Accepted Manuscript

Impacts of wastewater treatment plant effluent on energetics and stress response of rainbow darter (Etheostoma caeruleum) in the Grand River watershed

Hossein Mehdi, Fiona H. Dickson, Leslie M. Bragg, Mark R. Servos, Paul M. Craig

PII: S1096-4959(17)30184-7
DOI: doi:10.1016/j.cbpb.2017.11.011
Reference: CBB 10147

To appear in:

Received date: 28 August 2017
Revised date: 13 November 2017
Accepted date: 20 November 2017

Please cite this article as: Hossein Mehdi, Fiona H. Dickson, Leslie M. Bragg, Mark R. Servos, Paul M. Craig , Impacts of wastewater treatment plant effluent on energetics and stress response of rainbow darter (Etheostoma caeruleum) in the Grand River watershed. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Cbb(2017), doi:10.1016/j.cbpb.2017.11.011

Impacts of wastewater treatment plant effluent on energetics and stress response of rainbow darter (*Etheostoma caeruleum*) in the Grand River watershed

Hossein Mehdi*, Fiona H. Dickson, Leslie M. Bragg, Mark R. Servos, and Paul M. Craig

Department of Biology, University of Waterloo, N2L 3G1, Waterloo, ON, Canada

*Corresponding author:
Hossein Mehdi
Department of Biology, Faculty of Science, University of Waterloo
200 University Ave W
N2L 3G1 Waterloo, ON, Canada
Tel: 519 888 4567
h2mehdi@uwaterloo.ca

Abstract

The objective of this study was to assess the effects of municipal wastewater treatment plant effluent on the energetics and stress response of rainbow darter (*Etheostoma caeruleum*). Male and female rainbow darter were collected upstream and downstream of the Waterloo WWTP in the Grand River watershed, ON, Canada. To assess the effects of wastewater treatment plant effluent on whole-body and tissue specific metabolic capacity, closed-chamber respirometry and muscle-enzyme activity analyses were performed. Plasma cortisol was also collected from fish before and after an acute air-exposure stressor to evaluate the cortisol stress response in fish exposed to additional stressors. Male and female rainbow darter collected downstream of the effluent had higher oxygen consumption rates, while differences in enzyme activities were primarily associated with sex rather than collection site. No impairment in the cortisol stress response between downstream and upstream fish was observed, however baseline cortisol levels in female fish from the downstream site were significantly higher compared to other baseline groups. Stress-induced cortisol levels were also higher in female fish from both sites when compared to their male counterparts. Overall, this study demonstrates that chronic exposure to WWTP effluent impacts whole-body metabolic performance. This study was also able to demonstrate that sex-differences are a key determinant of various metabolic changes in response to physiological stress, thereby, providing a novel avenue to be considered and further explored.

Key words: rainbow darter, energetics, stress response, wastewater treatment plant effluent, metabolism.

1. Introduction

Wastewater treatment plant (WWTP) effluents are an emerging source of multiple environmental stressors of potential concern (Kaplan, 2013). WWTP effluents can cause significant perturbations in aquatic environments, including but not limited to: increase in eutrophication and decrease in dissolved oxygen due to nutrient influx, entrance of chemicals of emerging concern such as pharmaceuticals and personal care products (PPCPs), pesticides, and natural substances (Carey and Migliaccio, 2009; Guillette and Gunderson, 2001; Hotchkiss et al., 2008). Although significant technological improvements in wastewater treatment facilities have improved the quality of discharged effluent, many of these chemicals still persist in effluent and as a result, they are introduced into surface waters of effluent-receiving watersheds (Daughton and Ternes, 1999; Kolpin et al., 2002; Luo et al., 2014). While these chemicals are detected at low concentrations (ng/L to µg/L range) in receiving waters, their reported impact on non-target species has been significant (Boxall et al., 2012; Cleuvers, 2004, 2003; Galus et al., 2013; Rudd et al., 2014). These chemicals are now being referred to as chemicals or contaminants of emerging concern (CECs). This is not only because they are resilient to conventional wastewater treatment, but also because many are endocrine, neuroendocrine, and/or metabolic disruptors (Lange et al., 2009; Mennigen et al., 2011; Schulte et al., 2011; Sumpter, 2005).

