± 0.24^{A}**	**2.35 ± 0.55^{A}**	**3.21 ± 0.29^{B}**
hsp70 constitutive	**Cell stress/Molecular chaperone**	**S85730**	**0.04**	**1.00 ± 0.88^{A}**	**5.14 ± 0.62^{B}**	**2.29 ± 1.76^{A}**	**0.58 ± 1.13^{A}**
hsp 90	**Cell stress/Molecular chaperone**	**AB196457**	**0.044**	**1.00 ± 0.21^{A}**	**2.65 ± 0.21^{B}**	**1.54 ± 0.27^{A}**	**2.00 ± 0.64^{AB}**
vitelline envelope protein alpha	Developmental processes	AF231706	0.048	1.00 ± 1.65^{AB}	-1.55 ± 0.68^{A}	1.18 ± 1.53^{AB}	5.10 ± 0.82^{B}
vitelline envelope protein beta	Developmental processes	AF231707	0.025	1.00 ± 4.03^{A}	10.31 ± 4.67^{A}	-60.81 ± 29.03^{B}	-6.16 ± 3.55^{AB}
myc	Oncogene/Regulatory	S79770	0.043	1.00 ± 1.7^{A}	-12.21 ± 4.32^{B}	-2.37 ± 0.83^{AB}	-1.76 ± 2.685^{AB}

immune function were decreased, including low molecular mass protein 2, major histocompatibility complex 1, Mx2 and Sox24, while betaglobin 2 transcripts were higher. There were changes in genes encoding proteins involved in xenobiotic metabolism, including arylhydrocarbon receptor a + b (AhR), and CYP1A1. AhR transcripts decreased with exposure to MWWE, while CYP1A1 transcripts increased. There were changes in the expression of estrogen responsive genes, including vitelline envelope protein α and vitelline envelope protein β. The changes in the vitelline envelope proteins mRNA abundances were not consistent, with vitelline envelope protein α significantly higher and vitelline envelope protein β lower in the effluent groups compared to the upstream control (Table 1).

2.4.3 Quantitative real-time RT-PCR

Quantitative real-time PCR was carried out on select genes to confirm expression patterns seen with microarray analysis (Fig. 2). All genes tested showed similar pattern to those seen in the microarray. Transcript abundance for both isoforms of GR, GR1 and GR2, significantly increased with exposure to MWWEs in the 50% and 100% treatments ($p=0.039$ and $p=0.029$, respectively: Fig. 2A). TRβ transcript levels increased with increasing effluent concentration ($p= 0.019$; Fig. 2B) compared to the upstream control. GLUT2 transcripts were significantly lower at all concentrations of effluent ($p\leq0.001$) compared to the upstream control (Fig. 2C). HSP90 transcript levels were higher in the effluent groups, except at 50% effluent, compared to the upstream site ($p=0.003$; Fig. 2D). IRC was significantly higher in the 10% effluent concentration, and remained elevated but variable at higher concentrations ($p=0.041$; Fig. 2E) compared to the upstream control. IGF1 and IGF2 was not significantly

Fig. 2. Effect of municipal wastewater effluent (MWWE) exposure on liver glucocorticoid receptor 1 and 2 (GR1, GR2; A), thyroid hormone receptor β (TRβ; B), glucose transporter 2 (GLUT2; C), heat shock protein 90 (HSP90; D), insulin receptor c (IRC; E) and insulin-like growth factor 1 and 2 (IGF1, IGF2; F) gene expression (quantitative real-time PCR) in rainbow trout. Values were normalized to β-actin and represent percent of upstream control site. Bars represent mean +/- SEM (n=4 independent samples). Different letters indicate significant differences.


A.
GR1
GR2
GR1 & GR2 mRNA (% control)
Effluent concentration (%)
B.
TRβ mRNA (% control)
Effluent concentration (%)
C.
GLUT2 mRNA (% control)
Effluent concentration (%)
D.
HSP90 mRNA (% control)
Effluent concentration (%)
E
IRC mRNA (% control)
Effluent concentration (%)
F.
IGF-1
IGF-2
IGF-1 & IGF-2 mRNA (% control)
Effluent concentration (%)

different in the effluent groups (p=0.101 and 0.282, respectively; Fig. 2F) compared to the upstream control and confirms the results seen with the microarray analysis.

2.4.4 SDS-PAGE and Western Blotting

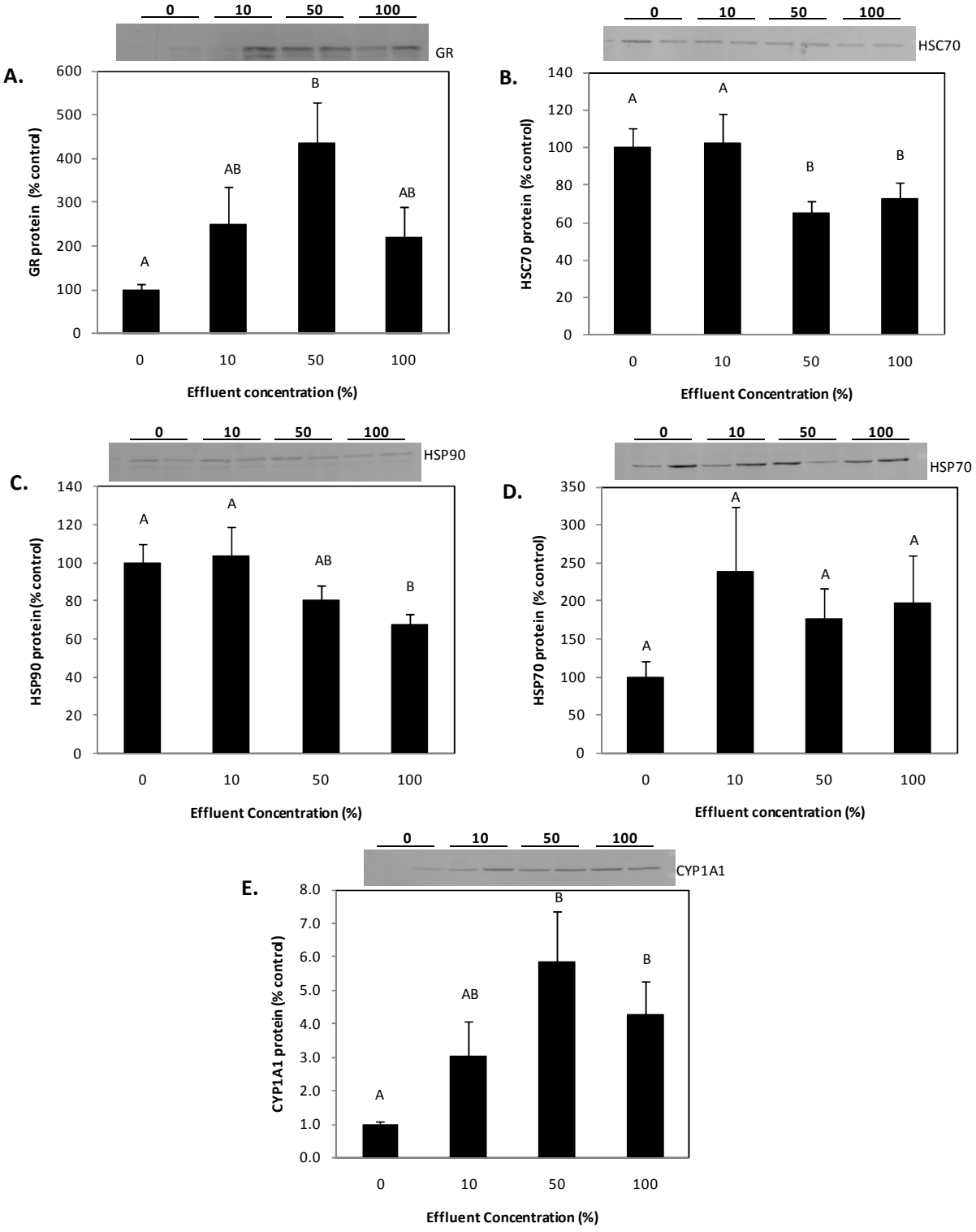
Select hepatic genes observed to change significantly using microarray were examined for protein expression using SDS-PAGE and western blotting (Fig. 3). GR protein expression was higher in the effluent groups but was statistically significant (p=0.033) only at the 50% effluent concentration compared to the upstream control (Fig. 3A). Of the heat shock proteins, HSC70 (p=0.021; Fig. 3B) and HSP90 (p=0.05; Fig. 3C) expression were significantly lower with increasing MWWE concentration, while HSP70 expression was not significantly different (p=0.421; Fig. 3D) compared to the reference group. CYPIA1 expression was significantly higher (p=0.012; Fig. 3E) in the 50 and 100% MWWE groups compared to the upstream control group.

2.5 Discussion

Our study for the first time demonstrates that exposure to a tertiary-treated MWWE, considered to be relatively clean compared to treatment plants utilizing only secondary treatment (*24*), elicits organismal and cellular stress responses in rainbow trout. This was clearly evident from the elevated plasma cortisol and glucose levels, as well as changes in several stress-related gene and protein expression in trout liver.

Elevated plasma cortisol response to stress is an evolutionarily conserved adaptive response that allows the animal to regain homeostasis (*25*). The cortisol levels usually return to pre-stress levels after the stressor is removed and/or if the animal adapts to the stressor and this is mediated via a negative feedback regulation of the hypothalamus-pituitary-interrenal

Fig 3. Effect of municipal wastewater effluent (MWWE) exposure on glucocorticoid receptor (GR; A), heat shock protein 70 constitutive (HSC70; B), heat shock protein 90 (HSP90; C), heat shock protein 70 inducible (HSP70; D) and cytochrome P450 IA1 (CYP1A1; E) protein expression in rainbow trout. A representative western blot is shown above each histogram. Bars represent mean +/- SEM (n=8 fish) and represent percent of upstream control site. Different letters indicate significant differences ($P<0.05$; ANOVA). See methods for western blot details.


A.
0
10
50
100
GR
GR protein (% control)
600
500
400
300
200
100
0
A
AB
B
AB
0
10
50
100
Effluent concentration (%)
B.
0
10
50
100
HSC70
HSC70 protein (% control)
140
120
100
80
60
40
20
0
A
A
B
B
0
10
50
100
Effluent Concentration (%)
C.
0
10
50
100
HSP90
HSP90 protein (% control)
140
120
100
80
60
40
20
0
A
A
AB
B
0
10
50
100
Effluent Concentration (%)
D.
0
10
50
100
HSP70
HSP70 protein (% control)
350
300
250
200
150
100
50
0
A
A
A
A
0
10
50
100
Effluent concentration (%)
E.
0
10
50
100
CYP1A1
CYP1A1 protein (% control)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
A
AB
B
B
0
10
50
100
Effluent Concentration (%)

(HPI) axis functioning. The significant concentration-related increase in plasma cortisol levels in MWWE exposed fish even after 14 d suggests disruption of the HPI axis functioning in rainbow trout. As MWWEs are a complex mixture of chemicals several factors, including the chemical composition may contribute to this altered cortisol response. To our knowledge little is known about the effects of MWWEs on plasma cortisol levels and HPI axis functioning. However, studies have shown that chemicals that are part of the complex mixture in MWWEs, including pharmaceuticals and xenoestrogens, affect plasma cortisol response to stress in fish (*26, 27*), and may be involved in the abnormal HPI axis functioning. Nevertheless, chronic elevation in plasma cortisol levels in the effluent groups suggests an enhanced metabolic demand in these animals, especially given that stress and/or cortisol stimulation increases the metabolic rate in fish (*28*). This cortisol response appears over and above that experienced by the control fish, as all these fish were confined in cages without food and also handled during sampling.

A key metabolic response to cortisol stimulation is the elevation in plasma glucose levels, which is a key fuel to meet the increased energy substrate demand associated with stress (*25*). Indeed the elevated plasma glucose levels in the MWWE exposed fish supports a higher energy demand in the present study. This did not correspond with changes in liver glycogen content suggesting gluconeogenesis as a likely factor for the hyperglycemia. This notion is supported by the elevated plasma cortisol levels as this steroid is a key stimulator of gluconeogenesis in fish (*25*). Also, the higher cortisol levels corresponded with higher GR mRNA abundances in fish exposed to MWWEs, supporting enhanced target tissue cortisol signaling as previous studies have shown that elevated cortisol levels upregulates GR gene

expression in trout liver (*23,29*). Also, the elevation in GR protein expression seen in the 50% effluent group supports an overall increase in GR signaling in response to MWWE exposure.

We cannot rule out the possibility that elevated glucose may also be due to changes in the turnover of the metabolite. For instance, a significant finding is the reduction in glucose transporter 2 (GLUT2; the primary glucose transporter in liver), gene expression in trout exposed to MWWEs, suggesting a reduction in glucose output capacity from the liver. GLUT2 is a bidirectional, low affinity, transporter that shuttles both D-glucose and D-fructose into and out of the liver, depending on the concentrations (*30*). It is thought to participate in glucose sensing and is regulated by levels of glucose itself, although other hormones may also be involved (*31*). While we cannot rule out the possibility that the suppression of GLUT2 expression in the MWWE groups may be due to the elevated glucose levels, other factors including a direct effect of chemicals on this transporter expression remains to be tested. The changes in growth hormone, thyroid hormone receptor and insulin receptor gene expression in the liver of effluent exposed fish along with changes in GR gene and protein expression suggests that disruption of some of the key metabolic hormones (*25*) may be involved in the abnormal glucose regulation in fish exposed to MWWEs. This is in agreement with a recent study on carp (*Cyprinus carpio*) using microarray analysis showing that industrial effluents impact energy metabolism and glucose dynamics (*32*). Taken together, our results suggest that chronic exposure to MWWEs may lead to disruptions in multiple endocrine signaling pathways and may compromise the energy substrate re-partitioning that is essential for coping with stress and also for maintaining fish performance.

This is further reflected in the altered expression of liver heat shock proteins (HSPs) in MWWE-exposed trout supporting a disturbance in the cellular stress response. As in mammals, a number of HSP families have been identified in fish and these molecular chaperones are quintessential for maintaining cellular function in unstressed as well as in stressed cells (*33*). The majority of studies in fish have focused on the expression of 70 (HSP70) and 90 (HSP90) kilodalton family of HSPs (*26, 33, 34*). Indeed, the inducible isoform of HSP70, but not the constitutive isoform (HSC70), are highly expressed in response to acute contaminant exposure in fish, and this is thought to play a key role in reestablishing protein homeostasis in response to the chemical insult (*33, 35*). The absence of a significant change in HSP70 protein expression (inducible isoform) in the present study in the effluent groups suggests a lack of an acute cellular stress response. However, the suppression of liver HSC70 and HSP90 protein expression in the 50 and 100% effluent concentrations points to a reduced cellular stress performance as these proteins are constitutively expressed even in unstressed cells and are critical for maintaining protein homeostasis and the cellular stress threshold (*28, 33*). Both these proteins are involved in various aspects of cellular protein transport and metabolism, including a key role for HSP90 in cell (steroid) signaling (*23, 33*) and HSC70 in the folding of nascent polypeptide chains and degradation of denatured proteins (*33, 35, 36*). Consequently, the lack of HSP70 protein expression (inducible isoform) in the MWWE exposed fish along with the reduction in the cognate HSC70 and HSP90 protein levels points to an impaired cellular stress threshold.

The higher expression of HSP70 and HSP90 mRNA abundances in the 100% effluent group, despite the lack of change in the protein expression (HSP70) or a reduction in protein

expression (HSP90) may reflect alterations also in mRNA stability, but this remains to be verified. Altogether, these findings highlight a disturbance in liver metabolism along with an altered cellular stress threshold in fish exposed to MWWE. The consequences due to this dysfunction may be apparent in response to secondary stressors and such studies are warranted to understand the impact of MWWEs on fish performance.

Other stress-related proteins including those involved in contaminant metabolism, including cytochrome P450s and arylhydrocarbon receptor, were also affected by exposure to MWWEs. This is in agreement with recent studies suggesting the presence of aryl hydrocarbon receptor agonists downstream from sewage treatment plants (*37*) and revealing higher tissue cytochrome P450 IA1 (CYP1A1) activity in MWWE exposed trout (*38*). Indeed there was a significant upregulation in cytochrome CYP1A1 protein expression in our MWWE exposed fish, indicating the presence of either polycyclic aromatic hydrocarbons (PAHs) and dioxins or other ligands that can activate AhR signaling in trout.

While our study did not reveal a clear MWWE effect on sex steroid levels, the lower plasma testosterone levels in fish exposed to 10% effluent concentration, but not in the higher effluent concentrations, supports previous studies showing decreases in sex steroid levels with exposure to MWWEs as well as estrogenic compounds (*8*). The testosterone disruption may be due to compounds in the effluents interfering with either the negative feedback regulation and/or regulation of endogenous steroid production (*39*). The elevated VTG levels seen in the present study suggest that there is estrogenic activity in this tertiary-treated MWWE with the highest VTG levels seen in the plasma of fish in the two lower effluent concentrations. Contaminants such as alkylphenol polyethoxylates can biodegrade

into chemicals that are more estrogenic than the original compounds introduced into the wastewater (*4*). Furthermore, the microarray analysis also showed changes in the mRNA abundances of two zona radiata proteins supporting the presence of (xeno)estrogens. These proteins are oocyte membrane proteins under the direct control of 17β-estradiol, while the lack of any change in plasma E2 levels in the MWWE groups, support the presence of (xeno)estrogenic activity in the effluent. It has been demonstrated previously that even well treated municipal effluent contain a variety of endocrine active compounds such as natural and synthetic estrogens (*40*). Interestingly, the mRNA abundances of the two zona radiata protein isoforms were different, one was significantly upregulated while the other was significantly downregulated, emphasizing the need for a multi-tiered approach, using gene and protein expression, rather than just gene array to characterize the effects associated with MWWEs exposure. This is also supported by recent studies looking at the effects of MWWE, as well as model estrogens and androgen, in fathead minnows (*41-42*). Although the MWWE-exposed fish showed similarities in expression patterns to both the estrogen and androgen exposed fish, there were also a number of differences, highlighting that the complex effluent may affect fish through numerous modes of action, and that the biological impacts of these mixtures cannot be easily modeled and predicted (*41-42*).

A novel observation was the notable decrease in mRNA abundance of genes involved in immune function in trout liver exposed to MWWEs (Table 1). Few studies have examined the effects of MWWEs on the immune system in fish, but a recent and highly relevant study by Garcia-Reyero et al. (*41*) showed immune function to be a significant target of MWWE exposure in fathead minnows. Filby et al. (*42*) identified immunotoxicity as a potential target

of MWWE exposure in fathead minnow, and our results are in agreement with recent studies in mussels showing that MWWEs and pharmaceuticals modulate immune function (*43, 44*). Also, upregulation of major histocompatibility complex II gene expression was observed in brown trout (*Salmo trutta*) following exposure to the non-steroidal anti-inflammatory drug declofenac (*45*). Although the immune-related gene numbers are relatively low in this study, the consistent suppression observed with these genes leads us to hypothesize that MWWE exposure suppresses immune function in fish. It remains to be seen if chronic elevation in plasma cortisol levels observed in the MWWE fish may be playing a role in the suppression of immune responsive genes (25).

In conclusion, using a multi-tiered response approach, including gene and protein expression, our results highlight disruptions of multiple endocrine pathways not previously associated with MWWE exposure in fish. While it is difficult to identify the causative agent(s) from the complex mixture, our results confirm previous studies indicating that MWWEs contain xenoestrogens and AhR ligands. Also, we show for the first time that a tertiary-treated MWWEs exposure affects multiple stress-related pathways in fish, including the heat shock protein responses, immune function, glucose homeostasis and energy substrate transport, growth and metabolism. Our study proposes a metabolic hypothesis - increased energy demand to cope with the stress of effluent exposure - that can compromise the energy substrate re-partitioning required for animal performance, including physiological adjustments to subsequent stressors. Future studies should be aimed at understanding the impact of MWWEs on fish performance, and we hypothesize that the effluent effect may differ with degree and type of wastewater treatment.

2.6 Acknowledgements

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Chapter 3

Exposure to municipal wastewater effluent impacts stress performance in rainbow trout

3.1 Overview

The objective of this 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), 11 β-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 the 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 1h 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.

3.2 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 (HSC) 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 the

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 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 Hale, 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 (MWWE) 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 (phosphoenolpyruvate 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.

3.3 Materials and Methods

3.3.1 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 heparinized needles by caudal puncture in quick succession. Fish were killed by spinal severance, weighed and fork length measured. Blood was centrifuged at 3000 x 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^{0}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.

3.3.2 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).

3.3.3 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 were separated using an 8% polyacrylamide gel along with a pre-stained 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 20V 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 4^0C. Membranes were washed in TTBS (3 x 10 min) and incubated in secondary antibody (goat-anti-rabbit IgG coupled to horseradish peroxidase; BioRad, Hercules, CA, USA) for 1 h. Membranes were again washed in TTBS (3 x 10 min) and then washed a final time in TBS (20 mM Tris, pH 7.5, 300 mM NaCl) for 10 min. Band detection was carried out using an ECL-Plus western blotting detection system (GE Healthcare Life Sciences, 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 samples were confirmed by probing the blots with β-actin (Cy3-coupled monoclonal primary

antibody produced in mouse, 1:1000; Sigma, St. Louis, MO, USA). All samples showed equal loading based on β-actin levels.

3.3.4 Quantitative Real-time RT-PCR

RNA extraction. Total RNA was extracted from head kidney tissue using the RNeasy extraction kit (Qiagen, Mississauga, ON, CAN) and treated with DNase. The concentration of total RNA was determined spectrophotometrically at 260/280 nm using a NanoDrop™ spectrophotometer (Thermo Scientific, Nepean, ON, CAN).

cDNA synthesis. First-strand cDNA was synthesized from 1 µg total RNA using the First-Strand cDNA Synthesis kit (MBI-Fermentas, Burlington, ON, CAN). Briefly, 1 µg of RNA was combined with 0.5 µg Oligo(dT) and heated for 5 min at 70^0C and then cooled on ice. A master mix containing 1 mM of each dNTP, 20 U of ribonuclease inhibitor and 40 U of M-MLV reverse transcriptase was added and the reaction was incubated for 1 h at 37^0C, and then stopped by incubation for 10 min at 70^0C.

Quantification. Samples were quantified using a SYBR green fluorescent dye master mix in an iCycler real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The genes of interest were melanocortin2 receptor (MC2R), steroidogenic acute regulatory protein (StAR), cytochrome P450 side chain cleavage (P450scc) and 11β-hydroxylase, while β-actin was the housekeeping gene. Primer information for these genes can be found in Table 1. Threshold cycle values for each sample were calculated using iCycler iQ real-time detection software (Bio-Rad). Briefly, each sample was assayed in triplicate. A master mix containing 3 µL cDNA, 3 µL of 10 mM primer pair, 40 µL of SYBR green mix and 34 µL of nuclease-free water was prepared for each sample, after which 25 µL was added to each of three wells.

Table 1: Forward (F) and reverse (R) sequences, amplicon size, accession number and annealing temperature for primers used in quantitative real-time RT-PCR. Primers include β-actin, melanocortin 2 receptor (MC2R), steroidogenic acute regulatory protein (StAR), cytochrome P450 side chain cleavage (P450scc) and 11β-hydroxylase (11beta).

Primer	Sequence	Amplicon size (bp)	Accession #	Annealing Temp (^{o}C)
β-actin F	5'-TGTCCCTGTATGCCTCTGGT-3'	130	AF157514	60
β-actin R	5'-AAGTCCAGACGGAGGATGG-3'			
MC2R F	5'-GAGAACCTGTTGGTGGTGGT-3'	105	EU119870	60
MC2R R	5'-GAGGGAGGAGATGGTGTTGA-3'			
StAR F	5'-TGGGGAAGGTGTTTAAGCTG-3'	101	AB047032	60
StAR R	5-AGGGTTCCAGTCTCCCATCT-3'			
P450scc	5'-GCTTCATCCAGTTGCAGTCA-3'	140	S57305.1	60
P450scc	5'-CAGGTCTGGGGAACACATC-3'			
11beta F	5'-CTTCCTCAAGAACGGGACAG-3'	130	AF179894.1	60
11beta R	5'-CTAGCAGACGGCAGAATCC-3'			

Each sample was assayed for the genes of interest and the housekeeping gene. The following PCR conditions were used for amplification: 95^{0}C for 3 min; 40 cycles of 95^{0}C for 20 s and annealing temperature (see Table 1) for 20 s; 95^{0}C for 1 min; 55^{0}C for 1 min followed by melt curve analysis starting at 55^{0}C and increasing in 0.5^{0}C increments to 95^{0}C every 10 s. Copy number of transcripts for each gene was determined with the threshold cycles (CT) using plasmid standard curves and were normalized to β-actin. The abundance of β-actin was unchanged between treatment groups and, therefore, used as a housekeeping gene in this experiment.

3.3.5 Liver Glycogen and Enzyme Activities

Liver samples were homogenized exactly as described above for SDS-PAGE and western blotting. Glycogen content was determined in the 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 liver per 100 g fish.

An aliquot of the liver homogenate 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) for enzyme activity determination. The enzyme activities were measured in 50 mM imidazole-buffered enzyme reagent (pH 7.4) at 22 ºC by continuous spectrophotometry at 340 nm using a microplate reader (VersaMax; Molecular Devices Corp., Palo Alto, CA) exactly as described before (Vijayan et al. 2006). Homogenate volumes were optimized for each enzyme prior to carrying out the assay and the blank and samples were run in duplicate. The following assay conditions were used:

- Hexokinase (HK: EC 2.7.1.1): 1 mM glucose, 5 mM MgCl2, 10 mM KCl, 0.25 mM NADH, 2.5 mM phosphoenolpyruvate (PEP), 5 U/mL lactate dehydrogenase (LDH) and 2.5 U/mL pyruvate kinase; reaction started with 1 mM ATP.
- Glucokinase (GK:EC 2.7.1.2): 15 mM glucose, 5 mM MgCl2, 10 mM KCl, 0.25 mM NADH, 2.5 mM phosphoenolpyruvate (PEP), 5 U/mL lactate dehydrogenase (LDH) and 2.5 U/mL pyruvate kinase; reaction started with 1 mM ATP.
- Pyruvate kinase (PK: EC 2.7.1.40): 3 mM KCl, 10 mM MgCl2, 0.12 mM NADH, 2.5 mM ADP, 20 UmL LDH; reaction started with 2.5 mM PEP.
- Lactate dehydrogenase (LDH: EC 1.1.1.27): 0.12 mM NADH and reaction initiated with 1 mM pyruvic acid.
- Phosphoenolpyruvate carboxykinase (PEPCK: EC 4.1.1.32): 20 mM NaHCO3, 1 mM MnCl2, 0.5 mM phosphoenolpyruvate, and 0.12 mM NADH; reaction started with 5 mM deoxyguanosine diphosphate.
- Alanine aminotransferase (AlaAT: EC 2.6.1.2): 0.12 mM NADH, 200 mM l-alanine, 0.025 mM pyridoxal 5-phosphate, and 12 U/mL LDH; reaction started with 10.5 mM α-ketoglutarate.
- Aspartate aminotransferase (AspAT: EC 2.6.1.1):7 mM a-ketoglutarate, 0.025 mM pyridoxal 5-phosphate, 0.12 mM NADH, and 8 U/mL malate dehydrogenase; reaction started with 40 mM aspartic acid.

The enzyme activity is represented as micromoles of substrate consumed or product liberated per minute (U) per gram protein.

3.3.6 Statistical Analysis

Comparisons over time and treatment (plasma measurements and mRNA levels) were carried out with two-way analysis of variance using SPSS statistical software, followed by the Tukey's post-hoc test. If the ANOVA showed significant interaction, treatments were compared within each time point using one-way ANOVA followed by the Dunnett's post-hoc test. All other comparisons (GR protein levels, liver glycogen and enzyme activities) were carried out with one-way ANOVA followed by the Tukey's post-hoc test. Data were log transformed, wherever necessary, to meet the assumptions of parametric statistics, including equal variance and normal distribution. A significance level of α=0.05 was used.

3.4 Results

3.4.1 Plasma Cortisol, Glucose and Lactate Levels

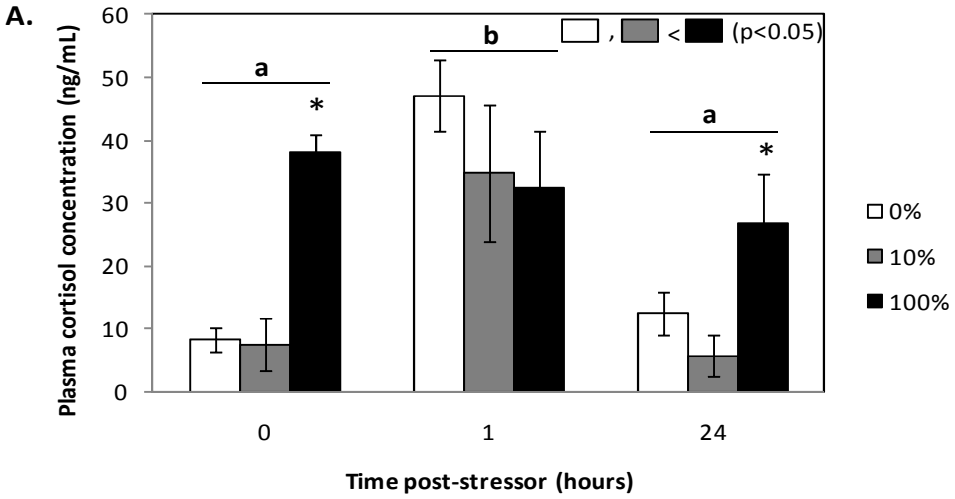
Cortisol levels were significantly increased in the 100% MWWE treatment group after 14 d exposure (Fig. 1A). Following the acute stressor, plasma cortisol levels increased significantly in the upstream control group and the 10% MWWE group, but did not change significantly in the 100% MWWE group. Overall, plasma cortisol level in the 100% group was significantly higher compared to the other two groups. Plasma glucose levels were not statistically different between the different effluent groups after 14 d exposure (Fig. 1B). Acute stressor significantly elevated plasma glucose at 1 h and the levels dropped to pre-stress levels at 24 h in the control and 10% groups but not in the 100% group. In the high effluent group, plasma glucose levels were significantly higher than the control and 10% group at 24 h post-stressor exposure. Overall, plasma glucose levels were significantly higher in the 100% MWWE group compared to the upstream control group but not the 10% group

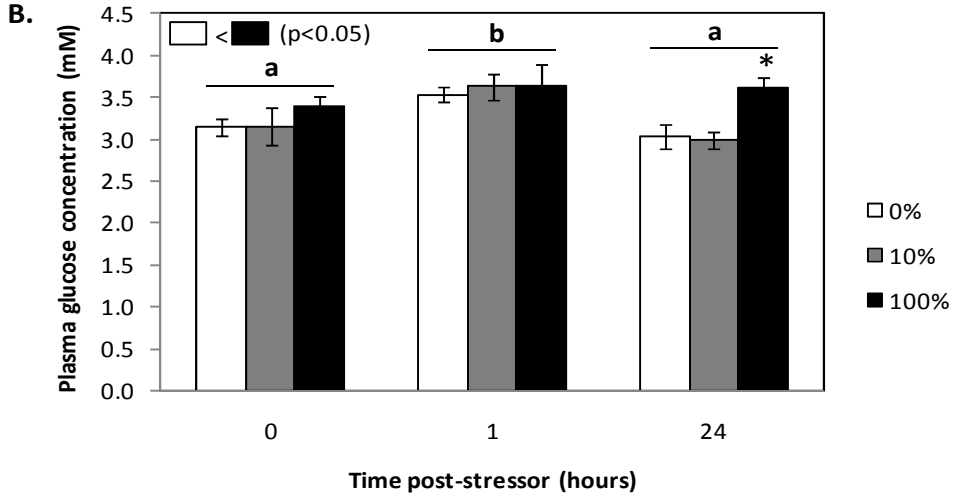
(Fig. 1B). Plasma lactate levels were significantly elevated in the 100% MWWE group compared to the upstream control group and the 10% group after 14 d exposure (Fig. 1C). Acute stressor significantly elevated plasma lactate at 1 h and the levels dropped to pre-stress levels at 24 h in the control and 10% groups but not in the 100% group. In the high effluent group, plasma lactate levels did not change after 1 h compared to the pre-stressor levels. Plasma lactate levels decreased significantly after 24 h compared to other time points, and the 100% effluent group was significantly lower compared to the upstream control group and the 10% effluent group. Overall, plasma lactate levels were significantly higher in the 100% group compared to the upstream control group and the 10% group (Fig. 1C).

3.4.2 Genes Involved in Corticosteroidogenesis

Following the 14 d exposure to MWWE, there were no significant differences among treatment groups in the transcript levels of MC2R (Fig. 2A). Acute stressor did not statistically change transcript levels 1 h post-stressor, but a significant decrease was observed at 24 h post-stressor in the 100% effluent group compared to the upstream control and the 10% effluent group (Fig. 2A). StAR transcript levels were significantly elevated in the 10% and 100% effluent groups compared to the upstream control following 14 d exposure (Fig. 2B). Acute stressor significantly elevated StAR transcripts in the 100% effluent group compared to the upstream control after 1 h, but transcripts were significantly depressed compared to both the upstream control and the 10% effluent treatment 24 h post-stressor (Fig. 2B). No change was seen in P450scc transcripts after the 14 d exposure (Fig. 2C). Acute stressor did not significantly change transcript abundance after 1 h, but transcripts were significantly decreased in the 100% effluent treatment compared to the upstream

Fig. 1: Effects of municipal wastewater effluent on plasma cortisol (A), glucose (B) and lactate (C) concentrations in rainbow trout prior to (0 h) and after an acute stressor (1 and 24 h). Bars represent mean +/- SEM (n = 6-12 fish). Different letters indicate significant differences between time-points ($P<0.05$; two-way ANOVA). Asterisks indicate significant differences from control within the time-point ($P<0.05$; one-way ANOVA). Inset indicates significant differences between treatments ($P<0.05$; two-way ANOVA).


A.
Plasma cortisol concentration (ng/mL)
60
50
40
30
20
10
0
, < (p<0.05)
a
b
a
*
*
0
1
24
Time post-stressor (hours)
0%
10%
100%


B.
Plasma glucose concentration (mM)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
< (p<0.05)
a
b
a
*
0
1
24
Time post-stressor (hours)
0%
10%
100%

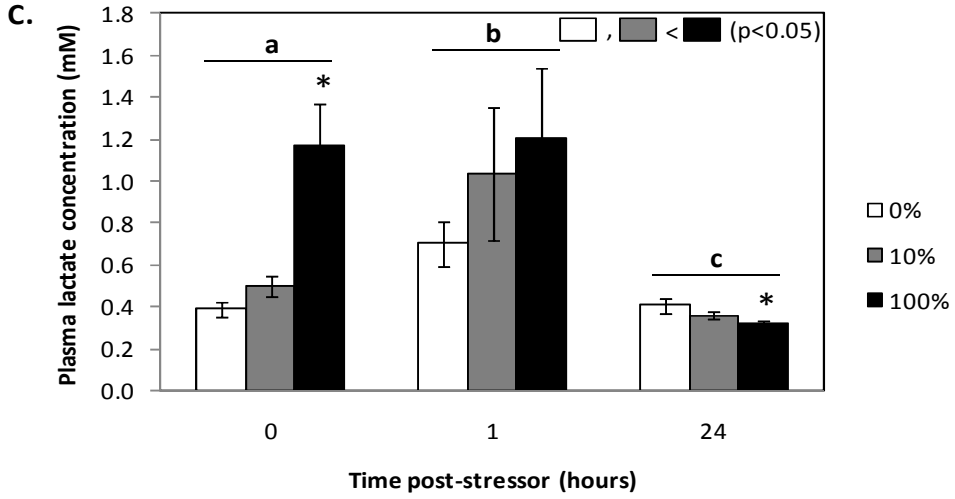
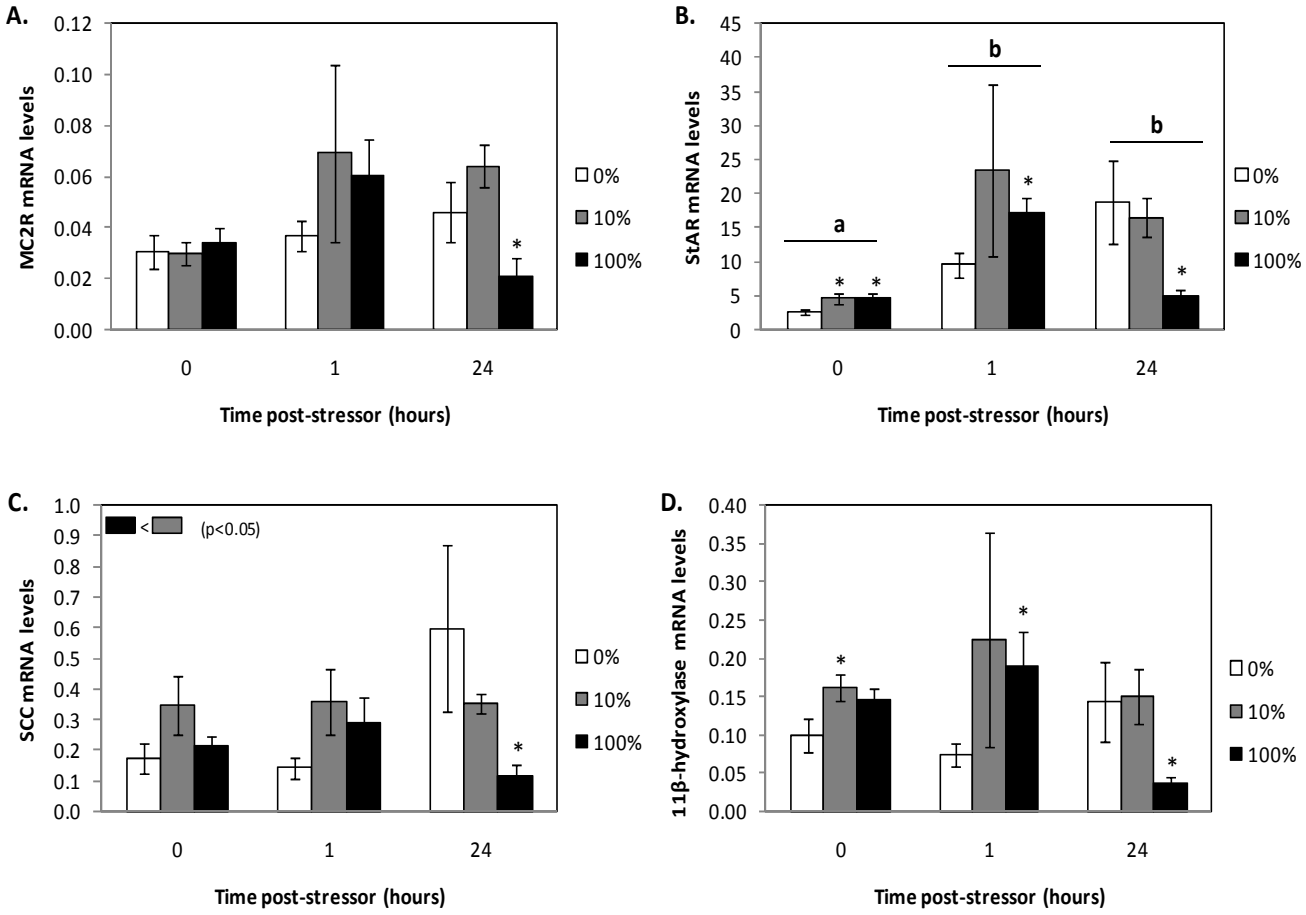

C.
Plasma lactate concentration (mM)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
, < (p<0.05)
a
b
c
*
*
0
1
24
Time post-stressor (hours)
0%
10%
100%

Fig. 2: Effects of municipal wastewater effluent on head kidney melanocortin 2 receptor (MC2R; A), steroidogenic acute regulatory protein (StAR; B), cytochrome P450 side chain cleavage (SCC; C), and 11β-hydroxylase (D) mRNA levels in rainbow trout prior to (0 h) and after an acute stressor (1 and 24 h). All mRNA levels were normalized to β-actin mRNA levels. Bars represent mean +/- SEM (n = 6 fish). Different letters indicate significant differences between time-points ($P<0.05$; two-way ANOVA). Asterisks indicate significant differences from control within the time-point ($P<0.05$; one-way ANOVA). Inset indicates significant differences between treatments ($P<0.05$; two-way ANOVA).


A.
MC2R mRNA levels
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
1
24
Time post-stressor (hours)
0%
10%
100%
*
B.
StAR mRNA levels
45
40
35
30
25
20
15
10
5
0
a
b
b
*
*
*
*
0
1
24
Time post-stressor (hours)
0%
10%
100%
C.
SCC mRNA levels
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
< (p<0.05)
*
0
1
24
Time post-stressor (hours)
0%
10%
100%
D.
11β-hydroxylase mRNA levels
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
*
*
0
1
24
Time post-stressor (hours)
0%
10%
100%

control after 24 h. Overall, the transcript response in the 10% group was significantly different from the response in the 100% group (Fig. 2C). Exposure to MWWE for 14 d significantly increased 11β-hydroxylase transcripts in the 10% effluent group compared to the upstream control (Fig. 2D). Following the acute stressor, transcripts were significantly elevated after 1 h, but significantly decreased after 24 h, in the 100% effluent group compared to the upstream control (Fig. 2D).

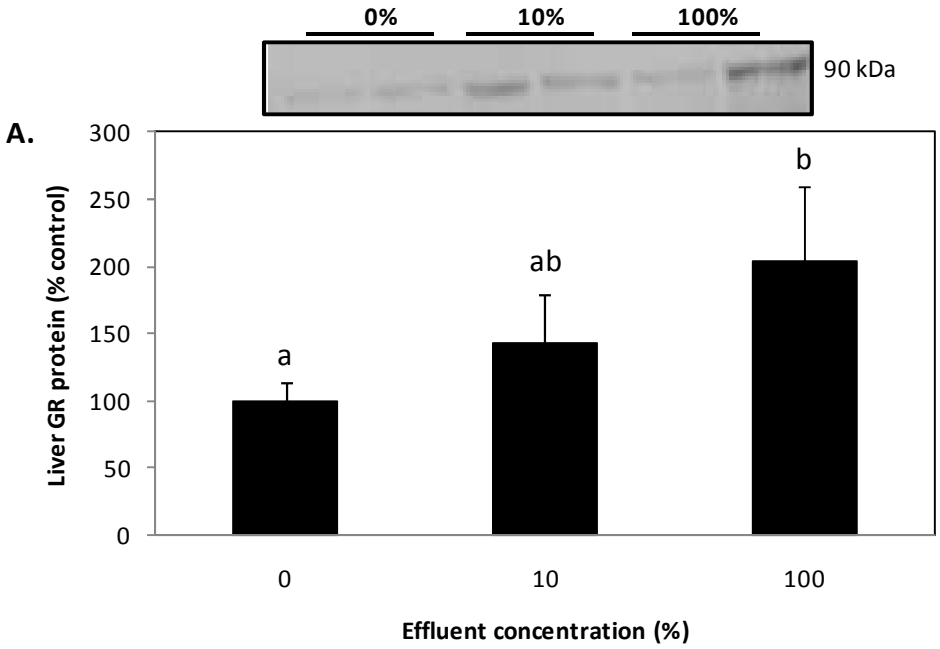
3.4.3 Glucocorticoid Receptor (GR) Protein Levels

A significant increase in liver GR protein levels was seen in fish exposed to 100% MWWE for 14 d compared to fish caged at the upstream control site, but not the 10% group (Fig. 3A). There was no significant difference in brain GR protein levels between the effluent exposed and control groups (Fig. 3B).

3.4.4 Liver Glycogen Content and Enzyme Activities

Liver glycogen content and activities of PK, LDH, PEPCK, AlaAT and AspAT were not significantly different between the effluent exposed and control sites (Table 2). There was a significant decrease in liver hexokinase (HK) and glucokinase (GK) activities in the 100% MWWE treatment group compared to the upstream control and the 10% MWWE treatment groups (Table 2).

Fig. 3: Effects of municipal wastewater effluent on liver glucocorticoid receptor (GR; A) and brain GR (B) protein levels in rainbow trout. Bars represent mean +/- SEM. (n = 6 fish). Different letters indicate significant differences ($P<0.05$; one-way ANOVA).


0%
10%
100%
90 kDa
A.
Liver GR protein (% control)
300
250
200
150
100
50
0
a
ab
b
0
10
100
Effluent concentration (%)

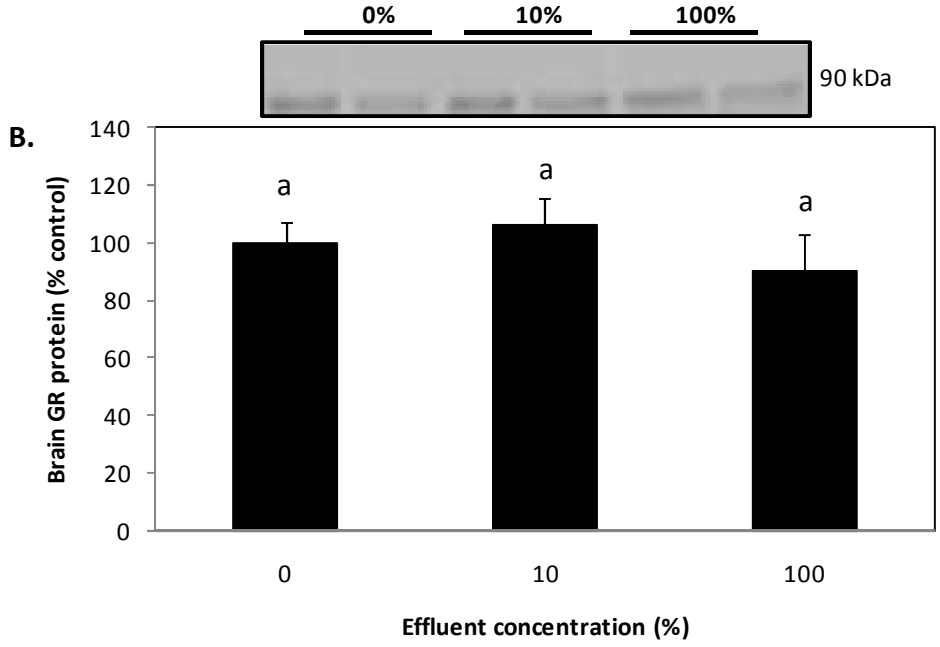

0%
10%
100%
90 kDa
B.
Brain GR protein (% control)
140
120
100
80
60
40
20
0
a
a
a
0
10
100
Effluent concentration (%)

Table 2: Effect of municipal wastewater effluent (0, 10 or 100%) on liver glycogen content (μmol glucosyl units/liver/100 g fish) and activities of hexokinase (HK), glucokinase (GK), pyruvate kinase (PK), phosphoenolpyruvate carboxykinase (PEPCK), lactate dehydrogenase (LDH), alanine aminotransferase (AlaAT) and aspartate aminotransferase (AspAT) (μmol/min/g protein) in rainbow trout. Values represent mean +/- SEM. (n=6 fish). Different letters indicate significant differences ($P<0.05$; one-way ANOVA).

	0%	10%	100%
Glycogen	25.2 ± 1.61^{a}	22.2 ± 2.40^{a}	19.5 ± 2.56^{a}
HK	10.8 ± 0.40^{a}	11.1 ± 0.92^{a}	8.6 ± 0.40^{b}
GK	10.8 ± 0.50^{a}	10.6 ± 0.96^{a}	8.8 ± 0.48^{b}
PK	8.4 ± 0.45^{a}	9.6 ± 1.16^{a}	8.0 ± 0.91^{a}
PEPCK	6.5 ± 0.26^{a}	6.6 ± 0.41^{a}	6.1 ± 0.38^{a}
LDH	691.2 ± 25.9^{a}	647.9 ± 59.8^{a}	674.6 ± 31.1^{a}
Ala AT	87.9 ± 6.42^{a}	95.8 ± 8.62^{a}	97.9 ± 12.63^{a}
Asp AT	179.5 ± 5.4^{a}	191.5 ± 15.7^{a}	187.3 ± 11.5^{a}

3.5 Discussion

The results of this study demonstrate for the first time that exposure to MWWEs for 14 d subjects fish to chronic stress and disrupts the cortisol and metabolite responses to a secondary stressor. The stressor-induced elevation in plasma cortisol levels is a highly conserved adaptive response that is important for the metabolic adjustments to cope with the increased energy demand associated with stress in fish (Iwama et al., 2006; Mommsen et al., 1999; Vijayan et al., 2010). While fish from the upstream control and 10% effluent sites showed a clear transient elevation in plasma cortisol levels to an acute stressor, the absence of a similar response in the 100% effluent site points to perturbations in the functioning of the hypothalamus-pituitary-interrenal axis in rainbow trout. The maintenance of higher plasma cortisol levels in the 100% MWWE group at 24 h, despite a reduction in the transcript levels of genes involved in corticosteroidogenesis post-stressor exposure, leads us to propose that the clearance of this stress steroid may also be disrupted. The impairment in the acute stress performance with high MWWE exposure suggests that these fish may not be able to cope with additional stressors that are routinely encountered in the wild, leading to a reduction in the overall fitness of the animal.

Recently it was shown that chronic MWWE exposure (same effluent source as this study) for 14 d upregulated several stress-related genes and protein levels in rainbow trout supporting an enhanced energy demand in this group (Ings et al., 2011). The present study confirms the higher plasma cortisol levels seen in response to chronic MWWE exposure in trout (Ings et al., 2011), while the higher StAR mRNA levels with MWWEs exposure supports an increased steroid biosynthetic capacity, as this transcript abundance correlates

with elevated cortisol production capacity in trout (Aluru and Vijayan, 2006; Gravel and Vijayan, 2007). While the chemical(s) that may be involved in the impairment of the HPI axis functioning is unclear given the complex mixture of chemicals present in MWWEs, our recent results do confirm the presence of xenoestrogens and AhR ligands in this effluent (Ings et al., 2011). It has been demonstrated previously that xenoestrogens upregulate StAR mRNA levels (Arukwe, 2008; Vang et al., 2007), while AhR ligands suppress this key rate limiting step in steroid biosynthesis (Aluru et al., 2005; Aluru and Vijayan, 2006). It remains to be seen if the elevated StAR mRNA levels in the head kidney tissues of trout was due to a higher concentration of xenoestrogens in the effluent relative to chemicals that suppress this gene expression, including AhR ligands and salicylates (Aluru and Vijayan, 2006; Gravel and Vijayan, 2006). Despite the higher StAR mRNA levels in both the effluent groups, the plasma cortisol levels at 14 d were elevated only in the 100% effluent group suggesting other factor(s), including food-deprivation during the experimental period, may be playing a role in impairing this steroid stress axis.

No changes in brain GR protein levels were observed in the effluent groups, which argues against disturbance in negative feedback regulation as a factor for the elevated cortisol levels in the 100% effluent group. For instance, abnormal cortisol response to stressor in PCB-exposed fish correlated with altered GR protein levels in the brain suggesting disturbance in the feedback regulation of plasma cortisol levels (Aluru and Vijayan, 2004). Interestingly, we are seeing elevated liver GR protein levels in the 100% effluent site implicating a tissue-specific effect of MWWEs on this receptor protein levels. Previous studies have shown that chronically elevated plasma cortisol levels suppress liver GR protein

levels in trout (Sathiyaa and Vijayan, 2003; Vijayan et al., 2003). However, the higher GR protein levels, despite elevated plasma cortisol levels, leads us to propose that 100% MWWE exposure may be disrupting liver responsiveness to cortisol stimulation in rainbow trout. Further support for this argument arises from the lack of an elevated glucose response or changes in liver PEPCK (a key gluconeogenic enzyme) activity in the 100% MWWE group, especially given that stressor exposure and/or cortisol treatment elevates plasma glucose levels in fish (Iwama et al., 2006; Mommsen et al., 1999; Vijayan et al., 2010). This involves cortisol-mediated enhancement of liver gluconeogenic capacity, including upregulation of PEPCK mRNA levels and enzyme activity in trout (Vijayan et al., 2003). All this leads us to conclude that chronic elevation of cortisol in the 100% MWWEs may be due to the disruption of the cortisol clearance mechanism in trout and making this steroid unavailable for tissue action. A similar lack of target tissue cortisol responsiveness despite elevated steroid levels was also seen in the liver of trout chronically exposed to 3,3',4,4'-tetrachlorobiphenyl supporting altered plasma cortisol clearance (Vijayan et al., 1997). Indeed, higher liver CYP1A protein levels in the 100% MWWE group was seen in previous studies with this effluent (Ings et al., 2011), confirming the presence of AhR ligands. We hypothesize that MWWEs disrupts plasma cortisol clearance mechanism and target tissue cortisol responsiveness in rainbow trout.