PPCPs are designed to produce a therapeutic response through physiological changes in target species upon consumption (Overturf et al., 2015). Many of the processes and side effects that are caused in target species by PPCPs may be evident in non-target organisms across taxa, especially those with high homology to target species (Brown et al., 2014; Gunnarsson et al., 2008; Huggett et al., 2003). Over the last 10-15 years, there has been growing interest in studies investigating the risks posed by PPCPs on non-target species such as fish and other aquatic organisms (Overturf et al., 2015). Much of the research that has been conducted to date has investigated the effects of WWTP effluent and different classes of PPCPs on the reproductive fitness in fish through laboratory and field studies (Bahamonde et al., 2015; Chen et al., 2016; Fuzzen et al., 2015; Niemuth and Klaper, 2015; Tetreault et al., 2011). Comparatively, little research has been devoted to study the impacts of WWTP effluent on

other physiological aspects of non-target organisms. Investigating other endpoints, such as stress and metabolic physiology, can provide a more holistic and accurate view of the sublethal effects of contaminant exposure on fish and other aquatic organisms living in watersheds that are highly impacted by WWTP effluents.

1.1 Cortisol stress response

Most research assessing the effects of CECs on the endocrine system of fishes has been focused on the disruption of the reproductive endocrine axis (Mills and Chichester, 2005). Only recently has research considered the impacts of CECs on the cortisol stress axis, although most studies have investigated stress axis impairment from a single stressor or single contaminant exposure under laboratory settings. Little is known regarding the impacts of CECs on the cortisol stress response in fishes under natural environments, where fish are exposed to multiple stressors simultaneously (Vijayan et al., 2010). Fish living in sites heavily impacted by WWTP effluent are chronically exposed to a wide variety of chemicals that may have negative effects on the hypothalamus-pituitary-interrenal (HPI) axis which in turn can have negative effects on linked processes such as reproduction, growth, immune function, and metabolism (Filby et al., 2007; Ings et al., 2011b; Mommsen et al., 1999; Pickering and Pottinger, 1989; Pottinger et al., 2013; Vijayan et al., 2005, 2010.; Wendelaar Bonga, 1997). Cortisol is involved in the mobilization of fuels, regulation of metabolism, and overall maintenance of homeostasis in response to stress. Cortisol measurements are widely used as a marker of stress experienced by fish, allowing us to draw valuable conclusions regarding the effects of environmental factors on stress physiology (Barton and Iwama, 1991; Wendelaar Bonga, 1997). Chronic exposure to contaminants found in polluted environments can have deleterious effects on the endocrine system in fishes and impair their ability to mount a successful stress response (Pickering and Pottinger, 1987; Ytrestoyl et al., 2001). The aim of this study was to investigate how the stress response in rainbow darter (*Etheostoma caeruleum*) is affected by WWTP effluent exposure, which can provide understanding towards the sublethal impacts of contaminant exposure on the health and well-being of fishes.

1.2 Metabolic response

Contaminants associated with WWTPs and urbanization can negatively influence the metabolism of fish and other non-target organisms. Aquatic pollutants can cause metabolic dysfunction at many physiological levels, thereby influencing more ecologically-relevant processes such as behaviour, which are directly related to an animal's fitness (Alanärä et al., 1998; Biro and Stamps, 2010; Brodin et al., 2014; Scott and Sloman, 2004; Sloman et al., 2000). Exposure to contaminants can affect fish energy allocation for growth, reproduction, and other basal costs. Metabolic costs associated with detoxification processes result in energy allocation trade-offs, thereby impeding energy allocation to other basal processes (Handy et al., 1999; Scott and Sloman, 2004). Additionally, exposure to stressful environments, such as those affected by WWTP effluent discharge, can greatly influence the dynamics of metabolism in animals. Fish exposed to stressors undergo a variety of responses to maintain homeostasis. One of these responses is the mobilization and utilization of energy stores in the body. In our study, we used whole-body metabolic rate and muscle enzyme activities to assess the effects of WWTP effluent exposure on overall energy expenditure. Changes in enzyme activities can provide useful measures of organismal health and condition in response to environmental stressors (Goede and Barton, 1990). Enzyme activities provide a sensitive indicator as to how energy is being utilized and allocated in fish living in multi-stressed environments, such as those influenced by wastewater contamination. Previous studies have demonstrated changes in aerobic and anaerobic enzyme activities in fishes exposed to environmental contaminants. Such effects have been recorded across a variety of fish species and environmental contaminants. Whole-body routine metabolic rates and swimming performance have been previously used as indices of stress and metabolic dysfunction, especially in fish exposed to toxicants (Scott and Sloman, 2004). Metabolic costs associated with toxicant exposures can also constrain fish from properly responding to additional biotic and abiotic stressors, such as predation, hypoxia, and elevated temperature (Kelly et al., 2014; Mandic et al., 2009).