The abnormally high plasma cortisol levels seen in the 100% MWWE group was not altered in response to a secondary acute stressor exposure suggesting a lack of further HPI axis activation. One possibility is that the chronic exposure to chemical mixtures and the associated stimulation of the HPI axis, as seen by the elevated cortisol levels at 14 d,

exhausted the corticotrophs leading to interrenal inactivation in response to an acute stressor. An attenuated cortisol stress response to a secondary handling disturbance was also seen in feral fish collected from contaminated sites supporting the above contention, and this corresponded with atrophied pituitary corticotrophs and interrenal tissue (Hontela et al., 1992, 1997; Hontela and Vijayan, 2008). However, in the current study this may not be the case as elevated plasma cortisol levels were maintained in the 100% effluent group over the entire duration post-stressor exposure. Also, StAR and P450scc mRNA levels were elevated in all groups after the acute stress supporting ACTH signaling (Aluru and Vijayan, 2006). The interesting observation that key corticosteroidogenesis genes were suppressed in the 100% MWWE group at 24 h suggests a decrease steroid biosynthetic capacity post-secondary stressor exposure. However, in spite of this, the maintenance of higher plasma cortisol levels in the 100% effluent group at 24 h post-stressor exposure suggests disruption in plasma clearance of this stress steroid. This may lead to a decreased stress performance in these animals as cortisol may be unavailable for tissue action, including energy substrate repartitioning, to metabolically adjust animals to subsequent stressors, a phenomenon that is commonly encountered by feral fish. The elevation in plasma glucose levels in the 100% MWWE group at 24 h supports an increased energy demand as glucose is the primary fuel utilized to cope with the increased energy demand associated with stress in fish (Mommsen et al., 1999). It remains to be seen if the elevated plasma glucose levels in the 100% effluent group may also involve altered plasma clearance of this metabolite. The suppression of liver HK and GK (rate-limiting step in glucose uptake) activities in the 100% effluent group suggests a lower tissue utilization of glucose. A similar suppression of liver glucose

transporter 2 mRNA levels observed previously in 100% MWWE exposure (Ings et al., 2011) further confirms a decreased liver capacity for glucose uptake supporting a reduced plasma clearance of glucose. Whether the impaired glucose utilization capacity seen in this study with MWWE is liver-specific or a general phenomenon awaits further study. The reason for lower plasma lactate levels at 24 h after stress in the 100% MWWE group may suggest limited muscle activity after a secondary acute stressor relative to the control and 10% MWWE groups. Whether this would restrict the escape response from predators in fish from the 100% effluent group remains to be determined.

It is becoming clear that the HPI axis may be an important target for environmental contaminants, including MWWEs (Vijayan et al., 2005; Hontela and Vijayan, 2008; Ings et al., 2011). In the current study, undiluted MWWE exposure acted as a chronic stressor in exposed rainbow trout, and impaired cortisol and metabolic responses to a subsequent acute stressor. Exposure of trout to MWWE resulted in increases in plasma cortisol levels, coupled with a lack of tissue responsiveness to cortisol, and a lack of response to a secondary acute stressor. Fish experience higher energy demand to help cope with the chronic stress, but there is evidence that uptake of glucose by tissues is impaired by exposure to MWWEs. Although the effective concentration was high, the MWWE source was a relatively clean tertiary treatment plant (Gunnarsson et al., 2008), which may have implications for treatment plants utilizing less advanced treatment. Overall, exposed fish have a decreased metabolic capacity, which affects their stress performance and, therefore, their ability to respond to multiple stressors in the wild. Interestingly, most of the affects seen with 100% tertiary-treated

effluent was absent in the 10% effluent group, suggesting that dilution of the effluent may abrogate the impact on stress performance in trout.

3.6 Acknowledgements

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Chapter 4

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

Ings, J.S., Oakes, K.D. Vijayan, M.M., Servos, M.R. 2011. Temporal changes in stress and tissue-specific metabolic responses to municipal wastewater effluent exposure in rainbow trout. Prepared for scientific journal submission.

4.1 Overview

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 two days, 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, heart and gills, as well as target tissues metabolic capacity. Plasma cortisol (except for a significantly higher level in the 20% group at 8 d), glucose and lactate levels were unaffected by exposure to MWWEs. 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 gill and brain of trout exposed to MWWE. Activity of liver gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) and alanine aminotransferase (AlaAT) activity were significantly affected by MWWE exposure in trout liver. The glycolytic enzymes pyruvate kinase (PK) and hexokinase (HK) activities were significantly altered by MWWE exposure in the gill and heart, but not in the liver and brain. Overall, a 14 d exposure

to MWWE did not affect plasma organismal stress response, but cellular stress responsive pathways were altered. The tissue-specific temporal changes in the gluconeogenic and glycolytic activities suggest enhanced energy demand in fish exposed to MWWE, which may eventually lead to reduced fitness.

4.2 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 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 (Vijayan et al., 2005; 2010; Filby et al. 2007), 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, including those commonly found in MWWEs (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 chronic 14 d *in situ* 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 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 (Mommsen et al., 1999), including increased transcript abundance and enzyme activity of phosphoenolpyruvate carboxykinase, a rate-limiting step catalyzing the conversion of oxaloacetate to phosphoenolpyruvate (Wiseman et al., 2007; Sathiyaa and Vijayan, 2003; Vijayan et al., 2003). Thus, disruptions in the cortisol response by environmental contaminants, including MWWEs, may present immense 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; Tintos et al., 2007; Gravel and Vijayan,

2007). The objective of this study was to determine if MWWE exposure compromises the organismal and cellular stress response and leads to tissue-specific alterations in metabolic capacities in juvenile rainbow trout.

4.3 Materials and Methods

4.3.1 Animals

Juvenile rainbow trout (average body mass of 23 ± 6 g) were obtained from Silvercreek Aquaculture (Erin, ON, CAN) and transported to the University of Guelph Hagen Aqualab (Guelph, ON, CAN). 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 hours was used. Water was changed completely every two days. This was carried out by transferring fish to a second identical set of tanks with the same treatments and continued for the duration of the experiment. Fish were not fed for the duration of the experiment.

4.3.2 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. 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.

Fish were anesthetized with buffered MS-222 and killed by spinal severance, weighed and total length measured. The fish were all immature. Blood was withdrawn with needles

and syringes coated in EDTA by caudal puncture and centrifuged at 3000 x g for 5 min to collect plasma, which was stored at -80ºC for steroid hormone, glucose and lactate analyses. Liver, brain, gill and heart were snap frozen in liquid nitrogen and stored at -80ºC 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.

4.3.3 Plasma Measurements

Plasma cortisol levels were measured 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).

4.3.4 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) at 20V for 25 min with a

transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 10% (vol/vol) 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% (vol/vol) Tween 20) 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, CAN), heat shock protein 90 (hsp90; polyclonal rabbit anti-fish, 1:5000, StressMarq, Victoria, BC, CAN) or glucocorticoid receptor (polyclonal rabbit anti-trout GR, 1:1000, (Sathiyaa and Vijayan, 2003) for another hour. Membranes were then washed in TTBS (3 x 10 min) and incubated in secondary antibody (BioRad, Hercules, CA, USA) coupled to horseradish peroxidase for 1 hour. Membranes were again washed in TTBS (3 x 10 min) and then washed a final time in TBS (20 mM Tris, pH 7.5, 300 mM NaCl) 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 not detected in the gill.

4.3.5 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 Na_2HPO_4, 0.5 mM EDTA-Na, 0.2% BSA, 5 mM β-mercaptoethanol, pH 7.5). The enzyme activities were measured in 50mM imidazole-buffered enzyme reagent (pH 7.4) at 22ºC by continuous spectrophotometry at 340 nm using a microplate reader (VersaMax; Molecular Devices Corp., Palo Alto, CA) 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 MgCl2, 10 mM KCl, 0.25 mM NADH, 2.5 mM phosphoenolpyruvate (PEP), 5 U/mL lactate dehydrogenase (LDH) and 2.5 U/mL pyruvate kinase; reaction started with 1 mM ATP.
- Glucokinase (GK:EC 2.7.1.2): 15 mM glucose, 5 mM MgCl2, 10 mM KCl, 0.25 mM NADH, 2.5 mM phosphoenolpyruvate (PEP), 5 U/mL lactate dehydrogenase (LDH) and 2.5 U/mL pyruvate kinase; reaction started with 1 mM ATP.
- Pyruvate kinase (PK: EC 2.7.1.40): 3 mM KCl, 10 mM MgCl2, 0.12 mM NADH, 2.5 mM ADP, 20 UmL LDH; reaction started with 2.5 mM PEP.
- Lactate dehydrogenase (LDH: EC 1.1.1.27): 0.12 mM NADH and reaction initiated with 1 mM pyruvic acid.
- Phosphoenolpyruvate carboxykinase (PEPCK: EC 4.1.1.32): 20 mM NaHCO3, 1 mM MnCl2, 0.5 mM phosphoenolpyruvate, and 0.12 mM NADH; reaction started with 5 mM deoxyguanosine diphosphate.

- Alanine aminotransferase (AlaAT: EC 2.6.1.2): 0.12 mM NADH, 200 mM l-alanine, 0.025 mM pyridoxal 5-phosphate, and 12 U/mL LDH; reaction started with 10.5 mM α-ketoglutarate.
- Aspartate aminotransferase (AspAT: EC 2.6.1.1):7 mM a-ketoglutarate, 0.025 mM pyridoxal 5-phosphate, 0.12 mM NADH, and 8 U/mL malate dehydrogenase; reaction started with 40 mM aspartic acid.
- Glucose 6-phosphate Dehydrogenase (G6PDH: EC 1.1.1.49): 7 mM MgCl2, 0.4 mM NADP, reaction started with1 mM glucose-6-phosphate.
- Malic Enzyme (ME: EC 1.1.1.40) : 7 mM MgCl2, 0.4 mM NADP, reaction started with1 mM malate.
- Isocitrate Dehydrogenase (ICDH: EC1.1.1.42): 7 mM MgCl2, 0.4 mM NADP, reaction started with 0.6 mM isocitrate.

The enzyme activity is expressed as micromoles of substrate consumed or product liberated per minute (U) per gram protein. All enzymes activities were measured in the liver, while HK, PK and LDH were measured also in the brain, heart and gill.

4.3.6 Statistical Analysis

Comparisons over time and treatment (plasma measurements and protein expression) were carried out with two-way analysis of variance using SPSS statistical software, followed by the Tukey's post-hoc test. Within a time-point, treatments were compared using one-way ANOVA, followed by the Dunnett's post-hoc test. Data were log transformed, if necessary,

to meet the assumptions of parametric statistics including equal variance and normal distribution. A significance level of α=0.05 was used.

4.4 Results

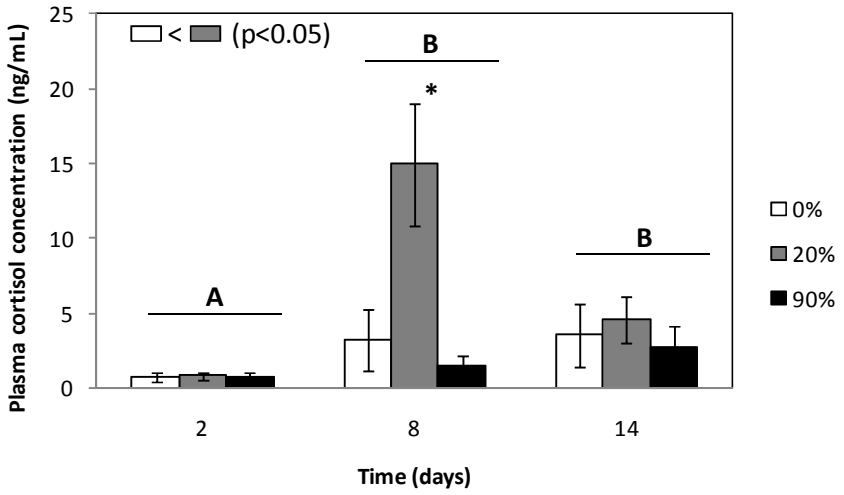
4.4.1 Plasma Cortisol, Glucose and Lactate Levels

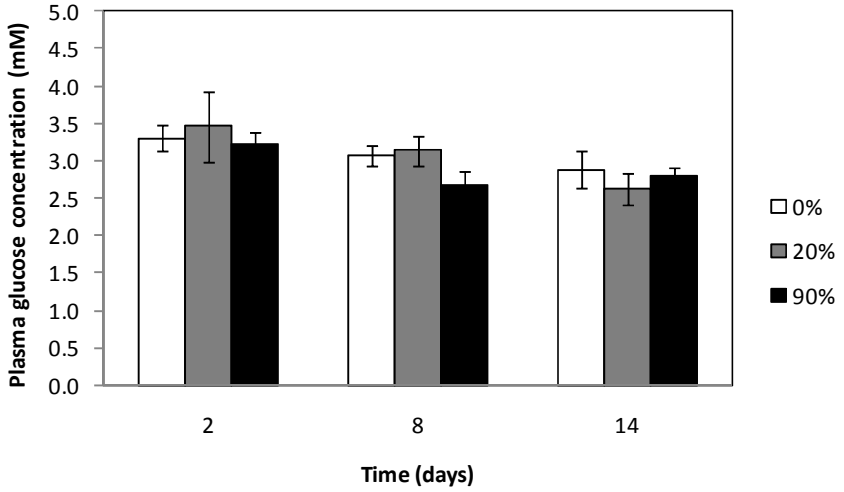
There were no significant differences in plasma cortisol levels between the treatment groups after 2 and 14 d exposure to MWWE. There was a significant elevation in plasma cortisol levels in the 20% effluent group after 8 d exposure compared to the control group (Fig. 1a). There were no significant differences in plasma glucose or lactate levels between the treatment groups at 2, 8 or 14 d exposure to MWWE (Figs. 1b and 1c). Plasma lactate, but not glucose, levels were significantly lower in all treatment groups at 14 d compared to other time points (Fig. 1c).

4.4.2 Glucocorticoid Receptor Protein Expression

A significant increase was observed in liver GR protein expression in the 90% effluent compared to the control after 2 d exposure to MWWE (Fig. 2a). After day 8, liver GR protein was significantly elevated in the 20% effluent group compared to the control, while on day 14, liver GR protein was significantly depressed in the 90% effluent group compared to the control (Fig. 2a). There was no significant difference in brain GR protein expression between treatment groups after 2, 8 or 14 d exposure to MWWE (Fig. 2b). There were no changes in heart GR protein expression after 2 and 8 d exposure to MWWE, while a

Fig. 1: Effects of municipal wastewater effluent on rainbow trout plasma cortisol (A), plasma glucose (B) and plasma lactate (C), after 2, 8, and 14 d chronic exposure (n=6 fish per treatment). Bars represent mean +/- SEM. Different letters indicate significant differences between time-points ($P<0.05$; two-way ANOVA). Asterisks indicate significant differences from control within the time-point ($P<0.05$; one-way ANOVA). Inset indicates significant differences between treatments ($P<0.05$; two-way ANOVA).


Plasma cortisol concentration (ng/mL)
25
20
15
10
5
0
< (p<0.05)
B
*
A
B
0%
20%
90%
2
8
14
Time (days)


Plasma glucose concentration (mM)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0%
20%
90%
2
8
14
Time (days)

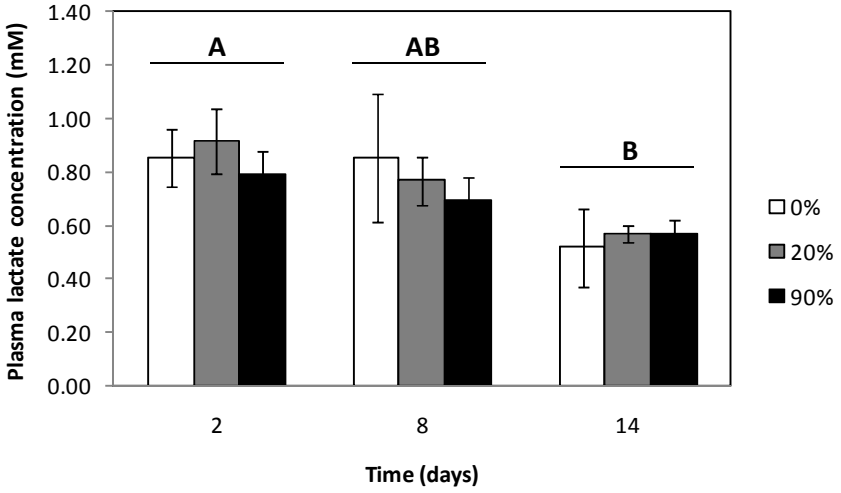
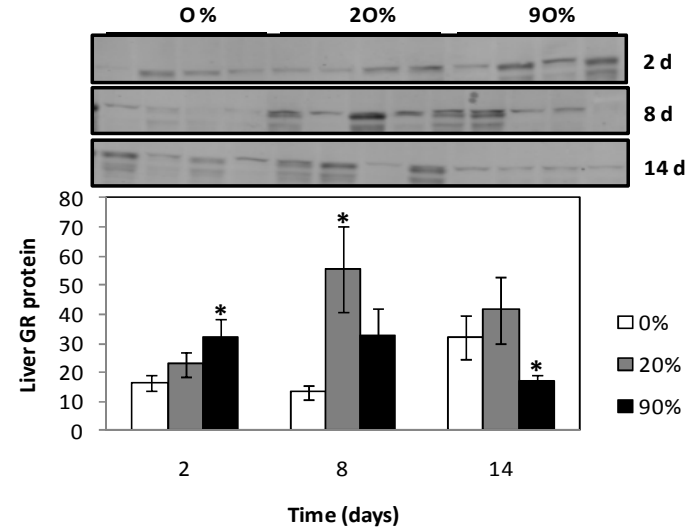
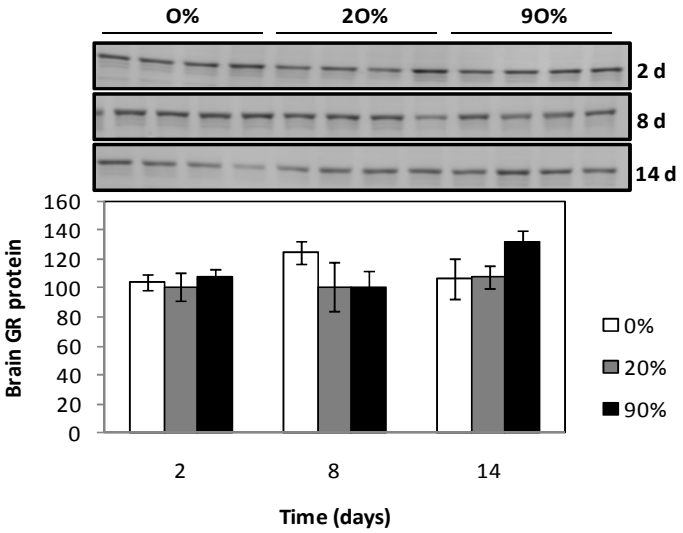
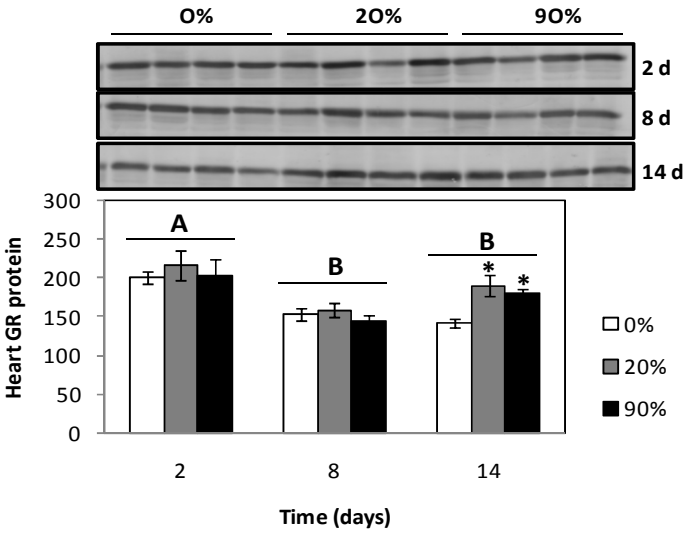

Plasma lactate concentration (mM)
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
A
AB
B
0%
20%
90%
2
8
14
Time (days)

Fig. 2: Effects of municipal wastewater effluent on rainbow trout glucocorticoid receptor (GR) protein expression over 2, 8, and 14 d chronic exposure in liver (A), brain (B) and heart (C) (n=6 fish per treatment). Bars represent mean +/- SEM. Different letters indicate significant differences between time-points ($P<0.05$; two-way ANOVA). Asterisks indicate significant differences from control within the time-point ($P<0.05$; one-way ANOVA).


O %
2O%
9O%
2 d
8 d
14 d
Liver GR protein
80
70
60
50
40
30
20
10
0
*
*
*
0%
20%
90%
2
8
14
Time (days)


O%
2O%
9O%
2 d
8 d
14 d
Brain GR protein
160
140
120
100
80
60
40
20
0
0%
20%
90%
2
8
14
Time (days)


O%
2O%
9O%
2 d
8 d
14 d
Heart GR protein
300
250
200
150
100
50
0
A
B
B
*
*
0%
20%
90%
2
8
14
Time (days)

the control

significant increase occurred after 14 d exposure in the 20% and 90% treatment groups compared to the control group (Fig. 2c). Overall, GR protein expression in the heart was significantly reduced at 8 and 14 d compared to 2 d exposure regardless of the treatment (Fig. 2c).

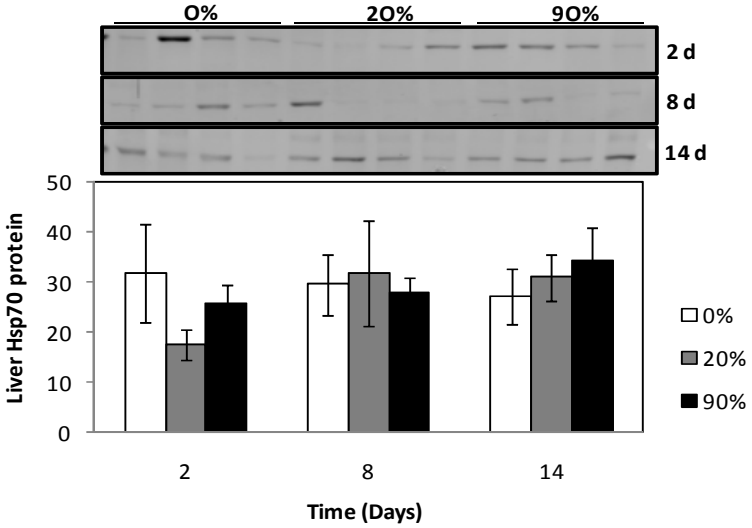
4.4.3 Liver Heat Shock Protein Expression

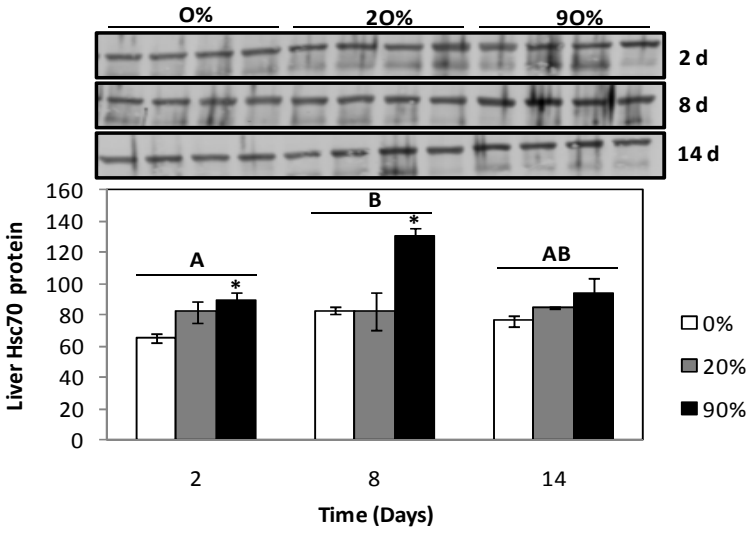
There were no significant differences in the inducible hsp70 protein expression between the treatments at 2, 8 or 14 d exposure to MWWE (Fig. 3a). On day 2 and day 8, there was a significant elevation in hsc70 protein levels in the 90% effluent group compared to the control group, but no significant differences were observed on day 14 (Fig. 3b). Overall, hsc70 levels were significantly different on day 8 compared to the 2 d sampling period but not the 14 d sampling period (Fig. 3b). Following 2 d exposure to MWWE, no differences were seen in hsp90 protein expression between treatment groups, but after 8 d exposure there was a significant elevation in hsp90 in the 90% effluent group compared to the control group (Fig. 3c). After the 14 d exposure, no differences were seen between the groups (Fig. 3c).

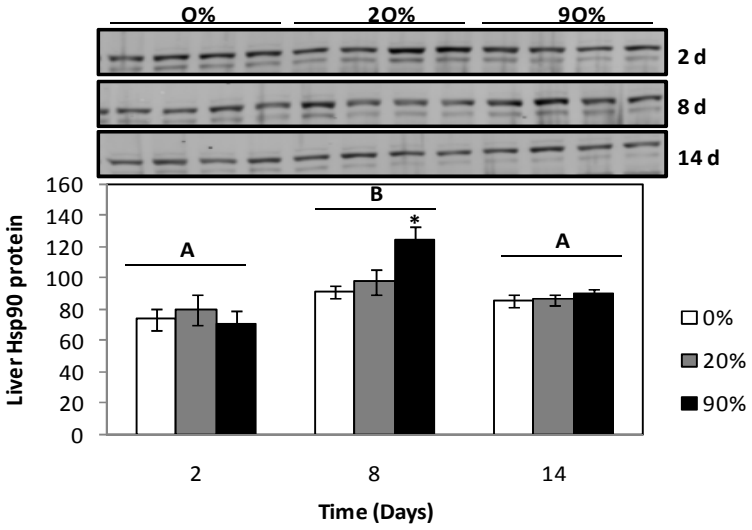
4.4.4 Liver Glycogen

There was a general decrease in liver glycogen content in all treatment groups at 8 and 14 d compared to the 2 d groups (Table 1). No significant treatment differences were seen in liver glycogen content on day 2 and 14, whereas on day 8 glycogen content was significantly lower in the 90% effluent compared to the control group (Table 1).

Fig. 3: Effects of municipal wastewater effluent on rainbow trout liver heat shock protein 70 (hsp70; A), heat shock cognate 70 (hsc70; B), and heat shock protein 90 (hsp90; C) protein expression over 2, 8, and 14 d chronic exposure (n=6 fish per treatment). Bars represent mean +/- SEM. Different letters indicate significant differences between time-points ($P<0.05$; two-way ANOVA). Asterisks indicate significant differences from control within the time-point ($P<0.05$; one-way ANOVA).







the 14 d exposure, no

4.4.5 Enzyme Activities

There were no differences in liver PEPCK activity between treatments after 2 d and 8 d exposure, but a significant increase was seen PEPCK activity in both the 20% effluent group and the 90% effluent group compared to the control after 14 d exposure (Table 1). Overall, PEPCK activity changed significantly over time, with PEPCK activity in both the 20% effluent and 90% effluent groups being significantly increased compared to the control group (Table 1). There were no significant differences between treatment in liver AlaAT activity after 2 d or 8 d exposure (Table 1). After 14 d exposure, there was a significant elevation in AlaAT activity in both the 20% and 90% effluent groups compared to the control (Table 1). There were no significant differences in liver AspAT activity between treatments after 2 d and 14 d exposure, but a transient increase in activity in the 20% effluent group compared to the control was seen after 8 d exposure (Table 1). Overall, there was a difference in AspAT activity patterns over time (Table 1). Liver HK was significantly decreased after 2 d exposure to MWWE in the 90% effluent group compared to the control, but there were no differences between treatments after 8 d and 14 d exposure (Table 1) with activity decreasing over time (Table 1). Liver GK activity followed the same pattern. Activity was significantly decreased in the 90% effluent group compared to the control after 2 d exposure, but no differences in activity were see between treatments after 8 d and 14 d exposure, and GK activity also decreased over time (Table 1). An overall decrease in liver PK activity was seen after 14 d exposure in all treatments, but no differences were observed between treatments at any time point (Table 1), while liver LDH activity did not change significantly between treatments at any time point, or over the duration of the experiment

Table 1: Effects of municipal wastewater effluent on liver glycogen (mmol/g protein) and enzyme activities (μmol/min/g protein), following 2, 8 and/or 14 days of exposure in juvenile rainbow trout. Values represent mean +/- SEM. Different letters indicate significant differences ((P<0.05; two-way ANOVA) between time-points (time effect column). Asterisks indicate significant differences from control within the time-point (P<0.05; one-way ANOVA). HK: Hexokinase; GK: Glucokinase; PK: Pyruvate kinase; LDH: Lactate dehydrogenase; PEPCK: Phosphoenolpyruvate carboxykinase; AlaAT: Alanine aminotransferase; AspAT: Aspartate aminotransferase; G6PDH: Glucose-6-phosphate dehydrogenase; ME: Malic enzyme; IDH: Isocitrate dehydrogenase.

Liver	Exposure Duration (Days)	0%	20%	90%	Time Effect
Glycogen	2	586.3 ± 143.4	459.7 ± 172.4	655.2 ± 237.1	A
	8	252.2 ± 49.4	222.3 ± 39.2	96.5 ± 4.2*	B
	14	195.5 ± 20.0	209.1 ± 31.5	160.0 ± 30.6	B
HK	2	4.05 ± 0.22	3.35 ± 0.30	3.12 ± 0.32*	A
	8	2.76 ± 0.19	3.23 ± 0.24	2.92 ± 0.17	B
	14	2.03 ± 0.19	2.39 ± 0.20	2.45 ± 0.13	C
GK	2	4.44 ± 0.38	3.54 ± 0.37	3.11 ± 0.34*	A
	8	3.08 ± 0.25	3.65 ± 0.46	3.35 ± 0.23	A
	14	2.21 ± 0.22	2.64 ± 0.26	3.00 ± 0.26	B
PK	2	23.4 ± 3.0	21.7 ± 4.0	19.4 ± 3. 0	A
	8	16.9 ± 2.5	19.0 ± 2.7	19.6 ± 2.7	A
	14	13.0 ± 0.66	12.5 ± 1.6	10.8 ± 0.53	B
LDH	2	251.4 ± 18.3	282.1 ± 20.0	271.4 ± 27.9	
	8	257.5 ± 26.3	300.2 ± 17.6	276.0 ± 28.3	No Change
	14	240.9 ± 17.3	260.8 ± 12.0	260.8 ± 31.9	
PEPCK	2	1.45 ± 0.13	1.77 ± 0.08	1.55 ± 0.20	A
	8	1.73 ± 0.18	1.71 ± 0.09	1.75 ± 0.13	AB
	14	1.57 ± 0.09	2.06 ± 0.08*	2.22 ± 0.17*	B
AlaAT	2	23.6 ± 3.45	31.1 ± 4.17	29.4 ± 4.78	
	8	31.6 ± 3.52	37.0 ± 2.94	31.0 ± 3.27	No Change
	14	23.4 ± 2.43	34.4 ± 3.77*	36.9 ± 4.61*	
AspAT	2	33.3 ± 2.45	42.0 ± 5.33	36.5 ± 2.81	A
	8	45.0 ± 1.99	53.0 ± 2.74*	46.2 ± 2.30	B
	14	50.0 ± 3.30	46.8 ± 3.17	47.3 ± 4.39	AB
G6PDH	14	15.8 ± 1.90	10.4 ± 0.97*	10.9 ± 1.10*	N/A
ME	14	2.00 ± 0.20	1.78 ± 0.18	2.74 ± 0.18*	N/A
IDH	14	12.4 ± 1.22	11.6 ± 1.01	12.2 ± 1.07	N/A

(Table 1). After 14 d exposure, there was a significant decrease in liver G6PDH activity in both the 20% and 90% effluent groups compared to the control (Table 1). Liver MDH activity increased significantly in the 90% effluent group compared to the control group and the 20% effluent group (Table 1). There was no difference in liver ICDH activity between treatments (Table 1).

Brain HK activity significantly decreased over the duration of the experiment, but no overall differences were observed between the treatment groups (Table 2). There was a transient decrease in HK activity after 8 d exposure in the 20% effluent group compared to the control, but there were no differences in activity between the treatments after 2 d or 14 d exposure to MWWE (Table 2). Brain PK activity was significantly decreased after 14 d exposure, with no differences seen between treatments at any time point (Table 2). Significant differences in overall LDH activity patterns were seen in the brain, but there were no differences in LDH activities between treatment groups (Table 2).

Heart HK activity remained largely unchanged throughout the duration of the experiment, with a small decrease in activity occurring in all treatments after 8 d exposure (Table 3). No differences were seen between treatments at any time point (Table 3). Both the 20% and 90% effluent groups had increased heart PK activity compared to the control group after 14 d exposure, but there were no differences in PK activity between groups after 2 d and 8 d exposure (Table 3). A transient increase in heart LDH activity was seen in the 90% effluent group compared to the control group was seen after 2 d exposure, but no changes were seen after 8 d or 14 d exposure (Table 3). Again, overall differences in activity levels existed between days (Table 3).

Table 2: Effects of municipal wastewater effluent on brain enzyme activities (µmol/min/g protein) following 2, 8 and/or 14 days of exposure in juvenile rainbow trout. Values represent mean +/- SEM. Different letters indicate significant differences ((P<0.05; two-way ANOVA) between time-points (time effect column). Asterisks indicate significant differences from control within the time-point (P<0.05; one-way ANOVA). HK: Hexokinase; PK: Pyruvate kinase; LDH: Lactate dehydrogenase.

Brain	Exposure Duration (Days)	0%	20%	90%	Time Effect
HK	2	31.5 ± 0.74	32.0 ± 1.9	33.2 ± 0.27	A
	8	30.1 ± 1.42	23.5 ± 2.89*	26.7 ± 1.39	B
	14	29.1 ± 0.78	29.8 ± 1.01	27.8 ± 0.96	B
PK	2	206.5 ± 15.8	222.1 ± 13.7	233.2 ± 20.2	A
	8	191.6 ± 12.0	200.7 ± 25.4	189.6 ± 10.5	AB
	14	180.4 ± 13.2	191.7 ± 7.3	175.4 ± 16.3	B
LDH	2	144.2 ± 10.3	157.1 ± 8.3	146.2 ± 3.9	A
	8	135.2 ± 6.1	124.6 ± 4.9	132.1 ± 9.7	AB
	14	130.1 ± 5.7	145.5 ± 6.0	116.7 ± 12.8	B

Table 3: Effects of municipal wastewater effluent on heart enzyme activities (µmol/min/g protein) following 2, 8 and/or 14 days of exposure in juvenile rainbow trout. Values represent mean +/- SEM. Different letters indicate significant differences ((P<0.05; two-way ANOVA) between time-points (time effect column). Asterisks indicate significant differences from control within the time-point (P<0.05; one-way ANOVA). HK: Hexokinase; PK: Pyruvate kinase; LDH: Lactate dehydrogenase.

Heart	Exposure Duration (Days)	0%	20%	90%
HK	2	21.7 ± 1.73	20.8 ± 1.33	21.7 ± 1.21
	8	18.6 ± 1.63	18.0 ± 0.73	17.2 ± 0.55
	14	18.9 ± 1.10	20.8 ± 1.65	20.2 ± 1.74
PK	2	218.6 ± 15.5	220.7 ± 19.3	230.5 ± 16.4
	8	229.0 ± 18.2	251.3 ± 17.1	186.1 ± 16.9
	14	141.1 ± 11.2	188.7 ± 9.6*	240.8 ± 15.8*
LDH	2	341.3 ± 29.4	385.8 ± 28.0	449.0 ± 24.5*
	8	347.6 ± 27.0	334.9 ± 28.6	343.4 ± 20.5
	14	426.1 ± 32.7	435.9 ± 33.3	418.5 ± 45.0

There were no differences in HK activity between treatments in the gill after 2 d exposure, but HK activity in both treatments was elevated compared to the control after 8 d exposure and decreased compared to the control after 14 d exposure (Table 4). Gill PK activity decreased in the 90% effluent group compared to the control after 14 d exposure, but no significant differences were seen between treatments at the other time points (Table 4). Overall, there was difference in the activity levels after 14 d compared to 8 d exposure (Table 4). Gill LDH activity showed overall differences in activity between days, but no significant differences between treatment groups at any time point (Table 4).

4.5 Discussion

Our study demonstrates that chronic exposure to MWWE disrupts the liver stress response and alters the tissue-specific metabolic capacity in rainbow trout. An important aspect of adapting to environmental stressors is the activation of a suite of highly conserved stress proteins belonging to the hsp family (Vijayan et al., 2005; Deane and Woo, 2010). The 70 kDa family of hsps have been widely studied as a marker of cellular stress and these proteins are present either as constitutive proteins (hsc70), essential for protein homeostasis in “unstressed cells”, and/or induced (hsp70) acutely in response to stressor-mediated proteotoxicity (Vijayan et al., 2005; Deane and Woo, 2010). The hsp90 expression is also widely studied as a marker of proteotoxicity, especially as a target for disruption of cellular signaling, including glucocorticoid receptor signaling in fish (Vijayan et al., 2005). The hsp70 is thought to have a protective role in defending cells against contaminant-mediated proteotoxicity and is used as a biomarker of contaminant-derived cellular stress in the environment (Vijayan et al., 2005; Iwama et al., 2006; Deane and Woo, 2010).

Table 4: Effects of municipal wastewater effluent on gill enzyme activities (µmol/min/g protein) following 2, 8 and/or 14 days of exposure in juvenile rainbow trout. Values represent mean +/- SEM. Different letters indicate significant differences ((P<0.05; two-way ANOVA) between time-points (time effect column). Asterisks indicate significant differences from control within the time-point (P<0.05; one-way ANOVA). HK: Hexokinase; PK: Pyruvate kinase; LDH: Lactate dehydrogenase.

Gill	Exposure Duration (Days)	0%	20%	90%	Time Effect
HK	2	14.7 ± 0.95	14.3 ± 0.76	13.9 ± 0.48	No change
	8	12.5 ± 0.15	14.2 ± 0.78*	14.4 ± 0.36*	
	14	15.7 ± 0.81	13.2 ± 0.54*	13.4 ± 0.76*	
PK	2	58.3 ± 4.77	63.6 ± 4.7 0	68.7 ± 2.85	AB
	8	65.3 ± 1.32	65.0 ± 3.71	69.6 ± 3.48	A
	14	61.2 ± 2.25	60.2 ± 2.28	52.5 ± 3.02*	B
LDH	2	65.3 ±	72.5 ± 2.90	72.8 ± 3.54	AB
	8	73.3 ± 4.25	81.3 ± 5.58	75.6 ± 3.39	A
	14	65.1 ± 3.48	70.3 ± 3.59	62.3 ± 3.09	B

Previous studies have suggested that MWWE exposure *in situ* elicits an organismal stress response in rainbow trout (Ings et al., 2011a), but very little is known about the impact of chronic exposure to MWWE on the cellular stress response. Here we show that MWWE exposure may alter the cellular stress responsein the liver by modulating the expression of hsps in trout.

In the present study chronic exposure to MWWE did not impact the expression of hsp70 in trout and this response is consistent with other studies showing a lack of inducible hsp70 expression with MWWE exposure in fish (Porter and Janz, 2003; Korkea-aho et al., 2008). However, there was a clear temporal effect of 90% MWWE exposure on the constitutive proteins, including liver hsc70 (2 and 8 d) and hsp90 protein expression (8 d) suggesting increased energy demand for protein synthesis in response to effluent exposure. An increase in the expression of these constitutive proteins also suggests disturbance to liver protein homeostasis, leading to altered cellular stress threshold to subsequent stressors (Vijayan et al., 2010), but this remains to be tested. Our results underscore an overall metabolic reorganization, suggesting enhanced energy substrate demand in fish liver exposed to MWWE. The longer-term maintenance of this tissue-specific metabolic response to MWWE may compromise other energy demanding pathways, including growth and reproduction.

A key metabolic role for liver is the production of glucose to meet the tissue energetic requirements in response to increased energy demands during stress. This glucose production is met by either epinephrine-mediated acute regulation of glycogenolysis and/or cortisol-mediated sustained production by gluconeogenesis (Vijayan et al., 1994; Vijayan et al., 2003;

Vijayan et al., 2010). There is increasing evidence that the cortisol stress axis, including target tissue cortisol action mediated by GR, are likely the target of environmental contaminants, including MWWEs (Vijayan et al., 2005; 2010; Ings et al., 2011a,b). In the current study, exposure to MWWEs had a minimal effect on plasma cortisol. After 8 d exposure, there was a significant increase in cortisol in the 20% MWWE exposed fish, but this increase was not seen after 14 d, and was not seen in the 90% MWWE exposed fish. Although no clear explanation for this increase is apparent, it may be due to changes in effluent composition as studies have shown that the MWWE composition is variable and dependent on a number of factors, including influent composition, season, temperature, and treatment process (Hemming et al., 2004; Clara et al., 2005; Vieno et al., 2005; Servos et al. 2003). This was shown in the effluent used in the present study, with differences in several pharmaceuticals and other contaminants measured throughout the course of the experiment (Wang, 2010). Interestingly, the higher plasma cortisol levels in the diluted MWWE at 8 d corresponded with higher liver GR protein expression suggesting a clear impact on the stress axis in that group. Previous studies have shown suppression in GR protein content with elevated plasma cortisol levels (Vijayan et al., 2003; Sathiyaa and Vijayan, 2003). This raises the possibility that despite elevated steroid levels, the tissue responsiveness was not affected, suggesting altered plasma clearance as a likely reason for the elevated cortisol concentration in the diluted MWWE group at 8 d.

While plasma cortisol levels did not show any clear pattern, the tissue-specific changes in GR protein expression points to alterations in cortisol signaling in fish upon exposure to MWWE. Previously, we have shown an elevation in GR protein in the liver in

the presence of elevated cortisol after 14 d exposure to MWWE, suggesting higher tissue sensitivity to cortisol (Ings et al., 2011b). The current study supports a temporal change in cortisol signaling in response to MWWE, while the lack of a change in either plasma glucose or lactate response in the MWWE group points to a lack of sustained organismal secondary stress response (Iwama et al., 2006). However, the changes in liver glycogen content and enzyme activities in response to MWWE clearly implicate an altered liver metabolic capacity.

The liver glycogen drop was significantly greater in the high effluent concentration group compared to the other groups after 8 d, supporting an increased energy substrate utilization to cope with the enhanced energy demand associated with MWWE exposure. This glycogen drop may be due to a reduced glucose phosphorylation capacity at 2 d in the liver of MWWE exposed fish. Interestingly, this liver glycogen depletion was recovered by the next sampling period, and was commensurate with significant increases in the activities of enzymes involved in gluconeogenesis and amino acid metabolism, including PEPCK and AlaAT, respectively. As these two enzymes are cortisol responsive in fish (Mommsen et al., 1999; Vijayan et al., 2005), our results support the notion that altered liver responsiveness to cortisol may be involved in the transient metabolic adjustments to cope with increased energy demand associated with MWWE exposure. An increase in the activity of the lipogenic enzyme G6PDH, involved in providing reducing energy in the form of NADPH (Panserat et al., 2009; Vijayan et al., 1991), further supports this hypothesis, and the reducing equivalents also play an important role in xenobiotic biotransformation and protection against oxidative stress (Winzer et al., 2002).

Very few studies have examined the target tissue glucocorticoid receptor expression in response to contaminant exposure in fish (Vijayan et al., 2005). We demonstrate for the first time that GR protein expression in the heart is impacted by MWWE exposure. After 14 d exposure to MWWE, there was a significant increase in GR protein expression in both effluent groups compared to the control. This suggests a tissue specific effect of MWWE, and leads us to propose that the heart is a key target for MWWE impact in fish. The heart plays an important role in the stress response, as it is essential for the delivery of glucose and oxygen to the tissues. The increase cardiac pyruvate kinase (PK) activity in the MWWE group points to an enhanced glycolytic capacity, suggesting an enhanced metabolic capacity and glucose utilization to cope with the effluent stressor.

Aside from in the heart, none of the other tissues tested showed consistent elevation in the glycolytic capacity. The brain, generally considered to have a high demand for glucose during stress, had minimal changes in the activity of glycolytic enzymes aside from a transient decrease in HK activity in the 20% MWWE group after 8 d. In the gill, there was a significant increase in gill HK activity after 8 d, but this was reversed by 14 d. The gill may be more impacted by exposure to MWWEs than the other tissues as it interacts directly with the surrounding environment. The observed decrease in glycolytic capacity of the gills, with significant decreases occurring in HK and PK activity by the end of the exposure, is interesting as it might be expected that increased interaction with the effluent would increase the energetic requirements and, therefore, enhance glycolytic capacity in that tissue. However, this decrease may suggest that gill function may be impaired by longer exposures

to MWWE. Indeed, there is evidence that exposure to MWWE may cause histopathological damage to the gills suggesting disruption of gill function (Schlacher et al., 2007).

Overall, this study suggests a metabolic cost associated with chronic exposures to MWWE in trout. This involves increased expression of hsps in the liver, which may be an adaptive response to cope with proteotoxicity. The elevated energy demand in fish exposed to MWWE is met by an increased liver capacity for gluconeogenesis. However, a lack of change in plasma glucose levels with MWWE exposure may reflect alterations in the plasma turnover of this metabolite, but this remains to be tested. The elevated glycolytic enzyme activity in the heart points to a tissue-specific increased metabolic demand, including glucose utilization, to cope with MWWE exposure. Overall, exposure to MWWE is a chronic stressor and increases the tissue energy demand. This may compromise the energy substrate repartitioning that is essential for animals to cope with additional stressors and to reestablish homeostasis.

4.6 Acknowledgements

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Chapter 5

Tissue-specific metabolic changes in response to an acute handling disturbance in juvenile rainbow trout exposed to municipal wastewater effluent

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5.1 Overview

The objective of this study was to evaluate the effects of municipal wastewater effluent (MWWE) exposure on aspects of both organismal and cellular stress response in rainbow trout (*Oncorhynchus mykiss*). Juvenile rainbow trout were exposed for 14 d (2-d static renewal) to tertiary-treated MWWE at concentrations of 0%, 20% and 90%. Following the MWWE exposure, fish were subjected to an acute handling stress and sampled at 1, 4 and 24 h post-stressor, to evaluate the fish performance to additional stressors. Organismal stress response evaluation included measuring plasma cortisol, glucose and lactate concentrations, and tissue metabolic capacity, including gluconeogenic (liver) and glycolytic enzyme activities in the liver, brain, heart and gill. No significant differences between treatments were seen in plasma cortisol, glucose or lactate concentrations after 14 d exposure to MWWE. However, MWWE exposure significantly affected plasma cortisol and glucose response to the acute secondary stressor. Acute handling disturbance enhanced liver gluconeogenic capacity in the control group, but this response was altered in the MWWE exposed groups. MWWE exposure did not affect the acute stressor-mediated enhancement of brain or gill glycolytic capacity, but significantly reduced the glycolytic capacity of liver and heart in response to a secondary stressor compared to the control group. Altogether, chronic exposure to MWWE impacts the metabolic performances to a secondary stressor challenge, including disruptions in tissue-specific gluconeogenic and glycolytic capacities in rainbow trout.

5.2 Introduction

In fish, as in other animals, stress involves disruption in homeostasis as a result of intrinsic or extrinsic stimuli known as stressors. The stressors are wide ranging, including chemical, physical or biological (Iwama et al., 2006), and the fish elicits a coordinated set of behavioral and physiological responses in an attempt to regain homeostasis, which is called

the stress response. A well studied stress response is the activation of two neuroendocrine pathways, including the hypothalamic-sympathetic-chromaffin cell (HSC) axis and the hypothalamus-pituitary-interrenal (HPI) axis, leading to the release of catecholamines and corticosteroids, respectively, into the circulation. The functioning of the HPI axis has been the focus of most studies related to stress detection because, unlike catecholamines, it is possible to obtain resting levels of plasma cortisol (Iwama et al., 2006).

It is well established that following an acute stressor exposure, including handling and confinement, there is a rapid and transient increase in plasma cortisol concentration that returns to resting levels over a 24 h period (Mommsen et al., 1999; Vijayan et al., 2010). Cortisol has a wide ranging effect on the animal, including ion and osmoregulation, immune function, growth and metabolism, and reproduction. The physiological implications of this hormonal response during a stressful episode include mobilization of energy substrate to fuel the increased energy demand associated with stress (Vijayan et al., 2010). A well established metabolic response is the plasma elevation of glucose in response to stress and this is mediated in part by the stressor-induced initial catecholamine and cortisol surge (Wendelaar Bonga, 1997; Vijayan et al., 2010). The acute stressor-induced glucose release is initially mediated by catecholamine-induced glycogenolysis, whereas the maintenance of this response and repletion of liver glycogen stores involves cortisol-induced hepatic gluconeogenesis (Mommsen et al., 1999). Gluconeogenesis results in the formation of glucose from non-carbohydrate sources such as lactate, glycerol and amino acids, and is mediated by phosphoenolpyruvate carboxykinase (PEPCK), considered to be the rate limiting step and converts oxaloacetate into phosphoenolpyruvate. Studies have shown an

increase in PEPCK activity and gene expression, as well as enzymes involved in amino acid metabolism such as alanine aminotransferase (AlaAT) and aspartate aminotransferase (AspAT) in response to acute stress or cortisol (Vijayan et al., 1994; 2003; Gravel and Vijayan, 2007; Wiseman et al., 2007). Enzymes involved in glycolysis, such as the rate-limiting enzymes hexokinase (HK), and pyruvate kinase (PK), have also been measured in response to acute stress, and have shown increased activity (Gravel and Vijayan, 2007) or gene expression (Wiseman et al., 2007; Momoda et al., 2007) in the liver, likely due to increases in energy demand for stressor adaptation. Plasma cortisol and glucose levels are routinely measured as indicators of primary and secondary responses to stressor exposure in fish along with plasma lactate levels, which reflects the muscular activity of the animal (Wendelaar Bonga, 1997; Iwama et al., 2006). Changes in either gluconeogenic or glycolytic capacity can give valuable insight into the changes in energy repartitioning required to cope with stress, and modulation by environmental contaminants could interfere with stressor adaptation.

From an ecotoxicological stand-point, stress response pathways are sensitive targets for environmental contaminants (Hontela et al., 1992,1997; Vijayan et al., 1997a,b; Hontela and Vijayan, 2008). Recently, we showed that chronic exposure to municipal wastewater effluent (MWWE), which is a complex mixture of industrial chemicals, pharmaceuticals and personal care products and a major source of contamination in the aquatic environment (Chambers et al., 1997), impacts stress response in rainbow trout (Ings et al., 2011a,b). The objective of this study was to examine the impact of exposure to MWWE on the response to acute stress and subsequent tissue-specific metabolic consequences by measuring the activity

of key gluconeogenic (PEPCK, AlaAT, AspAT) and glycolytic (HK, PK, glucokinase (GK), lactate dehydrogenase (LDH)) enzymes in juvenile rainbow trout (*Oncorhynchus mykiss*). Fish were exposed to two doses of MWWE, plus a control, for two weeks in a controlled laboratory setting. Following the 14 d exposure, fish were subjected to a 5 min handling stress, and sampled at 1, 4 and 24 h post stressor exposure.

5.3 Materials and Methods

5.3.1 Animals

Juvenile rainbow trout (average body mass of 22.8 ± 5.85 g) were obtained from Silvercreek Aquaculture (Erin, ON, CAN) and transported to the University of Guelph Hagen Aqualab. Fish were held in a holding tank for one week, and fed to satiety every second day. Fish were then transferred to the experimental tanks for a one week acclimation period before the start of the experiment. A light:dark regiment of 16:8 hours was used. Water was changed completely every two days. Two identical sets of tanks were prepared so that the fish were held in one set of tanks for two days, and then transferred to the second set of tanks containing fresh control or treatment water for another two days in order to minimize stress in the fish due to water changes. This continued for the duration of the experiment to minimize handling stress and ammonia accumulation. Fish were not fed for the duration of the experiment.

5.3.2 Experimental Design

Fish were exposed to either control well water, or MWWE diluted with well water to a concentration of 20% or 90% effluent for 14 d in July, 2009. Fish were sampled after 1, 8

and 14 d of exposure to examine the animal response to chronic MWWE exposure and these results are published elsewhere (Ings et al. in preparation, Chapter 4), with fish sampled on day 14 used in that study, as well as the 0 h pre-acute stressor samples in the current study (6 fish per treatment). . Following the 14 d exposure, fish were subjected to an acute 5 min handling disturbance and sampled at 1, 4 and 24 h post-stressor. Three tanks containing 6 fish were set up for each treatment, and, following the stressor, two fish per tank were sampled at 1, 4 and 24 h, for a total of 6 fish per treatment per time point.

Fish were anesthetized with buffered MS-222 and killed by spinal severance, weighed and fork length measured. Blood was withdrawn with heparinized needles by caudal puncture and centrifuged at 3000 g for 5 min to collect plasma for cortisol, glucose and lactate analyses. The fish were all immature and liver weights were recorded. Liver, brain, heart and gill were snap frozen in liquid nitrogen and stored at -80^{0}C for protein and enzyme activity analysis. This experiment was conducted in accordance with animal use protocols approved by University of Waterloo Animal Care Committee, in accordance with the Canadian Council for Animal Care.

5.3.3 Plasma Measurements

Plasma level of cortisol was measured 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 plasma lactate (Trinity Biotech, St. Louis, MO, USA).

5.3.4 Enzyme Activities

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 a standard. The tissue homogenate 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) 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 MgCl2, 10 mM KCl, 0.25 mM NADH, 2.5 mM phosphoenolpyruvate (PEP), 5 U/mL lactate dehydrogenase (LDH) and 2.5 U/mL pyruvate kinase; reaction started with 1 mM ATP.

- Glucokinase (GK:EC 2.7.1.2): 15 mM glucose, 5 mM MgCl2, 10 mM KCl, 0.25 mM NADH, 2.5 mM phosphoenolpyruvate (PEP), 5 U/mL lactate dehydrogenase (LDH) and 2.5 U/mL pyruvate kinase; reaction started with 1 mM ATP.
- Pyruvate kinase (PK: EC 2.7.1.40): 3 mM KCl, 10 mM MgCl2, 0.12 mM NADH, 2.5 mM ADP, 20 UmL LDH; reaction started with 2.5 mM PEP.
- Lactate dehydrogenase (LDH: EC 1.1.1.27): 0.12 mM NADH and reaction initiated with 1 mM pyruvic acid.

- Phosphoenolpyruvate carboxykinase (PEPCK: EC 4.1.1.32): 20 mM NaHCO3, 1 mM MnCl2, 0.5 mM phosphoenolpyruvate, and 0.12 mM NADH; reaction started with 5 mM deoxyguanosine diphosphate.
- Alanine aminotransferase (AlaAT: EC 2.6.1.2): 0.12 mM NADH, 200 mM l-alanine, 0.025 mM pyridoxal 5-phosphate, and 12 U/mL LDH; reaction started with 10.5 mM α-ketoglutarate.
- Aspartate aminotransferase (AspAT: EC 2.6.1.1):7 mM a-ketoglutarate, 0.025 mM pyridoxal 5-phosphate, 0.12 mM NADH, and 8 U/mL malate dehydrogenase; reaction started with 40 mM aspartic acid.