While previous research has successfully demonstrated the impacts of single contaminants and/or stressors on metabolic physiology of fish, few studies have been dedicated to the study of multiple stressors associated with WWTP effluents. WWTP effluents provide a unique system

of multiple stressors as they consist of a complex mixture of chemicals. As a result of this complexity, it is essential to understand how the metabolic physiology and other sublethal endpoints of fish can be affected (Boxall et al., 2012; Rudd et al., 2014).

1.3 Study scope

The objectives for this study were to assess the impacts multiple stressors associated with WWTP effluent on stress response and metabolic physiology of fish, using rainbow darter as a model organism. The rainbow darter was selected as a model species for this study due to its high abundance in the Grand River watershed, its sensitivity to anthropogenic disturbances, and its high site fidelity, making it a sentinel biomarker in understanding the impacts of various environmental influences in watersheds (Bahamonde et al., 2015; Diamond et al., 2016; Fuzzen et al., 2016; Hicks and Servos, 2017). The Grand River is the longest river that is found exclusively in southern Ontario, Canada. The watershed serves a population of approximately 1,000,000 people where they benefit from the services of 30 WWTPs that discharge their effluent in the river. The chemical profile of the discharged effluent from WWTPs and surface waters has been well studied and reported in the past (Arlos et al., 2015; Metcalfe et al., 2010). This study hypothesized that effluent-exposed fish will have elevated baseline cortisol levels. Further, exposed fish will have dampened cortisol levels in response to additional stressors due to chronic exposure to stress causing an impaired stress response. Additionally, we hypothesized that routine metabolic rate will be higher in fish exposed to the effluent. This will be accompanied by elevated enzyme activity in the muscle tissue, likely as a compensatory mechanism for the added metabolic costs of living in highly stressed environments.

It should be noted that this study was performed with an exploratory mindset, and as a result, there were a few limitations. Due to logistical experimental design, only one sampling day was performed for each experiment, thus not allowing us to replicate the study. It should also be noted that this study was performed in only two sites along the Grand River watershed, upstream and downstream of the Waterloo WWTP, serving as a contaminated and reference sites respectively. Therefore, fish were not collected along a gradient of WWTP effluent concentrations. However, it was our intention to only sample two sites during the same day, as

sampling during different time points can potentially have confounding effects on our study, as environmental conditions are highly dynamic.

2. Materials and methods

2.1 Fish collection

In April 2017, male and female rainbow dater were collected from two sites in the Grand River, southwestern Ontario, Canada. This coincided with their breeding season (Fuzzen et al. 2016). The sites were located upstream (WMR) and downstream (EIT) of the Waterloo Municipal WWTP, serving as the reference and contaminated sites respectively (Fig. 1). Fish were collected using a backpack electrofisher (Smith Root, LR-20) and held in aerated buckets. A subset of the collected fish was used for enzyme activity analyses (experiment 3). The length (±0.1 cm), total, gonadal, and hepatic weights (±0.001 g) were recorded prior to the collection of epaxial muscle tissue. Muscle samples were collected within ~3hr of fish collection and later snapped-frozen in liquid nitrogen and stored at -80°C until further analysis. A second subset of the collected fish was brought back to the laboratory for cortisol stress response analysis (experiment 1). These fish were housed in 10 L acrylic tanks at a density of 1-1.5 fish/L segregated based on sex and collection site. Water temperature was maintained at 12°C (comparable to river temperature) under a 12hr:12hr light-dark cycle. Fish acclimated to these conditions while fasting for a maximum of 48 hours before experiments began, ensuring minimal contaminant depuration occurred while still providing enough time for fish to recover from field-handling stress. In September 2016, male and female rainbow darter were collected from the same sites and brought back to the laboratory using the same methods as described above, however, the water temperature in the lab was maintained at 20°C to mimic the conditions in the river. Fish collected in September were used in respirometry experiments (experiment 2) to measure whole-body routine metabolic rates, as this time period was outside of the breeding seasons, and therefore, confounding effects of mating and reproduction were avoided.