The enzyme activity is expressed as micromoles of substrate consumed or product liberated per minute (U) per gram protein. All enzymes were measured in the liver, while HK, PK and LDH were measured in the brain, heart and gill.

5.3.5 Statistical Analysis

Comparisons over time and treatment (plasma measurements and protein expression) were carried out with two-way analysis of variance (ANOVA) using SPSS statistical software (IBM, Somers, NY, USA) followed by the Tukey's post-hoc test. Within a time-point, treatments were compared using one-way ANOVA, followed by the Dunnett's post-hoc test. Data were log transformed, if necessary, to meet the assumptions of parametric statistics including equal variance and normal distribution. A significance level of $\alpha=0.05$ was used.

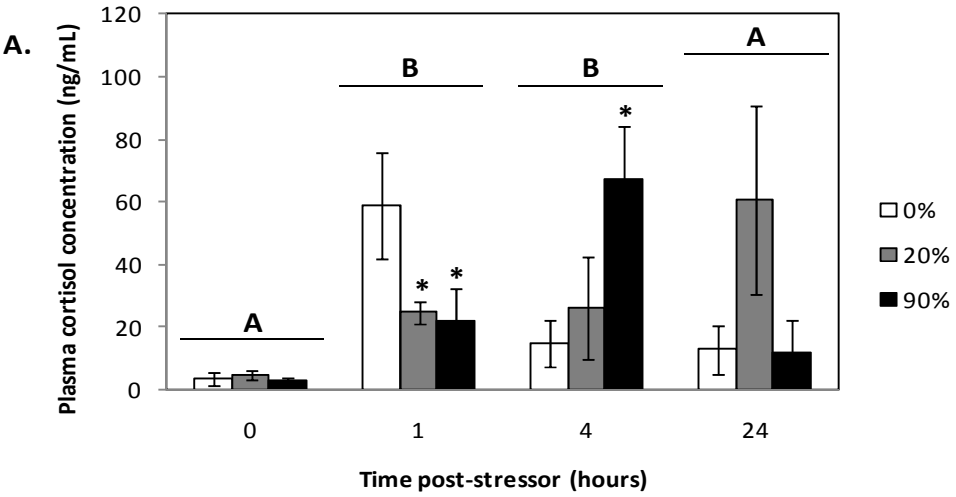
5.4 Results

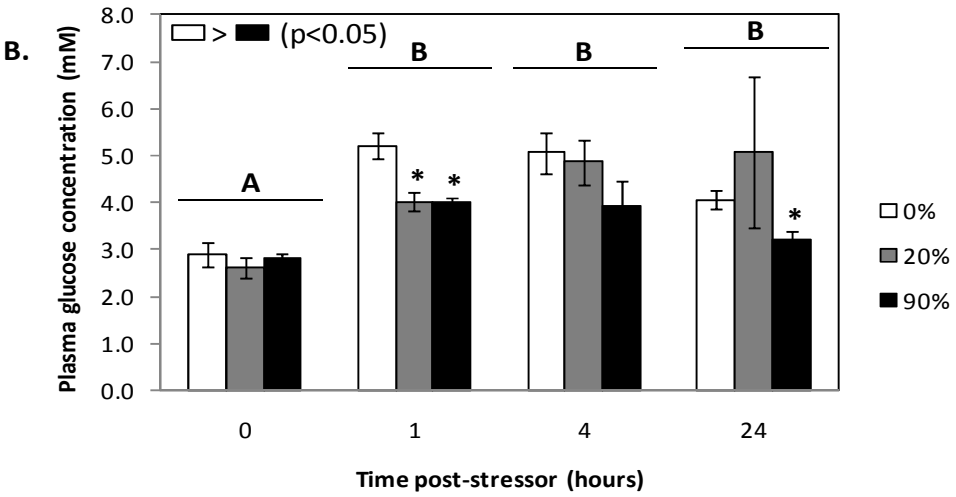
5.4.1 Stress Response

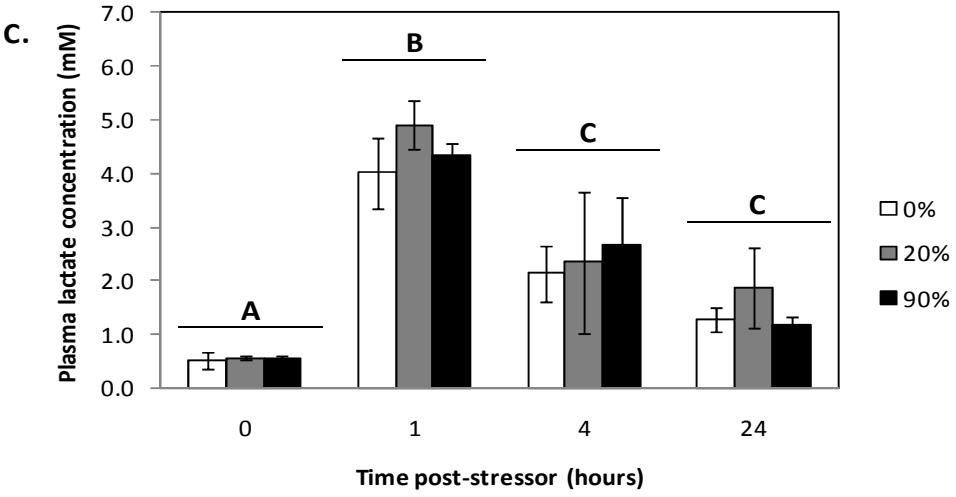
Following the 14 d exposure period, there were no differences in plasma cortisol concentration between the control fish and the MWWE-treated fish (Fig. 1a). Acute stressor exposure significantly elevated plasma cortisol levels in the control group after 1 h compared to pre-stressor values. A significant elevation was also seen in MWWE-treated fish compared to pre-stressor values, but this increase was significantly attenuated compared to control fish (Fig., 1a). Plasma cortisol concentrations returned to pre-stressor levels at 4 h and 24 h post-stressor in control fish as well as in the 20% MWWE group, although values were variable (Fig. 1a). In the 90% effluent group, values were elevated compared to the control group after 4 h, and returned to pre-stressor levels after 24 h (Fig. 1a).

No differences were seen in plasma glucose concentrations between control and MWWE-treated fish following the 14 d exposure period (Fig. 1b). Acute stressor exposure significantly elevated plasma glucose levels in control group after 1 h compared to pre-stressor levels. Plasma glucose was increased compared to pre-stressor concentrations in both the 20% and 90% MWWE treatment groups, but this increase was significantly less than in the control fish (Fig. 1b). Glucose concentration in control fish remained elevated 4 h post-stressor, but started to decrease to pre-stressor levels by 24 h (Fig. 1b). No differences were seen between treatments at 4 h post-stressor. After 24 h, no differences were seen between the 20% MWWE group and the control, but glucose levels in the 90% MWWE treated fish were significantly depressed (Fig. 1b).

Fig. 1: Effects of municipal wastewater effluent on rainbow trout plasma cortisol (A), plasma glucose (B) and plasma lactate (C), before an acute handling stress and 1, 4 and 24 h post secondary stressor (n=6 fish per treatment). Bars represent mean +/- SEM. Different letters indicate significant differences between time-points ($P<0.05$; two-way ANOVA). Asterisks indicate significant differences from control within the time-point ($P<0.05$; one-way ANOVA). Inset indicates significant differences between treatments ($P<0.05$; two-way ANOVA).


A.
Plasma cortisol concentration (ng/mL)
120
100
80
60
40
20
0
A
B
B
A
*
*
*
0
1
4
24
Time post-stressor (hours)
0%
20%
90%


B.
Plasma glucose concentration (mM)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
> (p<0.05)
A
B
B
B
*
*
*
0
1
4
24
Time post-stressor (hours)
0%
20%
90%


C.
Plasma lactate concentration (mM)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
A
B
C
C
0
1
4
24
Time post-stressor (hours)
0%
20%
90%

There were no differences in plasma lactate concentrations between control and MWWE-treated fish following the 14 d exposure period (Fig. 1c). Exposure to acute stress significantly elevated plasma lactate in all treatment groups 1 h post-stressor, with no differences between the treatments (Fig. 1c). Lactate levels in all treatments decreased in the 4 and 24 h time points compared to the 1 h time point, but remained elevated compared to the pre-acute stressor values (Fig. 1c).

5.4.2 Metabolic Response

Liver PEPCK activity was increased in both the 20% and 90% MWWE-treated fish following the 14 d exposure period compared to the control. Following the acute stressor, PEPCK activity increased significantly in all groups for the remaining time points in all treatments, except for a significant decrease after 4 h in the 90% MWWE fish (Table 1). Liver AlaAT activity also increased in the 90% MWWE group following the 14 d exposure, and activity increased in all treatments following the acute stressor. After 24 h post-exposure, the activity of AlaAT in the 90% MWWE treatment group was significantly higher than the other two treatment groups (Table 1). There were no significant differences between treatment groups at any time point in liver AspAT activity. By 24 h post-acute stressor, there was an overall increase in AspAT activity (Table 1). No changes were seen between treatments in liver HK, GK, PK and LDH activity following the 14 d MWWE exposure period, and activity of all enzymes increased post-acute stressor, regardless of treatment (Table 1). Significant decreases were observed in HK activity in the 20% MWWE treated fish after 1 h, and the 90% MWWE treated fish after 4 and 24 h compared to the control group (Table 1). GK activity decreased in the 20% and 90% MWWE-treated fish after 1 h

Table 1: Effects of municipal wastewater effluent on enzyme activities (μmol/min/g protein) after 14 d MWWE exposure (pre-stressor) and 1, 4 and 24 h post secondary acute handling stress in juvenile rainbow trout. Values represent mean +/- SEM. Different letters indicate significant differences ((P<0.05; two-way ANOVA) between time-points (time effect column). Asterisks indicate significant differences from control within the time-point (P<0.05; one-way ANOVA). HK: Hexokinase; GK: Glucokinase; PK: Pyruvate kinase; LDH: Lactate dehydrogenase; PEPCK: Phosphoenolpyruvate carboxykinase; AlaAT: Alanine aminotransferase; AspAT: Aspartate aminotransferase.

Liver	Time post-stressor	0%	20%	90%	Time Effect
HK	Pre-stressor	3.17 ± 0.07	3.62 ± 0.16*	3.52 ± 0.10	A
	1	6.80 0.35	5.15 ± 0.17*	6.29 ± 0.27	B
	4	6.27 ± 0.20	5.90 ± 0.40	5.40 ± 0.25*	B
	24	7.26 ± 0.27	6.88 ± 0.50	6.46 ± 0.11*	C
GK	Pre-stressor	3.46 ± 0.12	3.69 ± 0.13	3.50 ± 0.11	A
	1	7.09 ± 0.28	5.53 ± 0.03*	5.93 ± 0.22*	BC
	4	6.01 ± 0.20	6.45 ± 0.70	6.00 ± 0.24	B
	24	6.53 ± 0.50	6.98 ± 0.55	7.40 ± 0.36	C
PK	Pre-stressor	6.73 ± 0.35	8.59 ± 0.86	7.89 ± 0.51	A
	1	11.01 ± 2.15	9.93 ± 1.67	8.52 ± 0.64	AB
	4	14.89 ± 1.61	8.70 ± 1.76*	9.23 ± 1.00*	B
	24	10.80 ± 1.52	12.96 1.86	8.57 ± 0.58	B
LDH	Pre-stressor	153.5 ± 9.9	166.9 ± 7.2	160.1 ± 10.1	A
	1	197.6 ± 15.9	237.4 ± 8.6	238.5 ± 17.8	B
	4	245.9 ± 24.9	230.5 ± 21.8	174.1 ± 8.4*	B
	24	250.6 ± 12.0	241.1 ± 25.5	220.1 ± 14.2	B
PEPCK	Pre-stressor	1.57 ± 0.094	2.06 ± 0.083*	2.22 ± 0.17*	A
	1	3.50 ± 0.349	3.32 ± 0.094	3.68 ± 0.349	B
	4	3.79 ± 0.282	3.93 ± 0.428	2.74 ± 0.124*	B
	24	3.63 ± 0.201	3.74 ± 0.354	3.73 ± 0.163	B
AlaAT	Pre-stressor	23.4 ± 2.4	34.4 ± 3.8	36.9 ± 4.6*	A
	1	42.9 ± 5.3	60.2 ± 7.6	68.2 ± 9.5	B
	4	58.9 3.5	60.6 ± 7.8	53.3 ± 5.6	B
	24	62.2 ± 6.5	54.1 ± 5.0	82.1 ± 4.8*	B
AspAT	Pre-stressor	50.0 ± 3.3	46.8 ± 3.2	47.3 ± 4.4	A
	1	50.4 ± 3.1	57.6 ± 3.2	60.8 ± 4.7	AB
	4	57.9 ± 5.0	53.5 ± 4.9	53.5 ± 5.2	AB
	24	55.1 ± 5.4	55.4 ± 7.2	67.4 ± 4.6	B

compared to the control but not at the other time points (Table 1). PK activity decreased in the 20% and 90% MWWE-treated fish compared to the control after 4 h but not at the other time points (Table 1). LDH activity also decreased in the 90% MWWE-treated fish compared to the control after 4 h, but not at the other time points (Table 1).

In the brain, there were no differences in HK, PK and LDH activities between treatments following the 14 d MWWE exposure. After the acute stress exposure, HK activity decreased overall after 4 and 24 h post-stressor (Table 2). Brain PK and LDH activity increased 1 h post-acute stressor and returned to pre-stressor levels after 4 h, with activity levels decreasing even lower than pre-stressor activity levels for LDH (Table 2). There were no changes in brain HK, PK and LDH activity between treatment groups (Table 2).

In the heart, PK activity was elevated in both the 20% and 90% MWWE treated fish compared to the control fish after the 14 d exposure period, while no differences in HK and LDH were seen between treatment groups (Table 3). Following the acute stressor, heart HK activity increased after 4 and 24 h compared to pre-stressor levels. Overall, heart PK activity decreased post-stressor, while heart LDH activity did not change at all (Table 3). There were no treatment differences in HK activity, while PK activity significantly decreased in the 90% MWWE group compared to the control after 4 h, and both the 20% and 90% MWWE groups compared to the control after 24 h (Table 3). No treatment changes were seen in LDH activity (Table 3).

In the gill, HK activity decreased in both MWWE treatment groups compared to the control fish following the 14 d exposure period. Overall, HK activity decreased post stressor compared to pre-stressor values (Table 4). PK activity increased 24 h post-stressor, while

Table 2: Effects of municipal wastewater effluent on brain enzyme activities (µmol/min/g protein) after 14 d MWWE exposure (pre-stressor) and 1, 4 and 24 h post secondary acute handling stress in juvenile rainbow trout. Values represent mean +/- SEM. Different letters indicate significant differences ((P<0.05; two-way ANOVA) between time-points (time effect column). Asterisks indicate significant differences from control within the time-point (P<0.05; one-way ANOVA). HK: Hexokinase; PK: Pyruvate kinase; LDH: Lactate dehydrogenase.

Brain	Time post-stressor	0%	20%	90%	Time Effect
HK	Pre-stressor	35.1 ± 1.6	37.2 ± 1.3	34.7 ±1.2	A
	1	35.3 ± 0.9	37.6 ± 1.4	37.0 ± 2.0	A
	4	30.7 ± 0.8	35.3 ± 3.0	28.8 ± 1.2	B
	24	28.8 ± 2.0	30.4 ± 1.2	26.1 ± 0.6	B
PK	Pre-stressor	123.4 ± 6.4	114.8 ± 4.4	109.1 ± 5.2	A
	1	160.1 ± 9.8	146.7 ± 5.5	158.9 ± 10.2	B
	4	119.2 ± 6.7	123.2 ± 18.5	118.9 ± 15.7	A
	24	114.3 ± 8.8	114.1 ± 3.2	114.2 ± 9.0	A
LDH	Pre-stressor	83.5 ± 1.2	88.5 ± 2.5	79.9 ± 1.8	A
	1	104.6 ± 4.3	94.2 ± 12.7	98.4 ± 5.6	B
	4	84.9 ± 4.2	84.4 ± 9.2	72.7 ± 3.7	A
	24	72.6 ± 5.4	72.9 ± 3.2	62.7 ± 3.2	C

Table 3: Effects of municipal wastewater effluent on heart enzyme activities (µmol/min/g protein) after 14 d MWWE exposure (pre-stressor) and 1, 4 and 24 h post secondary acute handling stress in juvenile rainbow trout. Values represent mean +/- SEM. Different letters indicate significant differences ((P<0.05; two-way ANOVA) between time-points (time effect column). Asterisks indicate significant differences from control within the time-point (P<0.05; one-way ANOVA). HK: Hexokinase; PK: Pyruvate kinase; LDH: Lactate dehydrogenase.

Heart	Time post-stressor	0%	20%	90%	Time Effect
HK	Pre-stressor	18.9 ± 1.1	20.8 ± 1.6	20.2 ± 1.7	A
	1	25.1 ± 2.6	24.5 ± 1.6	20.1 ± 2.4	A
	4	26.7 ± 1.5	25.7 ± 1.0	24.2 ± 1.2	B
	24	25.0 ± 2.9	22.0 ± 2.8	22.6 ± 1.4	B
PK	Pre-stressor	141.1 ± 11.2	188.7 ± 9.6*	240.8 ± 15.8*	A
	1	161.5 ± 16.4	127.6 ± 9.4	125.1 ± 13.2	B
	4	133.9 ± 10.7	126.3 ± 5.9	105.1 ± 3.9*	B
	24	172.5 ± 27.0	116.7 ± 14.1*	126.7 ± 12.4*	B
LDH	Pre-stressor	236.6 ± 15.9	265.7 ± 24.1	265.7 ± 29.1	
	1	232.1 ± 17.4	207.1 ± 39.7	228.7 ± 34.5	No Change
	4	260.6 ± 49.7	222.0 ± 11.5	156.3 ± 25.2	
	24	227.7 ± 38.9	179.7 ± 27.0	173.4 ± 35.6	

Table 4: Effects of municipal wastewater effluent on gill enzyme activities (μmol/min/g protein) after 14 d MWWE exposure (pre-stressor) and 1, 4 and 24 h post secondary acute handling stress in juvenile rainbow trout. Values represent mean +/- SEM. Different letters indicate significant differences ((P<0.05; two-way ANOVA) between time-points (time effect column). Asterisks indicate significant differences from control within the time-point (P<0.05; one-way ANOVA). HK: Hexokinase; PK: Pyruvate kinase; LDH: Lactate dehydrogenase.

Gill	Time post-stressor	0%	20%	90%	Time Effect
HK	Pre-stressor	15.7 ± 0.81	13.2 ± 0.54*	13.4 ± 0.76*	A
	1	10.8 ± 1.07	9.2 ± 0.47	10.8 ± 0.36	B
	4	11.2 ± 0.61	11.8 ± 0.58	11.1 ± 0.39	BC
	24	11.9 ± 0.66	11.3 ± 0.46	13.5 ± 1.21	C
PK	Pre-stressor	33.9 ± 1.9	33.7 ± 1.9	31.0 ± 2.7	A
	1	32.5 ± 3.0	34.0 ± 1.7	35.9 ± 1.9	A
	4	35.9 ± 1.9	34.9 ± 2.8	34.5 ± 2.8	AB
	24	39.5 ± 3.1	34.9 ± 2.8	48.1 ± 4.3	B
LDH	Pre-stressor	53.9 ± 5.0	58.7 ± 3.4	51.0 ± 1.5	A
	1	47.0 ± 5.7	47.5 ± 3.4	41.3 ± 3.9	B
	4	41.2 ± 1.8	35.0 ± 3.4	36.9 ± 3.1	B
	24	42.9 ± 3.2	35.9 ± 3.7	55.6 ± 12.2	AB

LDH activity was significantly lower 1 and 4 h, but not 24 h, post stressor (Table 4). There were no significant differences between treatments in HK, PK and LDH activity post stressor (Table 4).

5.5 Discussion

The results of this study demonstrate that exposure to MWWE for 14 d disrupts the adaptive cortisol response, as well as impairs the metabolic readjustments essential to cope with a secondary stressor. Few studies have examined the tissue-specific metabolic readjustments associated with acute stressor exposure in fish (Sangiao-Alvarellos et al., 2005), while even fewer focused on contaminant effects on intermediary metabolism (Tintos et al., 2006; 2007; Vijayan et al., 2006; Gravel and Vijayan, 2007). It is well established that one of the primary roles of increased cortisol following a stressor is the mobilization and replenishment of energy stores to help provide metabolic fuel, usually in the form of glucose, to overcome a stress challenge (Mommsen at el., 1999). Consequently, there are metabolic consequences to a disruption in the cortisol response but few studies have looked at changes in tissue-specific metabolic capacity in fish following exposure to a stressor. The results reveal perturbations in the stress-mediated changes in tissue intermediary metabolism with MWWE exposure, pointing to an altered capacity for metabolic adjustments to stress in rainbow trout.

5.5.1 Metabolic Response to Acute Stress

One of the primary roles of elevated cortisol levels in response to stress is increased tissue metabolic capacity, including enhancement of activities of enzymes involved in intermediary metabolism. This along with other cellular metabolic changes leads to an

increased energy demand in stressed fish and will entail energy substrate repartitioning to metabolically adapt the animal to stress. As expected plasma cortisol levels were elevated transiently in response to an acute stressor exposure and this correlated with an elevated plasma glucose response (Barton et al., 2002; Iwama et al., 2006; Vijayan et al., 2010). Also, acute stress significantly increased the activity of three key enzymes in the liver involved in glucose production, including PEPCK (rate-limiting step in gluconeogenesis) and AlaAT and AspAT (enzymes involved in amino acid metabolism and providing C3 precursors for gluconeogenesis) supporting a stressor-mediated cortisol stimulation of liver gluconeogenic capacity in fish (Mommsen et al., 1999; Vijayan et al., 2010). The metabolic changes in the control group also points to an increased tissue glucose utilization (HK activity) and glycolytic capacity (PK activity) as an adaptive response to cope with the stressor. The stressor-mediated changes in the liver point to an enhanced gluconeogenic and glycolytic capacities, suggesting that this tissue not only produces glucose but also utilizes this fuel to cope with the increased energy demand. While liver is the primary producer of glucose in fish, this metabolic fuel is utilized by several key tissues, including heart, brain and gill. This is in agreement with the increased capacity for glucose utilization and glycolysis seen in the heart and brain of trout exposed to an acute stressor in the present study. The heart plays an important role in stress adaptation by increasing blood flow in the body and subsequently metabolic fuels and oxygen delivery for glycolysis and oxidative phosphorylation (Wendelaar Bonga, 1997). Interestingly, only PK activity increased in the brain, and this increase was transient, with activity returning to pre-stressor levels four h post-stressor, while there were no significant changes in gill metabolic capacity. These results suggest tissue-

specific metabolic adjustments in response to acute stress in fish. However, the changes in tissue metabolic capacity to stress may be dependent on several factors, including the species, type and intensity of the stressor and the nutritional state of the animal (Vijayan and Moon, 1992; Vijayan et al., 1997a; Soengas and Aldegunde, 2002; Sangiao-Alvarellos et al., 2005; Vijayan et al., 2010).

5.5.2 Effects of Municipal Wastewater Effluent

The organismal stress response and subsequent metabolic reorganization is a key component of the successful adaptation to a stressor, so a disruption to this process has important implications when it comes to trying to understand the effects on fish exposed to environmental contaminants such as MWWE in the natural environment. Despite this, few studies have looked at the stress response and the subsequent metabolic response as an endpoint to contaminant exposure (Tintos et al., 2006; 2007; Gravel and Vijayan, 2007; Benguira et al., 2002; Aluru and Vijayan, 2006; Mennigan et al., 2010). In the current study, no differences were seen in plasma cortisol or glucose levels between the control and MWWE treated fish prior to the acute handling stress. This is unlike studies in the field that have shown elevated plasma cortisol in response to MWWE exposure in rainbow trout (Ings et al., 2011a,b). The reason for this difference is not clear, but MWWE effluent is dynamic and composition changes depending on a number of factors including input composition, temperature, treatment processes and weather, all of which may affect the type and concentrations of chemical pollutants in the effluent (Servos et al., 2003; Vieno et al., 2005; Clara et al., 2005) In fact, the concentrations of several pharmaceuticals were shown to vary considerably in the MWWE over the course of the current study (Wang, 2010). However, the

stressor-mediated plasma cortisol and glucose levels were attenuated in the MWWE groups compared to the control, supporting a disruption of the organismal stress response in rainbow trout (Ings et al., 2011b).

A key finding from this study was that exposure to MWWE disrupts the tissue-specific metabolic adjustments essential to cope with a secondary stressor. Prior to the acute stress, there was a significant increase in the activity of PEPCK and AlaAT in the MWWE-treated fish liver compared to the controls, suggesting an enhanced glucose demand in MWWE-exposed fish compared to control fish. However, the decrease in liver PEPCK activity by MWWE post-stressor exposure clearly points to a reduced capacity for gluconeogenesis. The activity of AlaAT is actually increased by 24 h, suggesting a possible channeling of amino acids for oxidation to cope with the increased energy demand. This is further supported by the reduced liver glycolytic capacity post-stressor in the MWWE treatment groups. Altogether it appears that the liver capacity for glucose production in response to a secondary stressor is impacted by MWWE exposure, this also increases liver capacity for amino acid catabolism. Whether these changes are related to an attenuated cortisol response and/or cortisol signaling by MWWE or it is a direct effect of chemical mixtures on intermediary metabolic pathways remains to be determined.

PK activity in the hearts of MWWE-treated fish decreased significantly compared to control levels post-acute stressor, suggesting that MWWE exposure may decrease the glycolytic capacity of this tissue. This is interesting as the heart plays an important role in stressor adaptation (Wendelaar Bonga, 1997). There was a significant increase prior to the stressor, indicating that the heart was in a highly glycolytic state following the 14 d exposure,

but was unable to maintain the required sustained increase in activity post-stressor. It is possible this decrease is a direct response to the disruption of the cortisol response and the decrease in circulating glucose levels but remains to be tested. Decreased glycolytic capacity suggests there might be an insufficient supply of energy substrate, which may lead to inadequate performance ability in exposed fish when faced with a challenge. Interestingly, MWWE did not affect the glycolytic capacity of the gill or the brain post acute stressor, emphasizing the tissue-specific effects of MWWE exposure on glycolytic capacity. Although the reason for this difference is not clear at this point, it is possible that the energy supply, in the brain at least, is being protected in exposed fish in order to maintain adequate tissue function. It is possible that components of the MWWE are acting directly on the glycolytic pathway to decrease the activity of these enzymes in the affected tissues. Regardless of the mechanism, the end result will be decreased energy available for stressor adaptation, which may have long-term health consequences.

The adaptive stress response is essential to regain homeostasis following an acute stressor. A MWWE-induced disruption to this response may impair the fish performance when encountering one or more stressors in the natural environment. Exposure to MWWE affects both the cortisol stress response and causes a tissue-specific disruption in the subsequent stressor-induced metabolic reorganization in juvenile rainbow trout. Gluconeogenesis in the liver appears to be a primary target of exposure to MWWE, leading to a decrease in plasma glucose and associated with a decrease in the glycolytic capacity of the liver and heart. Overall, MWWE disrupts the ability to respond to the increase in energy

demand associated with a secondary acute stressor in rainbow trout leading to metabolic consequences with the potential to affect growth and survival in exposed fish.

5.6 Acknowledgements

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Chapter 6

Venlafaxine and atenolol disrupts epinephrine-stimulated glucose production in rainbow trout hepatocytes

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6.1 Overview

The beta-blocker atenolol (ATEN), and the selective serotonin and norepinephrine reuptake inhibitor, venlafaxine (VEN) are found in municipal waste water effluents, but little is known about the effect of these pharmaceuticals on aquatic animals. We tested the hypothesis that VEN and ATEN, at environmentally relevant concentrations, disrupt the acute stress mediated glucose production by fish liver. To this end, rainbow trout (Oncorhynchus mykiss) hepatocytes were exposed in vitro to different concentrations (0, 0.1, 10, 1000 nM) of VEN or ATEN and glucose production in response to either cortisol or epinephrine (two key stress hormones) stimulation ascertained. Both VEN and ATEN did not affect either the unstimulated or cortisol (100 ng/ml)-stimulated glucose release over a 24 h period. The acute (3 h) unstimulated glucose production by isolated hepatocytes in suspension was also not modified by ATEN, while VEN (100 and 1000 nM) reduced basal glucose release. However, ATEN, even at concentration as low as 0.01nM completely abolished epinephrine (1 μM)-induced glucose production in trout hepatocytes. Interestingly, VEN also suppressed the epinephrine-induced glucose production but only at higher concentrations (100 and 1000 nM). We also tested if these drug effects on glucose production were due to perturbations in β-adrenoceptor signaling by exposing hepatocytes to a cAMP analogue (8-bromo-cAMP). Also, cells were exposed to glucagon (a metabolic hormone that increases glucose production) to confirm if the observed changes in glucose production observed with the drugs were either non-specific or specific to beta-adrenergic signaling. Neither VEN nor ATEN significantly impacted either 8-bromo-cAMP- or glucagon-induced glucose production suggesting that the mode of action of the drugs involved blocking beta

adrenoceptor signaling. Also, ATEN but not VEN attenuated the epinephrine-induced increase in glucose transporter 2 (GLUT2) mRNA abundance in trout hepatocytes. Overall, VEN and ATEN are beta-blockers and may disrupt the adaptive acute glucose response to a secondary stressor in rainbow trout.

6.2 Introduction

A diverse range of pharmaceutical compounds have been detected in municipal wastewater effluent (MWWE), agricultural run-off, surface waters and even drinking water in Canada and throughout the world (Metcalfe et al., 2003; Servos et al., 2007; Richardson, 2009; Corcoran et al., 2010). Although these compounds have been detected at relatively low concentrations, due to their pharmacological properties and the fact that aquatic organisms, including fish are being continually exposed, they have the potential to cause adverse effects (Massarsky et al., 2011). Two classes of compounds of particular importance are the beta-blockers and anti-depressants that have been detected in the aquatic environment at levels approaching those that could be potentially biologically significant (Knacker and Metcalfe, 2010; Owen et al., 2007; Kreke and Deitrich, 2008).

Beta-blocking drugs are used in managing cardiovascular disorders and are widely prescribed worldwide. They have been detected in municipal wastewater effluents in the ng/L to low µg/L range, especially in highly urbanized areas (Alder et al. 2010; Wick et al. 2009; Fent et al., 2006). Pharmacologically, beta-blocking drugs are competitive antagonist of β-adrenergic receptors (Owen et al., 2007). One of the most commonly prescribed beta-blocker is the drug atenolol, which blocks the β1-adrenergic receptor (Rang et al., 2003). Minimal effects using traditional toxicological endpoints have been reported for

environmentally relevant levels of beta blockers in fish, and relatively few studies have looked at the impact of exposure to atenolol (Winter et al., 2008; Giltrow et al., 2009; Hampel et al., 2010; Bartram et al., 2011).

Anti-depressant drugs are the focus of an increasing amount of environmental research due to the large number of these drugs detected in the aquatic environment (Kreke and Deitrich, 2008). The majority of research thus far focused on the selective serotonin reuptake inhibitor, fluoxetine, due to its historically high prescription rate (IMS Health, 2006), and detection in the environment and potential effects on key components of ecosystems (Oakes et al., 2010). Fluoxetine has wide ranging effects in fish, including anorexigenic effects and weight loss (Mennigen et al., 2009, 2010a), effects on glucose metabolism (Mennigen et al., 2010a) and effects on fecundity, ovarian steroid hormone levels and gene expression (Lister et al., 2009). However, lately another selective serotonin and norepinephrine reuptake inhibitor, venlafaxine, is being increasingly prescribed and the concentration of this drug is detected at even higher levels in the environment (Meltcalfe et al., 2010). Reuptake inhibiting drugs function by elevating the levels of certain neurotransmitters in the synaptic cleft by blocking the transporters on the pre-synaptic membrane responsible for their removal. The studies mentioned above suggest a non-target effect of SSRIs on peripheral tissues of fish, but the mechanisms involved are unclear.

We recently showed that the glucose response to an acute stress is attenuated in fish exposed to MWWE (Ings et al., 2011a,b). This was accompanied by changes in liver metabolic capacity, including as alterations in the mRNA abundance of the primary liver glucose transporter GLUT2 in trout (Ings et al., 2011a,b). As both atenolol and venlafaxine

are the predominant beta blocker and SSNRI, respectively, in MWWEs (Küster et al., 2010; Metcalfe et al., 2010; Ings, 2011), we tested the hypothesis that these two pharmaceuticals suppress the stress hormones-induced glucose production by trout liver. Two hormones important in the acute glucose regulation during stress are epinephrine and cortisol and they increase glucose production by enhancing glycogenolysis and gluconeogenesis in the liver, respectively (Mommsen et al., 1999; Iwama et al., 2006). We tested this hypothesis by utilizing rainbow trout (Oncorhynchus mykiss) hepatocytes as an in vitro model system. Several studies have clearly established this cell system as an excellent model to understand hormone action on metabolism, including glucose production by epinephrine, cortisol, cAMP analogs and glucagon (Moon, 2004; Vijayan et al., 1994; Vijayan et al., 1997; Plisetskaya and Mommsen, 1996). Here we determined whether VEN and ATEN exposure impacted the hepatocyte responsiveness to hormone-stimulated glucose production. We also examined whether exposure to VEN and ATEN impacted GLUT2 gene expression in trout hepatocytes.

6.3 Materials and Methods

6.3.1 Animals

Juvenile trout (~100 g, <1 y), were obtained from Alma Research Station, Alma, Ontario, Canada, and held in a flow-through well water (in 500 L tank) at 13°C and the fish were maintained under a 12 h:12 h (light/dark) photoperiod. The animals were maintained in accordance with guidelines established by the Institutional Animal Care and Use Committee at the University of Waterloo.

6.3.2 Primary Culture of Trout Hepatocytes

Trout hepatocytes were isolated by in situ perfusion of the liver with collagenase exactly as described previously (Sathiyaa et al., 2001). Isolated hepatocytes were first washed with modified Hank's medium (110 mM NaCl, 3 mM KCl, 1.25 mM K2HPO4, 5 mM NaHCO3, 0.6 mM MgSO4, 1 mM MgCl2 and 10 mM Hepes; pH 7.63 at room temperature, medium A), followed by modified Hank's medium containing 1.5 mM CaCl2 and 2% defatted bovine serum albumin (BSA), pH 7.63 (medium C). L-15 media, supplemented with antibiotic and antimycotic agents, was used to resuspend the washed hepatocytes. Trypan blue dye exclusion method was used to determine hepatocyte viability and >95% cells were viable.

6.3.3 Cortisol Effects

Cells were plated in six-well tissue culture plates at a density of 1.5 million cells/well (0.75 million cells/mL) in L-15 media and were maintained at 13 °C for 24 h prior to the experiments. At the start of the experiment, media was changed and cells were treated with either VEN (0.1, 10, 1000 nM) or ATEN (0.1, 10, 1000 nM), along with the glucocorticoid receptor antagonist RU-486 (10 μM) in the presence or absence of 100 ng/mL cortisol. The RU486 and cortisol concentrations (stressed levels seen in trout) used were exactly as described before in trout hepatocytes (Aluru and Vijayan, 2007). Cells were exposed for 24 h, after which the medium was collected for the measurement of glucose release. The experiment was repeated using hepatocytes from eight independent fish (n=8).

6.3.4 Epinephrine Effects

Cells were settled for 1 h in a fresh aliquot of medium C, and were then plated in 500 μL aliquots in 24-well tissue culture plates at a concentration of 20 mg/mL in medium C. Cells were treated immediately with VEN (0.1, 10, 1000 nM), ATEN (0.001, 0.01, 0.1, 1, 10, 1000 nM) or the β-adrenoceptor antagonist propranolol (10 μM) and incubated for 2 h at 13 °C. Following the 2 h incubation, cells were treated with either 1 μM epinephrine or control (media only), and incubated at 13 °C for 1 h. The reaction was terminated by the addition of perchloric acid (2% final?), followed by centrifugation at 13000 rpm for 2 min, and the supernatant was collected for measurement of glucose concentration. A sample size of eight fish was used for this part of the experiment.

6.3.5 8-bromo-cAMP and Glucagon effects

Isolated hepatocytes were treated with either VEN (0.1-1000 nM) or ATEN (0.1-1000 nM) as described above for 2 h. Following this incubation, cells from each treatment group were either left as a control or exposed to epinephrine (1 μM), 8-bromo-cAMP (0.5 mM) or glucagon (0.1 μM) for 1 h. This concentration of 8-bromo-cAMP and glucagon have been shown previously to stimulate glucose production in trout hepatocytes (Vijayan et al., 1994; Plisetskaya and Mommsen, 1996). Cells were processed as described above and a sample size of four fish was used for this part of the experiment.

6.3.6 Hepatocyte Glut2 mRNA abundance

Cells were plated in six-well tissue culture plates at a density of 1.5 million cells/well (0.75 million cells/mL, stained with trypan blue and counted with a haemocytometer) in L-15 exactly as mentioned above (cortisol effect). As we were examining genomic effect, cells were allowed to form a monolayer prior to treatment. Consequently, Forty-eight hours after

plating, cells were treated with VEN (1 μM), ATEN (1 μM), or propranolol (10 μM), in the presence or absence of epinephrine (1 μM) for 24 h. Following 24 h incubation at 13 °C, cells were collected for RNA extraction for measurement of GLUT2 transcript levels using real-time quantitative reverse transcriptase polymerase chain reaction (qPCR). A sample size of four fish was used in this experiment.

6.3.7 Glucose and Glycogen Measurement

Glucose was measured using a commercially available colorimetric kit (Raichem, San Diego, CA, USA). Glycogen was determined by measuring glucose before and after amyloglucosidase digestion, following established methods (Vijayan et al., 2003).

6.3.8 Quantitative Real-time RT-PCR

Total RNA was extracted from hepatocytes using the RNeasy extraction kit (Qiagen, Mississauga, ON, CAN) and treated with DNase. The concentration of total RNA was determined spectrophotometrically at 260/280 nm using a NanoDrop™ spectrophotometer (Thermo Scientific, Napean, ON, CAN).

cDNA was synthesized from 1 μg total RNA using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Streetsville, ON, CAN). Briefly, 1 μg of RNA was combined with 0.5 μg random primers, 2 mM of each dNTP, and 50 U of Multiscribe™ reverse transcriptase in RT Buffer and incubated under the following conditions: 10 min at 25 ^{0}C, 120 min at 37 ^{0}C, and 5 min at 85 ^{0}C.

Samples were quantified using a SYBR green fluorescent dye master mix in an iCycler real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The gene of interest was GLUT2 and elongation factor 1α (EF1α) was used as a housekeeping gene. Threshold

cycle values for each sample were calculated using iCycler iQ real-time detection software (Bio-Rad). Briefly, each sample was assayed in triplicate. A master mix containing 3 μL cDNA, 3 μL of 10 mM primer pair, 40 μL of SYBR green mix and 34 μL of nuclease-free water was prepared for each sample, after which 25 μL was added to each of three wells. Each sample was assayed for the genes of interest and the housekeeping gene. The following PCR conditions were used for amplification: 95^0C for 3 min; 40 cycles of 95^0C for 20 s and annealing temperature (see Table 1) for 20 s; 95^0C for 1 min; 55^0C for 1 min followed by melt curve analysis starting at 55^0C and increasing in 0.5^0C increments to 95^0C every 10 s. Transcripts for each gene was determined by measuring the cycle where fluorescence passed a predetermined threshold level (threshold cycle) and converted to copy number using a standard curve made from plasmid vectors containing the target amplicon sequence. Transcript levels were normalized to a housekeeping gene (elongation factor 1α) and expressed as percent control.

6.3.9 Statistical Analysis

In each figure, the initial control levels, the positive controls and the stimulated (cortisol, epinephrine, 8-bromo-cAMP or glucagon) levels are shown where appropriate. All treatment effects are shown as a difference in the magnitude of change (difference between the control and hormone stimulation) in glucose production.All statistical analyses were conducted using repeated measures analysis of variance and paired t-tests, where appropriate. As post-hoc tests are not possible with repeated measures ANOVAs, t-tests were carried out post-ANOVA to determine differences between treatments, and a Bonferonni adjustment was used on the α-value for compensation.

6.4 Results

6.4.1 Cortisol Effects

Cortisol significantly increased media glucose levels when compared to basal control levels. RU-486 (GR antagonist) did not change unstimulated glucose levels, but significantly decreased cortisol-stimulated glucose production (Fig. 1a). The magnitude of change in glucose (difference in glucose production between the cortisol-stimulated cells and the control cells) response with cortisol was not altered by VEN treatment (Fig. 1b). Likewise, ATEN did not affect the magnitude of change in cortisol-stimulated glucose levels (Fig. 1c).

6.4.2 Epinephrine Effects

Epinephrine significantly elevated glucose production compared to basal control levels. Propranolol (a non-selective beta blocker) did not affect basal glucose release, but inhibited the epinephrine-induced glucose release in trout hepatocytes (Fig. 2a). VEN decreased the magnitude of change in glucose production seen with epinephrine compared to the control cells, but only at the highest concentrations (100 and 1000 nM; Fig. 2b). ATEN attenuated the epinephrine-induced glucose production at all three doses tested (Fig. 2c). An additional experiment to determine the lowest concentration of ATEN capable of attenuating epinephrine-stimulated glucose production (Fig 3a) revealed a significant inhibition at 0.01 nM and higher, but not at 0.001 nM (Fig 3b). A significant reduction in acute basal glucose levels in cells exposed to VEN was observed at higher concentrations (100 and 1000 nM; Fig. 4a), but there was no corresponding change in glycogen levels (Fig. 4b).

Fig. **1**: The effects of venlafaxine or atenolol on cortisol stimulated glucose release in rainbow trout hepatocytes in primary culture. A) Glucose release from hepatocytes in primary culture treated in either control conditions or with RU-486 (10 μM), with and without cortisol (100 ng/mL). B) Magnitude of change in glucose release following cortisol stimulation in cells treated with venlafaxine (0, 0.1, 10, 1000 nM). C) Magnitude of change in glucose release following cortisol stimulation in cells treated with atenolol (0, 0.1, 10, 1000 nM). Asterisk indicates significant difference from control. N=8 fish.

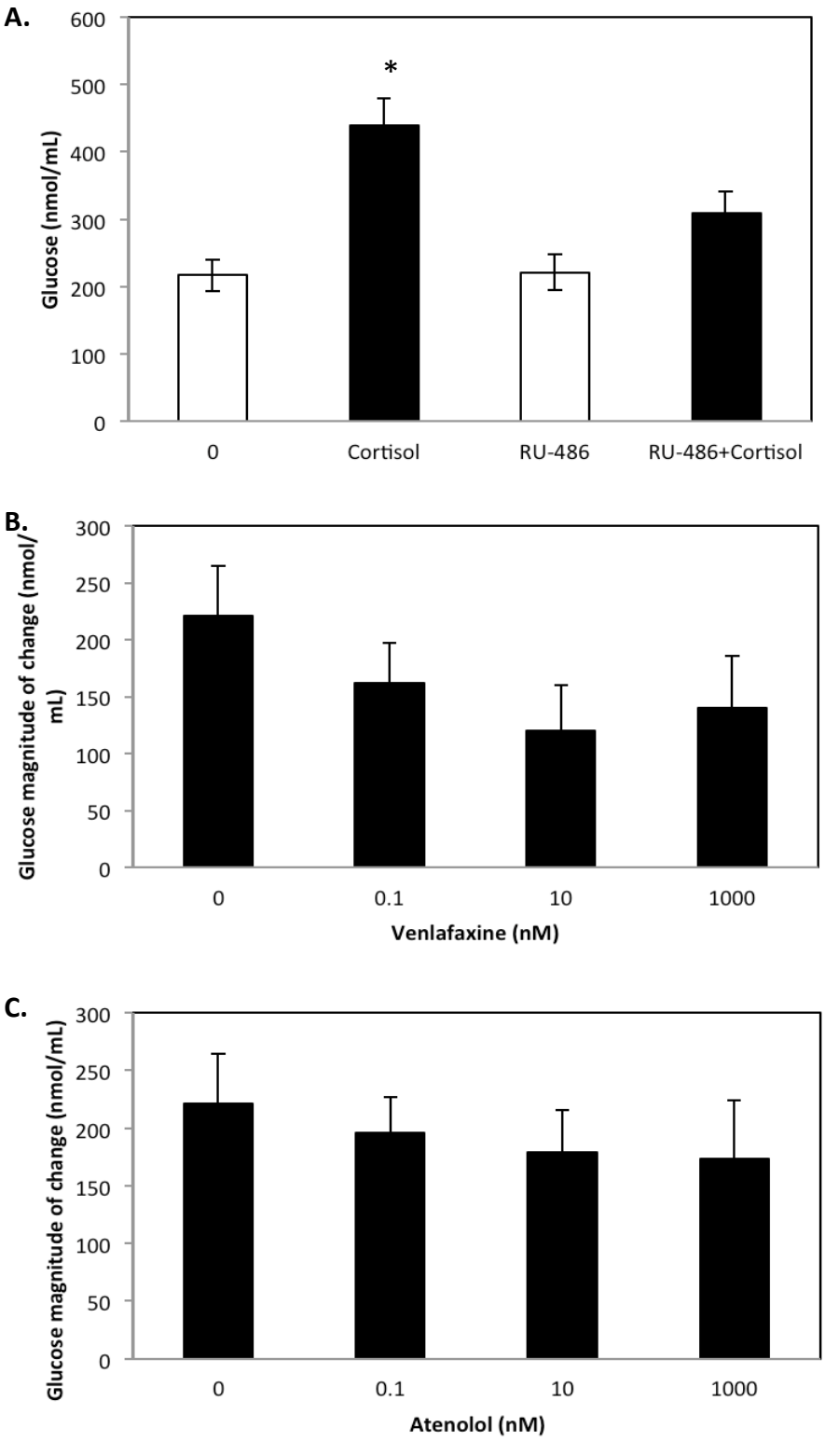

A.
600
500
400
300
200
100
0
Glucose (nmol/mL)
*
0
Cortisol
RU-486
RU-486+Cortisol
B.
300
250
200
150
100
50
0
Glucose magnitude of change (nmol/mL)
0
0.1
10
1000
Venlafaxine (nM)
C.
300
250
200
150
100
50
0
Glucose magnitude of change (nmol/mL)
0
0.1
10
1000
Atenolol (nM)

Fig. 2: The effects of venlafaxine or atenolol on epinephrine stimulated glucose content in rainbow trout hepatocytes in suspension. A) Glucose content in hepatocytes in suspension treated in either control conditions or with propranolol (10 μM), with and without epinephrine (1 μM). B) Magnitude of change in glucose content following epinephrine stimulation in cells treated with venlafaxine (0, 0.1, 10, 1000 nM). C) Magnitude of change in glucose content following epinephrine stimulation in cells treated with atenolol (0, 0.1, 10, 1000 nM). Asterisk indicates significant difference from control. N=8 fish.

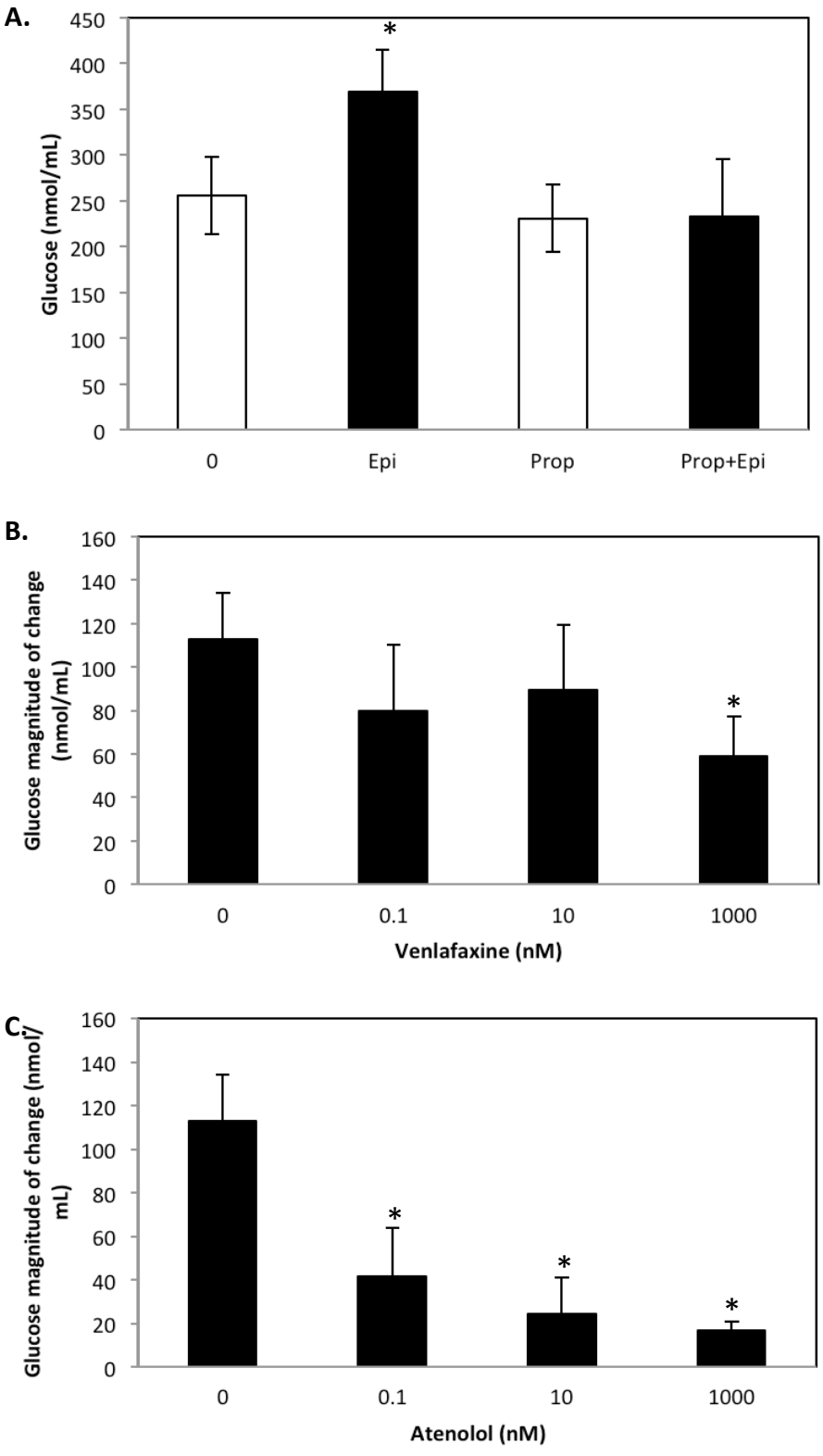

A.
Glucose (nmol/mL)
450
400
350
300
250
200
150
100
50
0
*
0
Epi
Prop
Prop+Epi
B.
Glucose magnitude of change (nmol/mL)
160
140
120
100
80
60
40
20
0
*
0
0.1
10
1000
Venlafaxine (nM)
C.
Glucose magnitude of change (nmol/mL)
160
140
120
100
80
60
40
20
0
*
*
*
0
0.1
10
1000
Atenolol (nM)

Fig. 3: The effects of low concentrations of atenolol on epinephrine stimulated glucose content in rainbow trout hepatocytes in suspension. A) Glucose content in hepatocytes in suspension treated in either control conditions or with epinephrine (1 μM). B) Magnitude of change in glucose content following epinephrine stimulation in cells treated with atenolol (0, 0.001, 0.01, 0.1, 1 nM). Asterisk indicates significant difference from control. N=5 fish.