Water quality parameters such as temperature, dissolved oxygen, pH, and conductivity were continuously recorded by the Grand River Conservation Authority in sites located upstream and downstream of the Waterloo WWTP and can be found in Table 1. All experimental and handling protocols followed the Canadian Council of Animal Care guidelines and were approved by the Animal Care Committee at the University of Waterloo (AUPP #15-03).

2.2 Exp. 1: Stress and cortisol analysis

Fish were randomly assigned into two groups: (i) baseline, where blood was collected without air exposure stress, or (ii) air-exposed group, where blood was collected following an air exposure stressor. There were 9 replicates for each group, sex, and collection site. Blood collection occurred between 08:00-12:00 to avoid diurnal effects on steroid variation. For baseline cortisol levels, fish were quickly caught with a hand net and immediately euthanized in an overdose of MS-222 (Sigma-Aldrich, Oakville, ON, Canada; 0.5 g/L). Total length (±0.1 cm) and weight (±0.001 g) were recorded, and blood was immediately collected as described below. For the air exposure test, fish were quickly caught in a hand net and left suspended in the air for 4 mins to elicit a stress response. Following the air exposure, fish were returned to their recovery tanks for 20 mins and then their blood was sampled following euthanasia and body measurements, similar to the baseline group. The air-exposure and recovery times were chosen in accordance with a similar study using round goby (*Neogobius melanostomus; Marentette et al., 2012*). In that study, round goby were subjected to a 4 min air-exposure stressor and plasma cortisol levels peaked between 10-30 mins post-stressor. Blood samples were collected using the retro-orbital technique described in (Vliegenthart et al., 2014). Briefly, following euthanasia, fish were dried gently, one eye was removed and 0.5 µL EDTA (2% stock) solution was pipetted into the open eye cavity to prevent blood from clotting. Blood was then collected using a 10 µL Eppendorf pipette and transferred into 0.5 mL microcentrifuge tubes. Following the first pipetting of pooled blood, gentle pressure was applied throughout the body of the fish from tail to head to accumulate more blood in the eye cavity for further collection, this technique was performed until no more blood would accumulate. Due to the applied pressure to the body of the fish, eggs or milt leaked out of the body and could not be collected, thus not allowing

proper measurements of gonadal weight for GSI calculations. This method of blood collection was appropriate due to the small body size of the rainbow darter. Even with using this method, some blood samples had to be pooled together to obtain enough volume for the cortisol EIA assay. Following blood collection, samples were centrifuged for 10 min at 8,000 g at 4°C. Plasma was then removed and stored at -80°C until further analysis. Plasma cortisol concentrations were determined using an enzyme-linked immunosorbent cortisol kit (StressMarq Biosciences, Inc., Cadboro Bay, Victoria, BC, Canada; Catalogue No: SKT-201). Each plasma sample was diluted 100x in provided assay buffer and run in duplicates following the explicit instructions of the manufacturer.

2.3 Exp. 2: Metabolic rate

Routine metabolic rate was measured in male and female rainbow darter collected from the upstream and downstream sites. 100-mL respirometry chambers were placed in bathing tanks where the water temperature was maintained at 20°C. Fish were individually placed in custom respirometry chambers and allowed to acclimate for 90 mins prior to measurements. During the acclimation period, a continuous supply of 100% oxygenated water was flowing through the chambers, ensuring oxygen depletion did not occur. Following acclimation, the chambers were closed off, and oxygen consumption was measured by the change of oxygen concentration over time using fiber-optic oxygen sensors (FireStingO_2, Pyro Science, Aachen, Germany). Oxygen consumption was measured over a period of 15 minute with oxygen concentration data being recorded every second using Pyro Oxygen Logger software (Pyro Science, Aachen, Germany). The 15-minute measurement period was chosen because it was previously observed in pilot studies that metabolic rate in rainbow darter remained fairly consistent after a ~90-minute acclimation period. This is possibly due to its benthic characteristics and low movement in the wild. Following oxygen consumption measurements, fish were weighed (±0.01 g) and returned to their housing tanks.