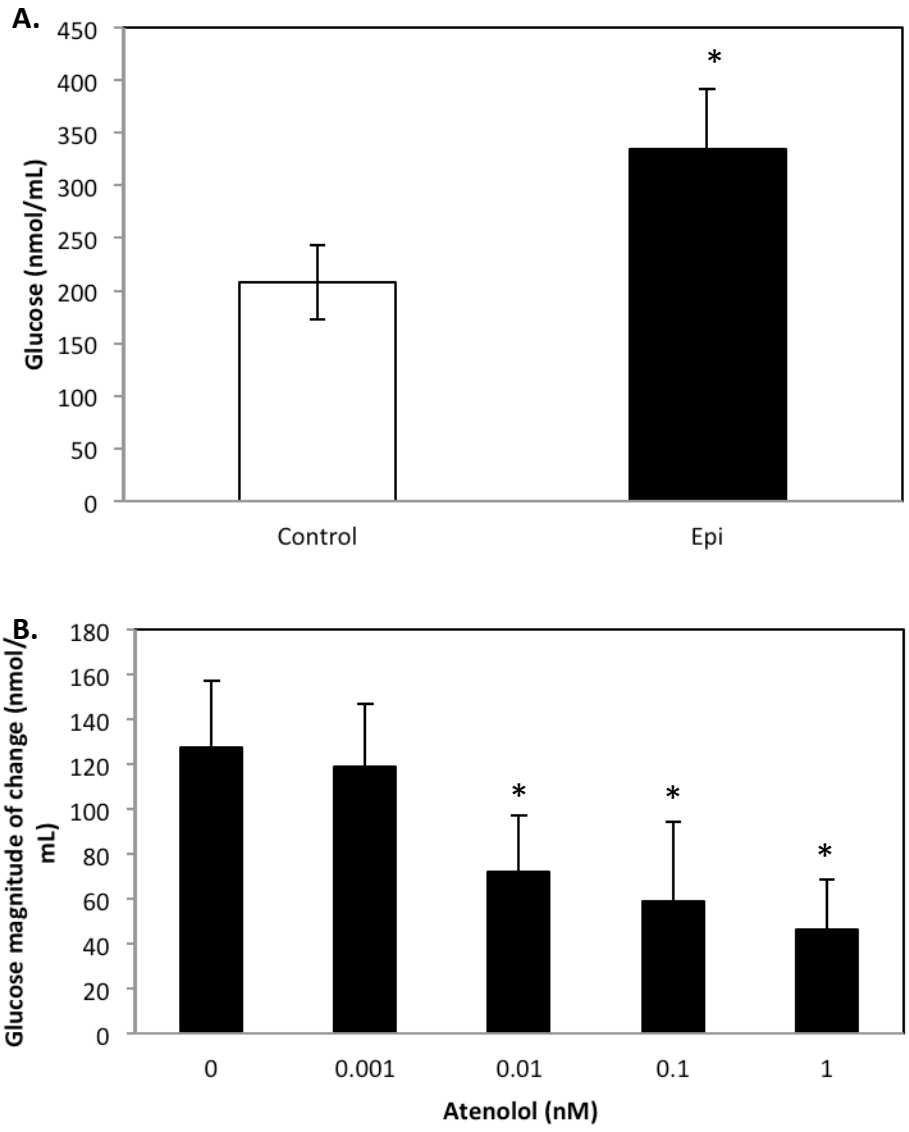
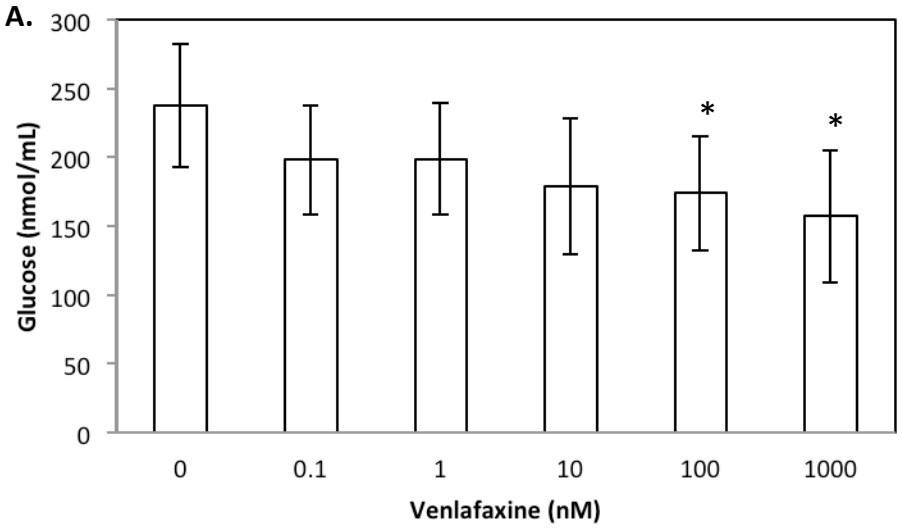
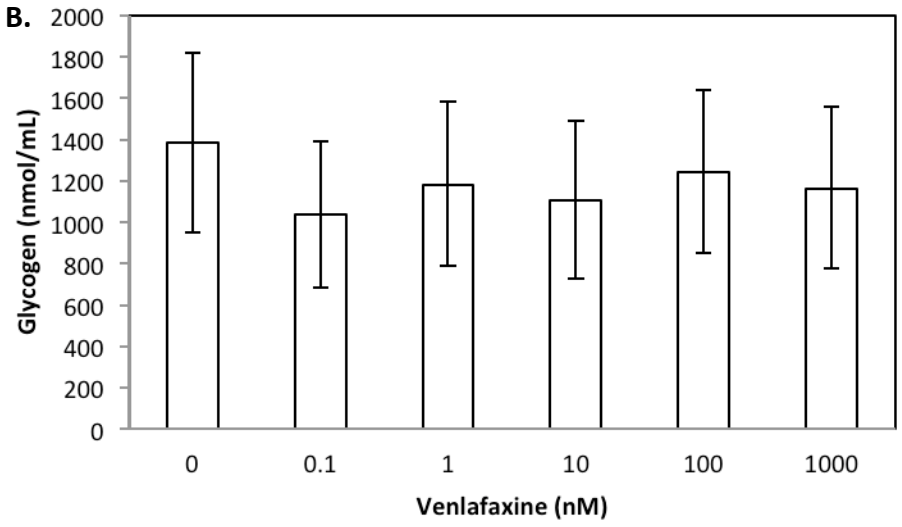

A.
Glucose (nmol/mL)
450
400
350
300
250
200
150
100
50
0
*
Control
Epi
B.
Glucose magnitude of change (nmol/mL)
180
160
140
120
100
80
60
40
20
0
*
*
*
0
0.001
0.01
0.1
1
Atenolol (nM)

Fig. 4: The effects of venlafaxine (0, 0.1, 1, 10, 100, 1000 nM) on A) basal glucose content and B) glycogen content in rainbow trout hepatocytes in suspension. Asterisk indicates significant difference from control. N=8 fish.


A.
300
250
200
150
100
50
0
Glucose (nmol/mL)
*
*
0
0.1
1
10
100
1000
Venlafaxine (nM)


B.
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Glycogen (nmol/mL)
0
0.1
1
10
100
1000
Venlafaxine (nM)

6.4.3 8-brom-cAMP and Glucagon Effects

Both 8-bromo-cAMP and glucagon stimulated glucose production in trout hepatocytes (Fig. 5a). There was no significant effect of VEN on either 8-bromo-cAMP-induced (Fig. 5b) or glucagon-induced (Fig. 5c) glucose production in trout hepatocytes. Similar to VEN, ATEN also did not significantly affect either 8-bromo-cAMP-induced (Fig. 5d) or glucagon-induced (Fig 5e) glucose production in trout hepatocytes.

6.4.4 Glut2 Response

Propranolol, ATEN and VEN had no significant effect on basal GLUT2 mRNA abundance (Fig. 6). Epinephrine elevated GLUT2 mRNA levels compared to the control, while propranolol and ATEN attenuated this epinephrine-induced GLUT2 mRNA abundance (Fig. 6). VEN had no effect on either unstimulated or epinephrine-stimulated GLUT2 mRNA abundance in trout hepatocytes (Fig. 6).

6.5 Discussion

This study demonstrates that VEN and ATEN disrupt liver capacity for glucose production in rainbow trout. This is important because both of these pharmaceuticals, as well as their metabolites and other related drugs, are commonly found in both MWWEs and in surface waters at levels approaching or exceeding 1 μg/L (Metcalfe et al., 2010). One of the primary goals of the adaptive stress response in fish is to increase plasma glucose concentration as this metabolic fuel is essential to cope with the increased energy demand associated with stress and reestablish homeostasis (Mommsen et al., 1999; Iwama et al.,

Fig. 5: The effects of venlafaxine or atenolol (0, 0.1, 1, 10, 100, 1000 nM) on epinephrine (1 μM), [8bromo] cAMP (500 μM), or glucagon (10 μM) glucagon stimulated glucose content in rainbow trout hepatocytes in suspension. A) Glucose content in hepatocytes in suspension treated in either control conditions or with epinephrine, 8-bromo cAMP, or glucagon. B) Magnitude of change in glucose content following 8-bromo cAMP stimulation in cells treated with venlafaxine (0, 0.1, 1, 10, 100, 1000 nM). C) Magnitude of change in glucose content following glucagon stimulation in cells treated with venlafaxine (0, 0.1, 1, 10, 100, 1000 nM). D) Magnitude of change in glucose content following 8-bromo cAMP stimulation in cells treated with atenolol (0, 0.1, 1, 10, 100, 1000 nM). E) Magnitude of change in glucose content following glucagon stimulation in cells treated with atenolol (0, 0.1, 1, 10, 100, 1000 nM). Asterisk indicates significant difference from control. N=4 fish.

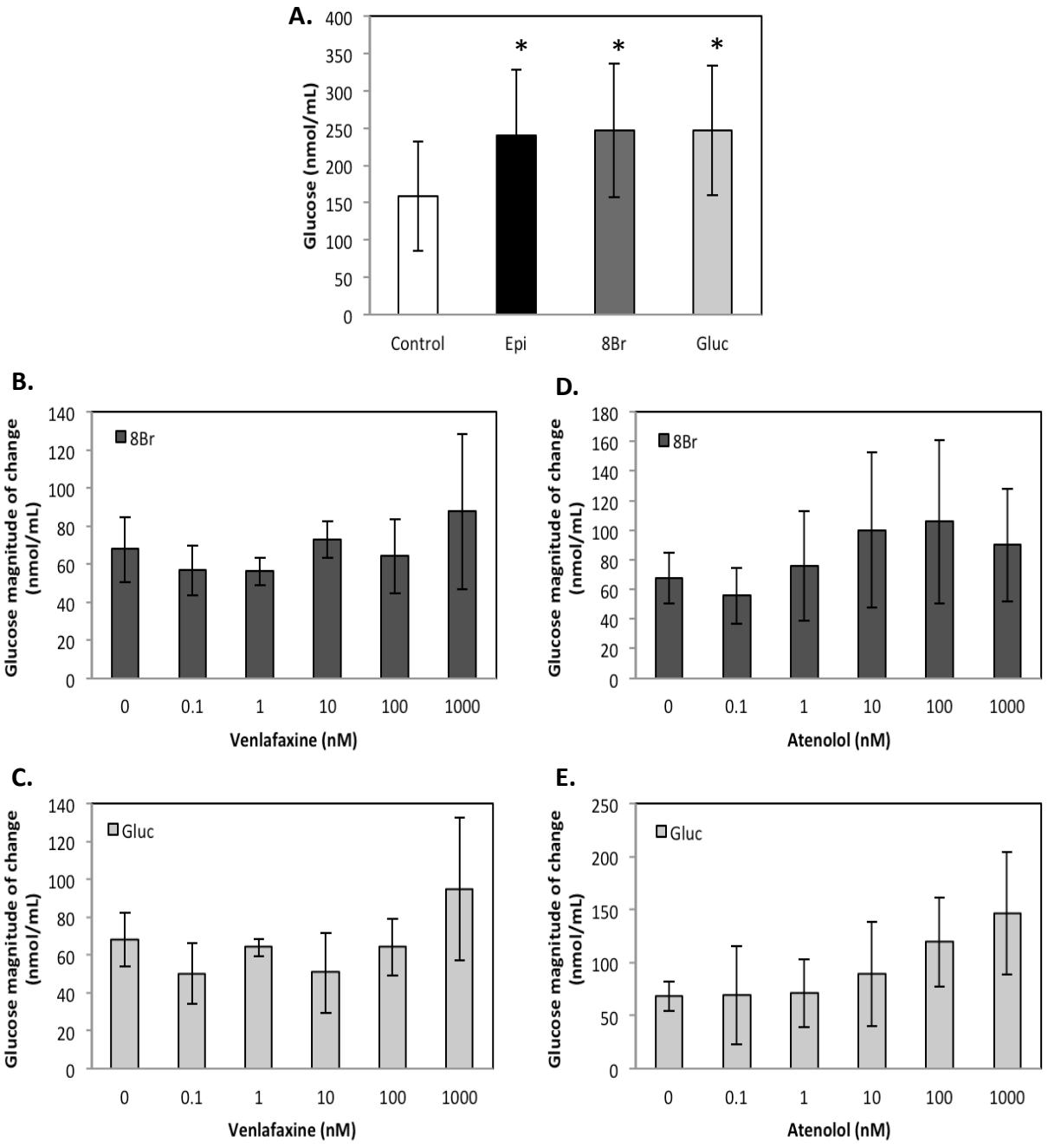
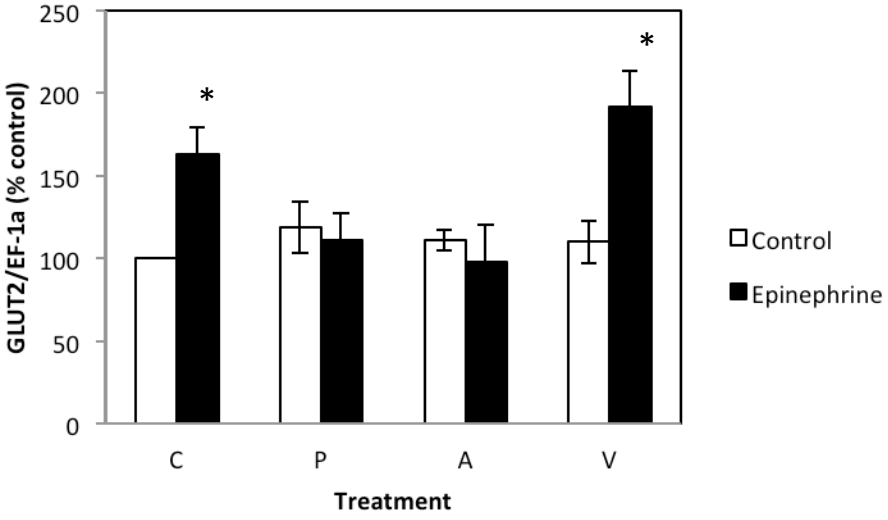

A.
Glucose (nmol/mL)
400
350
300
250
200
150
100
50
0
*
*
*
Control
Epi
8Br
Gluc
B.
Glucose magnitude of change (nmol/mL)
8Br
140
120
100
80
60
40
20
0
0
0.1
1
10
100
1000
Venlafaxine (nM)
D.
Glucose magnitude of change (nmol/mL)
8Br
180
160
140
120
100
80
60
40
20
0
0
0.1
1
10
100
1000
Atenolol (nM)
C.
Glucose magnitude of change (nmol/mL)
Gluc
140
120
100
80
60
40
20
0
0
0.1
1
10
100
1000
Venlafaxine (nM)
E.
Glucose magnitude of change (nmol/mL)
Gluc
250
200
150
100
50
0
0
0.1
1
10
100
1000
Atenolol (nM)

Fig. 6: The effects of control (C), venlafaxine (V: 1 μM), atenolol (A: 1 μM) or propranolol (P: 10 μM), with and without epinephrine (1 μM) on glucose transporter 2 (GLUT2) mRNA content (normalized to elongation factor 1α; EF-1α) in rainbow trout hepatocytes in primary culture. Asterisk indicates significant difference from control. N=4 fish.


250
200
150
100
50
0
GLUT2/EF-1a (% control)
*
*
C
P
A
V
Treatment
Control
Epinephrine

2006). Any impact on this adaptive metabolic response by contaminants may lead to compromised stress performance.

During the primary phase of the stress response, plasma glucose is quickly increased by the actions of the sympathetic nervous system, leading to the release of catacholamines, including epinephrine, into circulation from the chromaffin cells distributed mostly in the head kidney region of fish (Wendelaar Bonga, 1997; Reid et al., 1998). Epinephrine increases plasma glucose primarily through the rapid breakdown of glycogen stores in the liver (Weber and Shanghavi, 2000). The signaling pathway for this involves epinephrine binding to β-adrenergic receptors in the plasma membrane, activating adenylate cyclase and initiating a cAMP-mediated signal cascade that activates glycogen phosphorylase, a key enzyme involved in glycogenolysis (Reid et al., 1998). The acute glucose response during stress is sustained for longer periods by cortisol activation of glucocorticoid receptor signaling and simulation of hepatic gluconeogenesis (Mommsen et al., 1999; Vijayan et al., 2003; Aluru and Vijayan, 2007). Indeed the results of the current study confirm epinephrine- and cortisol-mediated glucose production in trout hepatocytes and this involves the activation of β-adrenoceptor and GR signaling, respectively.

Our results demonstrate that this conserved glucose response to stress hormones stimulation is disrupted by ATEN and VEN in trout hepatocytes. The effect of the drugs was specific to hepatocyte responsiveness to epinephrine, but not cortisol stimulation suggesting that these drugs may interfere with the acute glucose production capacity, a key component of the fight-or-flight response, during stress in fish. Although both drugs inhibited the stimulated glucose production, trout hepatocytes were more sensitive to ATEN than VEN.

The VEN effect was evident only at concentration that may not be environmentally realistic. In mammals, the mode of action of ATEN has been well-studied (Küster et al., 2010). ATEN is a specific antagonist of the β1 adrenoceptor. Blocking this receptor has been shown to lower heart rate, which decreases the effort by the heart and reallocates the blood flow to ischemic areas such as the kidney, which effectively lowers blood pressure (Rang et al., 2003). In fish and other non-target organisms, very few studies have looked at the effects of ATEN exposure, and to our knowledge, none have evaluated the effects on glucose dynamics at environmentally relevant concentrations. The mechanism of action of β-adrenoceptor antagonism has been fairly well supported for ATEN in several fish species. Several previous studies in other fish such as carp (Van Den Thillart et al., 2000) have shown interactions of ATEN with β-adrenoceptors and glucose levels. These studies have generally looked at high doses of ATEN to selectively block the β1-adrenoceptors for other purposes such as competitive binding studies (Fabbri et al., 2001; Mendonca and Gamperl, 2009).

A novel finding from this study was that ATEN at concentrations as low as 0.01 nM is capable of blocking epinephrine-stimulated glucose production in trout hepatocytes. Although, plasma atenolol concentrations have not been measured in fish, a recent study showed that trout exposed to environmentally relevant levels of propranolol had >1 nM concentration of this beta blocker in the plasma (Owen et al., 2009). These results clearly underscore the endocrine disruptive effects of environmentally relevant levels of atenolol, specifically leading to the inhibition of epinephrine action in fish. As epinephrine signaling is critical for acute stress adaptation, including metabolic and cardio-respiratory adjustments,

our results suggest that exposure to environmentally relevant levels of ATEN may lead to compromised fish stress performance.

The mode of action of VEN, a selective serotonin and norepinephrine reuptake inhibitor, in non-target organisms like fish is largely unknown. Very few studies have looked at VEN in an environmental context, despite the fact that it is being detected at relatively high concentrations (>1 μg/L) with increasing frequency, and its prescription rate is on the rise (Metcalfe et al., 2010). The effects of VEN on epinephrine-stimulated glucose production were less pronounced than ATEN, but a inhibition of beta-adrenergic signaling was seen at the higher concentrations tested. This effect has not previously been characterized in fish or other lower vertebrates, but studies in humans have suggested that SSRIs can bind to adrenoceptors in the brain (Baker, 1989), and may affect glucose homeostasis (McIntyre et al., 2006). The selective serotonin re-uptake inhibitor, fluoxetine, has been the focus of many more studies in fish due to its extensive use and detection in the environment. While the majority of research has focused on the effects of fluoxetine on reproduction (Lister et al., 2009, Mennigen et al., 2008, 2010b), a recent study in goldfish showed a significant decrease in plasma glucose levels in fish exposed to 540 ng/L fluoxetine (Mennigen et al., 2010a). It is well established in mammals that serotonin plays an important role in energy balance, and glucose homeostasis, and recent studies have suggested that this may be through a direct interaction with peripheral tissues (Lam and Heisler, 2007). Despite this, very few studies have looked at the interaction of antidepressants with glucose and energy metabolism in fish (Mennigen et al., 2009, 2010a). Our results for the first time demonstrates that VEN may potentially acts as a beta blocker at high concentrations in trout

peripheral tissues. However, it remains to be seen if the beta blocking effect of VEN on hepatic glucose production is evident at environmentally realistic levels. High concentration of VEN also showed a small but consistent decrease in acute unstimulated basal glucose production in trout hepatocytes. This was not commensurate with a significant decrease in glycogen levels, leading us to propose that this drug may also impact acute glucose release in trout hepatocytes.

We specifically asked the question whether ATEN and VEN impact on glucose production involved disruption of β-adrenergic receptor signaling. Consequently, we utilized 8-bromo-cAMP, a cAMP analogue, to stimulate membrane signal cascades while bypassing receptor binding and adenylate cyclase activation (Sandhu and Vijayan, 2011). In the current study, the decrease in epinephrine-stimulated glucose production in ATEN and VEN treated cells was not seen in the presence of 8-bromo-cAMP suggesting that the mechanism of action is upstream of cAMP signaling for both drugs. To further confirm if the effect was specific to β-adrenoceptor signaling and not a general effect of membrane receptors, the effect of these drugs on glucagon-induced glucose production was ascertained. As expected, glucagon exposure increased glucose production by trout hepatocytes. The mechanism of action of glucagon is very similar to that of epinephrine, including binding to G-protein coupled glucagon receptor in the liver, activating adenylate cyclase and initiating the cAMP signal cascade (Navarro et al., 1999). The absence of any effect of VEN or ATEN on glucagon-induced acute glucose production is a further confirmation that the inhibition observed in epinephrine-stimulated cells with both drugs is due to a specific antagonism of one or more β-adrenoceptors and not due to a general non-specific effect on membrane receptors.

A previous study showed that the primary glucose transporter in the liver, GLUT2, might be an important target of municipal wastewater effluent (Ings et al., 2011a). Our results did not show any changes to the steady state levels of GLUT2 mRNA for any of the treatments (data not shown), but did show a significant reduction in epinephrine-induced GLUT2 levels with exposure to both beta-blockers, ATEN and propranolol. VEN did not affect GLUT2 mRNA levels. No previous studies have looked at the link between epinephrine and the regulation of GLUT2 in fish, although several studies in rodents have suggested a direct link between sympathetic activity and increased GLUT2 expression (Miura et al., 2000; Schaan et al., 2005), although the mechanism is far from clear. These results lead us to propose that although both ATEN and VEN inhibit epinephrine-stimulated increases in glucose by targeting β-adrenergic signaling, they may also have distinct genomic effects. However, we cannot rule out the possibility that the regulation of GLUT2 by epinephrine may be receptor isoform specific, likely the β1-receptor as ATEN is a specific antagonist of this receptor in mammals (Rang et al., 2003), but this remains to be tested in fish.

Overall, both ATEN and VEN affect glucose production in rainbow trout hepatocytes, through interactions with epinephrine, but not cortisol action. This study suggests that the mechanism of action of both ATEN and VEN to inhibit epinephrine-stimulated increases in glucose may be through the antagonism of the β-adrenoceptors. We propose that ATEN is a more potent beta blocker compared to VEN and may impact epinephrine stimulated hepatocyte glucose production at concentrations that are environmental relevant. While our result leads us to hypothesize that these two drugs impacts acute glucose production by targeting the same signaling pathway, we cannot rule out the

possibility that the drug effects may also be distinct and receptor subtype specific. Since both pharmaceuticals are found consistently in municipal wastewater effluents along with other beta blockers, SSRIs and their metabolites, it is important to establish if the effects seen in the present study are modified by mixtures. Also, further research is needed to assess if these impacts in vitro translate to effects at the whole organism level. It is becoming clearer that energy balance and metabolism may be an important target of environmental contaminants. As activation of β-adrenoceptor signaling is essential not just for metabolic adjustments, but also for ion and osmoregulation and cardiovascular functions (Reid et al., 1998), our results highlight the potential impact of environmentally relevant levels of beta blockers and SS(N)RIs in disrupting homeostasis in fish.

6.6 Acknowledgements

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Chapter 7
General Conclusions

Experiments described in this thesis were designed to identify and then characterize non-reproductive endocrine and metabolic effects of exposure to municipal wastewater effluent (MWWE) in rainbow trout (*Oncorhynchus mykiss*), and to attempt to elucidate the mechanism of action of two pharmaceuticals found in MWWE, atenolol and venlafaxine. To this end, a series of field and laboratory *in vivo* studies and a series of *in vitro* studies were performed and the following conclusions were drawn.

1. Exposure to MWWE significantly impacts the transcript levels of a number of hepatic genes, spanning several functional categories, leading to the hypothesis that stress (cellular and organismal) and metabolism are major targets of one or more constituents of the effluent. (Chapter 2)
2. MWWE increases plasma cortisol and may act as a chronic stressor in exposed fish, while also impacting stress performance through the attenuation of the adaptive cortisol and glucose response to acute stress, as well as altered liver metabolic capacity. (Chapter 3)
3. Metabolic disruption is tissue-specific, and likely corresponds with MWWE-induced increases in energy demand and cellular stress in tissues, including the liver and the heart, which are key tissues in the adaptive metabolic response to stress. (Chapter 4)
4. The acute stressor-induced attenuation of plasma cortisol and glucose levels are associated with MWWE-mediated decreases in liver gluconeogenesis, as well as

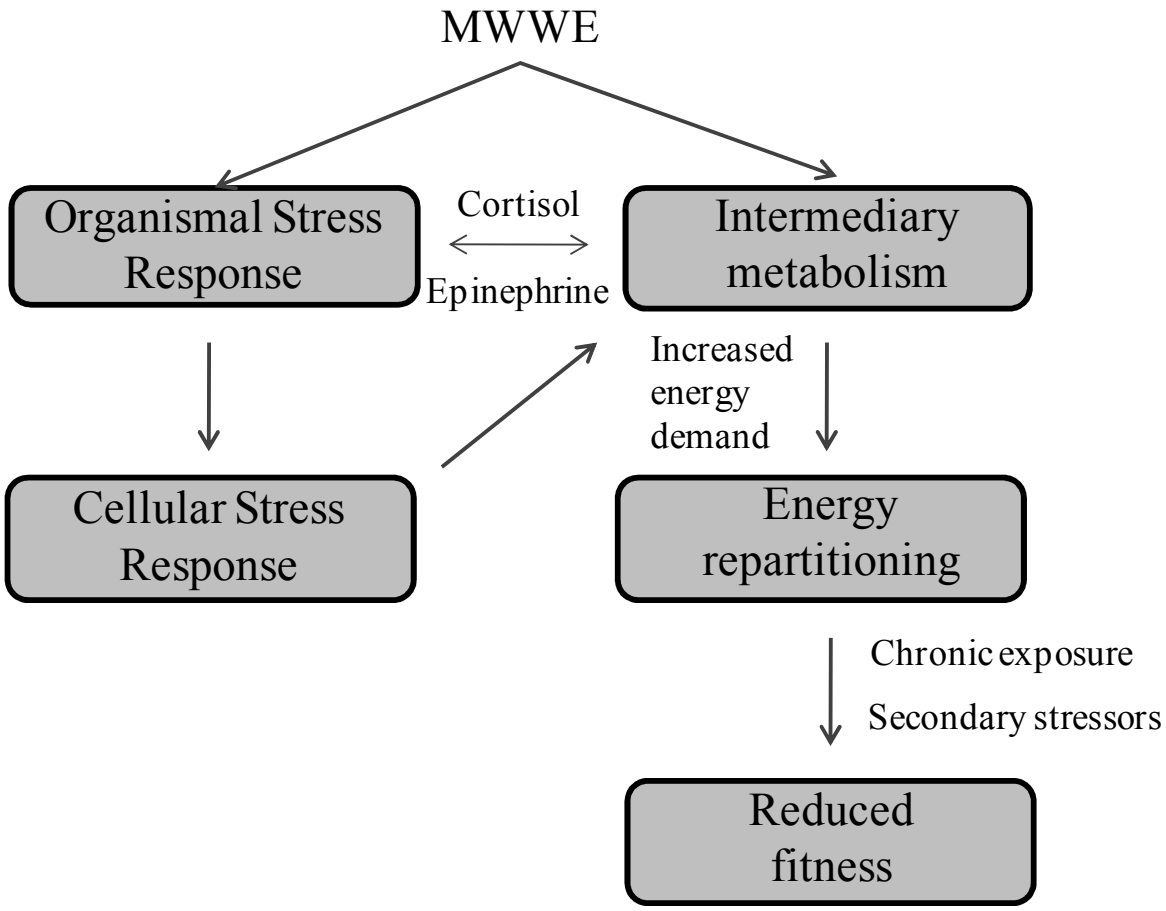
decreases in liver and heart glycolytic capacities in response to a secondary stressor. (Chapter 5)

5. Atenolol and venlafaxine impact epinephrine-induced but not cortisol-mediated glucose production in trout hepatocytes and the mechanism of action involves disruption of β-adrenoceptor signaling. (Chapter 6)

Taken together, these studies point to an overall metabolic disruption associated with MWWE exposure in rainbow trout (Fig. 1). MWWE can affect metabolic pathways either directly or indirectly by impacting HPI axis functioning and cortisol dynamics, which is an important regulator of metabolism. This has ramifications even beyond the specific observed effects, as cortisol plays an important role not only in metabolism but also in almost all relevant physiological processes, including reproduction, growth, immune function and osmoregulation. The goal of a functioning stress response is to provide energy through increased intermediary metabolism to fuel the increased demand associated with stressor exposure and regain homeostasis. If this adaptive process is compromised, it could lead to changes in energy partitioning that could impact fitness in the long run. Energy that would have been used for growth, reproduction and/or immune function will be diverted to chronic stress adaptation, compromising homeostasis and affecting animal survival.

This experiment used a model fish species and was conducted only at one wastewater treatment plant with the effluent undergoing tertiary treatment, which is among one of the most advanced treatment processes available on a large scale. There are many treatment plants in Canada and around the world using a variety of treatment processes that result in variations in effluent composition and quality. This may lead to unique effects between the

Fig 1. Conceptual diagram visualizing the effects of municipal wastewater effluent (MWWE) on the stress response and metabolism in rainbow trout, and how it leads to reduced fitness.


MWWE
Organismal Stress Response
Cortisol
Epinephrine
Intermediary metabolism
Increased energy demand
Cellular Stress Response
Energy repartitioning
Chronic exposure
Secondary stressors
Reduced fitness

different fish species exposed to MWWE. The treatment plant studied in this thesis (City of Guelph) represents an advanced treatment process that minimized confounding factors such as ammonia toxicity or low dissolved oxygen due to high biological oxygen demand. Sub-lethal effects of emerging contaminants including pharmaceuticals or other chemicals of concern (e.g. Bisphenol A, pesticides), may continue to affect fish performance even after considerable public investment in wastewater infrastructure. Because of this, understanding the mechanism of action of these compounds is becoming increasingly important. Furthermore, future studies must address the consequence of long term continuous exposure to MWWE in fish living downstream of treatment plants, and the role MWWE exposure plays in environments where fish are exposed to chemicals from multiple sources. It is also important to understand whether performance is compromised if an exposed fish is challenged by a natural secondary stressor (e.g. predator-prey relationships, seasonal variations in abiotic factors). Effects on the functioning of the stress response and on energy metabolism may present a chronic insult that could impair fish performance over a life time. It is important for future studies to determine whether these effects translate into observable effects in wild fish, and whether this leads to a failure to thrive, grow and even survive.

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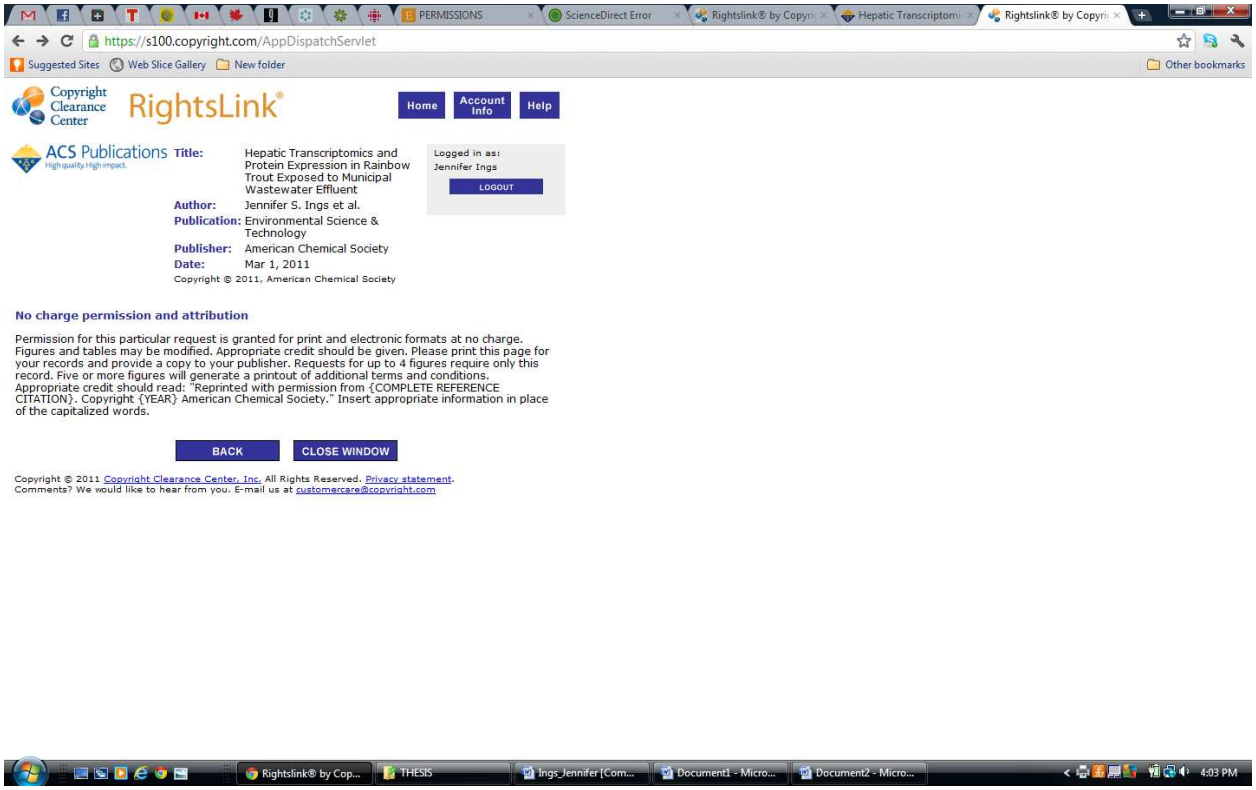

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Chapter 6

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Appendix A
Supporting Information for Chapter 2

Conductivity Measurement

Effluent concentration was estimated using conductivity as an indicator of the effluent plume. Conductivity (µS) was measured directly at the outfall pipe, considered 100% effluent. Conductivity was also measured upstream of the site, and considered to be background. Moving downstream, conductivity measurements were taken, the upstream background value was subtracted, and the net value was divided by the 100% effluent conductivity value minus the background and multiplied by 100 to give the approximate percentage. Cages were deployed in approximately 100% effluent, 50% effluent and 10% effluent. The following is a sample calculation:

[(1459 µS - 1110 µS) / (4080 µS - 1110 µS)] * 100 = 11.8% effluent

Microarray Sample Processing

cDNA synthesis and labeling. Thirty micrograms of intact total RNA (either treatment sample or reference sample RNA) were indirectly labeled with Cy5 and Cy3 following The Institute for Genomic Research (TIGR) protocol (http://www.tigr.org/tdb/microarray/protocolsTIGR.shtml) (Amersham Biosciences, Piscataway,NJ, USA). In brief, cDNA was synthesized using a commercial kit (Invitrogen, Carlsbad, CA, USA) by incorporating aminoallyl dUTP (AA-dUTP) at 42°C for 3 h. RNA was hydrolyzed with 10 µl of 1 M NaOH and 10 µl of 0.5 M EDTA for 15 min at 70°C and neutralized by adding 10 µl of 1 M HCl. To remove unincorporated AA-dUTP and free

amines, the reactions were purified with Qiagen columns using phosphate wash buffer and phosphate elution buffer. The purified sample was precipitated with Na-acetate, glycogen and 95% ethanol at -20^{0}C for 1h. Tubes were centrifuged at 13000 rpm for 30 min (4^{0}C) to collect the AA-cDNA pellet and placed in the fume hood for 15 min to dry. AA-cDNA was resuspended in 0.1 M sodium bicarbonate buffer. The AA-cDNA was coupled to either red fluorescent dye ester Cy5 or green fluorescent dye ester Cy3 (the dye chosen for the reference pool and sample were alternated for each array so that each dye was used on two samples per treatment). The coupling reaction was performed for 90 min in the dark at room temperature. The removal of uncoupled dye was performed using the Qiagen PCR purification kit.

Hybridization and Postprocessing. Before hybridization, the Cy3- and Cy5-labeled samples were combined and dried using Na-acetate, glycogen, and 95% ethanol precipitation, as described above. To the dried sample, 26 µl of hybridization buffer and 2 µl each of calf thymus DNA (10 mg/ml) and yeast tRNA (10 mg/ml) were added. The samples were denatured for 2 min at 95°C, snap cooled on ice for 1 min, and centrifuged for 1 min at maximum speed. The solution was pipetted onto a microarray slide underneath a silanized coverslip by capillary action. The slide was sealed into a hybridization chamber and hybridization was performed overnight at 37°C in a waterbath. After hybridization, the coverslip was removed by immersion in a low-stringency wash buffer (1% SSC, 0.2% SDS) at 48ºC. The slide was washed for 10 min in low-stringency wash buffer at 48ºC, 5 min in high stringency wash buffer (0.1% SSC, 0.2% SDS, and MilliQ water) at room temperature, and twice for 2.5 min in 0.1SSC at room temperature. Finally, the slides were quickly dipped

in MilliQ water and centrifuged at 1,000 g for 2 min in a 50-ml Falcon tube at room temperature for drying..

Image Analysis. Image analysis was carried out using VersArray ChipReader software version 3.1 (Bio-Rad). For each microarray slide, two images were produced by illuminating the array at 635 nm (excitation of Cy5) and 532 nm (Cy3). For both illuminations, photomultiplier tube (gain and light amplification) settings were at 900, and laser power was set at 90%. All images were captured in a TIFF format. Spot finding and quantification were carried out with ImaGene 3.0 microarray image analysis software (BioDiscovery, Los Angeles, CA). Briefly, a grid was created, taking into account the number of spots, the printing pattern, and the size of the spots. Spot finding was performed using a semiautomatic method in which each spot was checked for proper alignment within the grid manually. Finally, spot quantification was performed to obtain the expression level of each gene on the array.

Data Analysis. The data analysis was carried out using the microarray data analysis system (MIDAS). Poor or negative control spots were flagged as unreliable and were excluded from the analysis. For each spot, signal/noise (SN) threshold was calculated using the following formula: [I(A or B) Bkg(A or B)]/Bkg(A or B), where, respectively, I(A) and I(B) denote background-corrected signal intensity for channels A and B and Bkg(A) and Bkg(B) denote background intensity for channels A and B. The SN ratio for each channel was compared with the set threshold value of 2.0, and any spots 2.0 were marked as bad and excluded from downstream analysis. Data were then LOWESS normalized before calculation of the gene expression ratios. Data for each treatment (fold change relative to the RS) were

calculated as a ratio of normalized gene intensity for the sample to that of the reference RNA on each slide. Of the genes that were significantly different among the treatments, six were picked for gene quantification using quantitative real-time PCR (iCycler, Bio-Rad) to validate the reliability of gene expression patterns observed with the microarray.

Quantitative Real-time RT-PCR

cDNA synthesis. First-strand cDNA was synthesized from 1 µg total RNA from the pooled independent samples used in microarray analysis. This was carried out using the First-Strand cDNA Synthesis kit (MBI-Fermentas, Burlington, ON, CAN). Briefly, 1 µg of RNA was combined with 0.5 µg Oligo(dT) and heated for 5 min at 70ºC and then cooled on ice. A master mix containing 1 mM of each dNTP, 20 U of ribonuclease inhibitor and 40 U of M-MLV reverse transcriptase was added and the reaction was incubated for 1h at 37ºC, and then stopped by incubation for 10 min at 70ºC.

Quantification. Samples were quantified using a SYBR green fluorescent dye master mix in an iCycler real-time PCR detection system (Bio-Rad, Hercules, CA, USA) exactly as described before (18). Threshold cycle values for each sample were calculated using iCycler iQ real-time detection software (Bio-Rad). Briefly, each sample was assayed in triplicate. A master mix containing 3 µL cDNA, 3 µL of 10 mM primer pair, 40 µL of SYBR green mix and 34 µL of nuclease-free water was prepared for each sample, after which 25 µL was added to each of three wells. Each sample was assayed for the genes of interest as well as β-actin as an internal housekeeping standard. The following PCR conditions were used for amplification: 95ºC for 3 min; 40 cycles of 95ºC for 20 s and annealing temperature (See Supporting Information - Table 3) for 20 s; 95ºC for 1 min; 55ºC for 1 min followed by melt

curve analysis starting at 55ºC and increasing in 0.5ºC increments to 95ºC every 10 s. The melt curve predicted a single product for each primer pair. Relative abundance of transcripts for each gene was determined with the threshold cycles (C_T) using the relative standard curve, and were normalized to β-actin. The relative standard curve was made by serial dilution of pooled cDNA product to determine the efficiency of each primer pair. The sequences, amplification sizes, annealing temperatures and accession numbers for each primer pair is found in Supporting Information – Table 3. The efficiency of each primer pair was used in the analysis of gene expression. The relative abundance of β-actin was unchanged between treatment groups and, therefore, used as a housekeeping gene in this experiment.

SDS-PAGE and Western Blotting

The procedures for SDS-PAGE and western blotting followed established protocols (*31*) and the details are provided in the Supporting Information. Briefly, 40 µg of total protein were separated using an 8% polyacrylamide gel and along with a pre-stained molecular mass ladder (Bio-Rad Precision Plus prestained ladder (250 kDa, 150 kDa, 100 kKDa, 75 kDa, 50 kDa, 37 kDa, 25 kDa, 20 kDa, 15 kDa, 10 kDa)). The proteins were transferred onto a nitrocellulose membrane using a semi-dry transfer unit (Bio-Rad) at 20V for 25 min with a transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 10%(vol/vol) 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% (vol/vol) Tween 20) for 1 h followed by incubation in primary antibody for 1 h at room temperature. Membranes were then washed in TTBS (3 x 10 min) and incubated in secondary antibody (BioRad,

Hercules, CA, USA) coupled to horseradish peroxidase for 1 hour. Membranes were again washed in TTBS (3 x 10 min) and then washed a final time in TBS (20 mM Tris, pH 7.5, 300 mM NaCl) 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).

Table 1. Description of the City of Guelph wastewater treatment plant (City of Guelph Wastewater Treatment plant – Introduction to wastewater treatment: http://guelph.ca/uploads/ET_Group/wastewater/Introduction%20to%20Wastewater.pdf).

Parameter	Description
Population Served	115000
Capacity m^3/day	64000
Discharge m^3/day	54834
Secondary Treatment	Conventional and Extended Activated Sludge
Tertiary Treatment	Rotating Biological Contactors and Sand Filtration
Combined Sewers	No
Disinfectant	Sodium Hypochlorite
Dechlorination	Sodium Bisulphite
Current Upgrades	Various to reach effluent criteria for capacity of 73330 m^3/day

Table 2. Site characterization and water chemistry at each exposure site on the Speed River, Guelph, ON, CAN. Values represent the mean of 4 days, +/- SEM.

	Upstream	100%	50%	10%
Conductivity (µS)	1110	4080	2855	1459
Downstream (m)	-	10	20	40
pH	7.9 ± 0.06	7.8 ± 0.05	7.9 ± 0.07	7.9 ± 0.06
O2 (mg/L)	9.79 ± 0.56	9.44 ± 0.53	9.46 ± 0.53	9.79 ± 0.50
O2 saturation (%)	101.5 ± 7.12	103.5 ± 5.95	102.4 ± 5.45	104.3 ± 5.24
Temp (°C)	17.2 ± 1.03	19.8 ± 0.44	19.2 ± 0.54	18.5 ± 0.7

Table 3. Sequences, amplicon size, accession number and annealing temperature for primers used in quantitative real-time RT-PCR. Primers include thyroid hormone receptor β (TRβ), glucocorticoid receptor 1 and 2 (GR1, GR2), glucose transporter 2 (GLUT2), heat shock protein 90 (HSP90), insulin receptor C (IRC), insulin-like growth factor 1 and 2 (IGF1, IGF2) and β-actin (BACT).

Primer	Sequence	Amplicon size (bp)	Accession #	Annealing Temp (°C)
TRβ F	5'-TCACCTGTGAAGGATGCAAG-3'	152	AF146775	60
TRβ R	5'-GACAGCGATGCACTTCTTGA-3'			
GR1 F	5'-TTCCAAGTCCACCACATCAA-3'	115	Z54210	60
GR1 R	5'-GGAGAGCTCCATCTGAGTCG-3'			
GR2 F	5'-GGGGTGATCAAACAGGAGAA-3'	140	AY495372.1	60
GR2 R	5'-CTCACCCCACAGATGGAGAT-3'			
GLUT2 F	5'-GGCCATCTTCCTGTTTGTGT-3'	140	AF321816	60
GLUT2 R	5'-TGAAGTTGCTGGTCCAGTTG-3'			
HSP90 F	5'-TCCAGCAGCTGAAGGAGTTT-3'	135	AB196457.1/ AB196458.1	60
HSP90 R	5'-TGAGCTTGCAGAGGTTCTCA-3'			
IRC F	5'-GACCTGGCTGCTAGGAACTG-3'	149	AF062498	60
IRC R	5'-AGAGACTCAGGAGCCATCCA-3'			
IGF1 F	5'-TGGACACGCTGCAGTTTGTGTGT-3'	109	M95183.1	68
IGF1 R	5'-CACTCGTCCACAATACCACGGTT-3'			
IGF2 F	5'-CGGCAGAAACGCTATGTGGA-3'	91	M95184.1	58
IGF2 R	5'-TGCTGGTTGGCCTACTGAAA-3'			
BACT F	5'-TGTCCCTGTATGCCTCTGGT-3'	130	AF157514	60
BACT R	5'-AAGTCCAGACGGAGGATGG-3'			

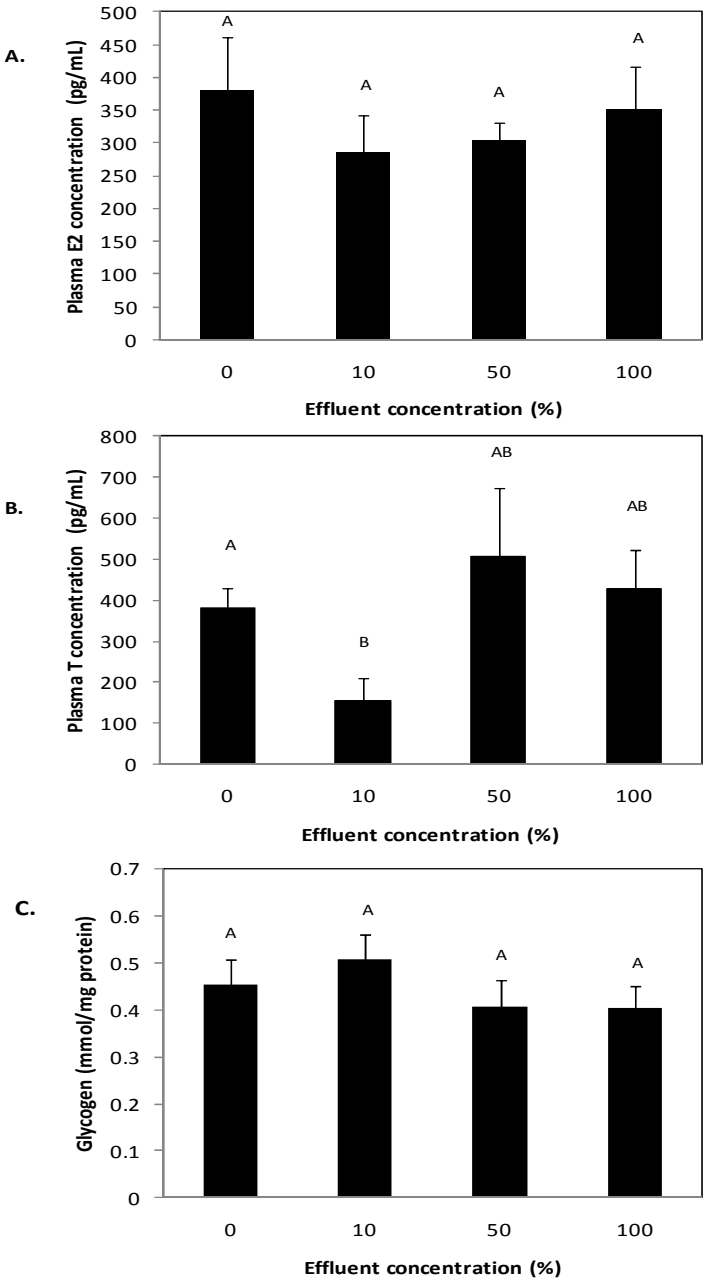

Figure 1. Effects of municipal wastewater effluent (MWWE) exposure on plasma 17β-estradiol (E2; n=9-16 fish; A), testosterone (T; n=9-16 fish; B) and liver glycogen content (n=8 fish; C) in rainbow trout. Bars represent mean +/- SEM. Different letters indicate significant differences ($P<0.05$, ANOVA).

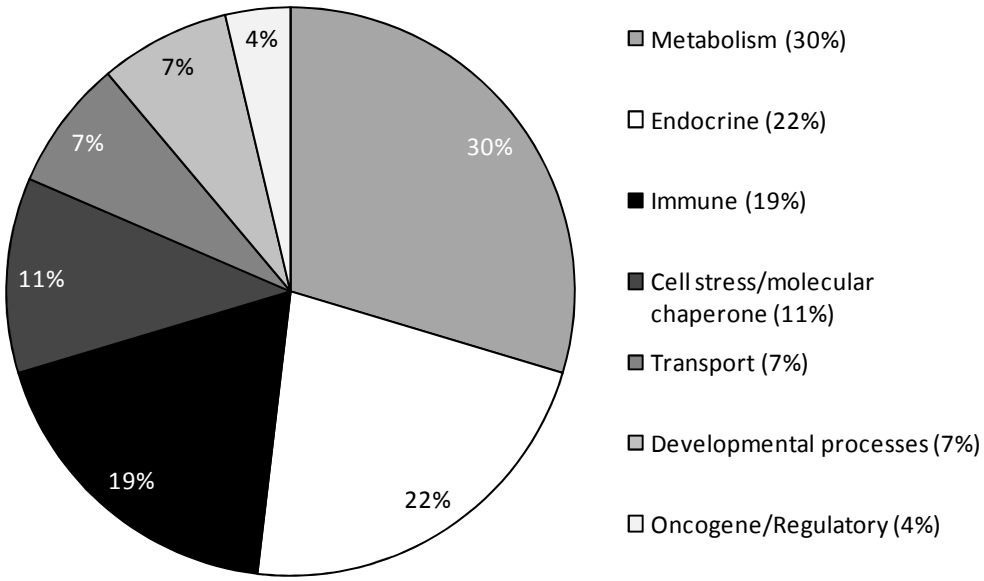

Figure 2. A pie chart showing the various functional categories that the significantly different genes fall into in response to municipal wastewater effluent (MWWE) exposure in rainbow trout.

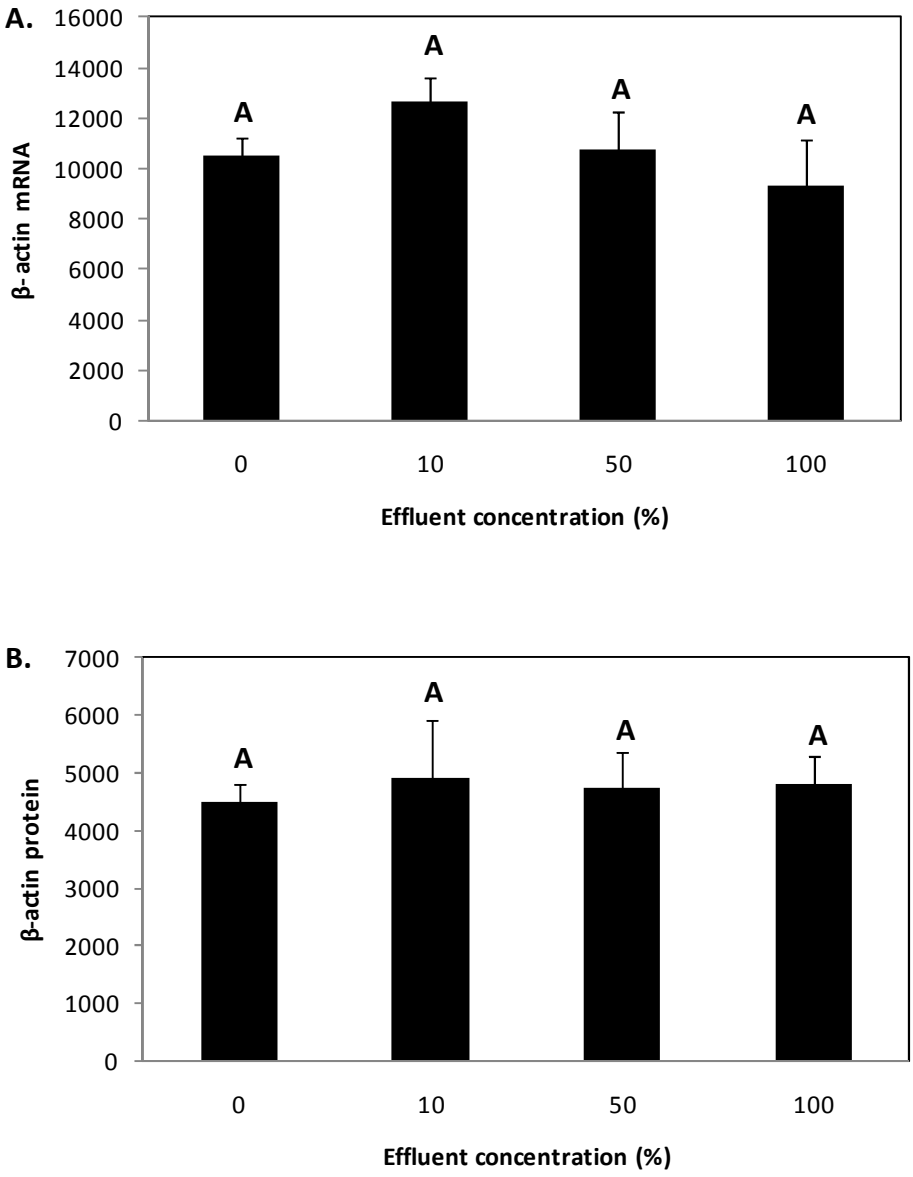

Figure 3. The effects of municipal wastewater effluent on rainbow trout hepatic β-actin transcript levels (A) and protein abundance (B). Bars represent mean +/- SEM. Different letters indicate significant differences ($P<0.05$, ANOVA).