2.4 Exp. 3: Enzyme activities

Frozen muscle tissue collected was powdered in liquid nitrogen using a mortar and pestle and ~50 mg of tissue was homogenized in 20 volumes of extraction buffer (20 mM Hepes, 1 mM EDTA, and 0.1% Triton X-100, pH 7.0) using an electric homogenizer (Omni tissue homogenizer, NW Kennesaw, GA, USA). Sample homogenates were then centrifuged (12,000 g, 10 min, 4°C), and supernatants were used for enzyme assays. Enzyme activities were assayed in 96-well microplates using a Molecular Devices Spectramax 190 spectrophotometer at room temperature at 340 nm unless stated otherwise. Pyruvate kinase (PK; E.C. 2.7.1.40), lactate dehydrogenase (LDH; E.C. 1.1.1.27), and 3-hydroxyacyl CoA dehydrogenase (HOAD; E.C. 1.1.1.35) were assayed on fresh homogenates, then samples were frozen at -80°C prior to the assays of citrate synthase (CS; E.C. 2.3.3.1), cytochrome c oxidase (COX; E.C. 1.9.3.1), and catalase (CAT; E.C. 1.11.1.6). The following are the reaction buffers used for each specific assay with all values in mM, unless otherwise indicated. *PK*: 5 ADP, 0.15 NADH, 10 fructose 1,6 bisphosphate, 100 KCl, 10 $MgCl_2$, 5 units/mL lactate dehydrogenase, and 50 imidazole buffer (pH 7.4). *LDH*: 0.15 NADH, 20 pyruvate, and 50 imidazole buffer (pH 7.4). *HOAD*: 0.15 NADH, 10 acetoacetyl CoA, and 50 imidazole buffer (pH 7.4). *CS*: 0.3 acetyl CoA, 0.1 DNTB, 0.5 oxaloacetate, and 50 Tris-HCl buffer (pH 8.0), assay was measured at 412 nm. *COX*: 0.05 cytochrome c from bovine heart reduced using sodium hydrosulfite, and 50 potassium phosphate buffer (pH 7.4), assay was measured at 550 nm. *CAT*: 20 H_2O_2 and 50 potassium phosphate buffer (pH 7.4), assay was measured at 240 nm. Enzyme activity was normalized to the protein concentration of individual homogenates, which was determined using bicinchoninic acid (BCA) assay with bovine serum being used as a standard. Chemicals used in assays were purchased from Sigma-Aldrich.

2.5 Statistical analyses

Data was analyzed using SigmaPlot 13.0 software (Systat Software Inc., San Jose, CA, USA). Data is presented as means ± 1 SEM. A two-way ANOVA and Tukey's post-hoc test were performed to determine significant differences between upstream and downstream sites and between males and females for the cortisol, metabolic rate, and enzyme data. Student's t-test was used

to compare morphological measurements (total length, total mass, HSI, GSI, and condition factor) between sites. The alpha value was set at 0.05 (i.e., $P<0.05$) for all tests.

3. Results

3.1 Morphological measurements

Rainbow darter used in *Exp. 1* demonstrated significant morphological differences between sites (Table 2). Males collected from the upstream site were significantly longer, heavier, and in better body condition, as indicated by their condition factor, K, than males collected from the downstream site. Further, rainbow darter used in *Exp. 3* displayed differences in morphological measurements. Males and females collected from the downstream site had larger gonadal investment, as indicated by their GSI, than fish from the upstream site. Whereas only males collected from the downstream site had higher hepatic investment, as indicated by their HSI, compared to males from the upstream site. No differences were observed in any of the remaining fish, regardless of sex or site.

3.2 Exp. 1: Cortisol stress response

Baseline plasma cortisol concentrations are presented in Fig. 2A. Female rainbow darter collected from the downstream site had a more than 2x higher baseline cortisol levels compared to males from the same site, and males and females from the upstream site. Following an air-exposure stressor, all fish from both sexes and sites were able to mount a stress response. Stress-induced cortisol levels were always significantly higher than baseline levels (Fig. 2A, B). Analysis revealed that female stress-induced cortisol levels were similar between upstream and downstream sites, however, they were significantly higher than their male counterparts from both sites.

3.3 Exp. 2: Metabolic rate

Differences in routine metabolic rate (RMR) were determined in male and female rainbow darter using closed-chamber respirometry (Fig. 3). There was a significant, ~2x increase in RMR

in both male and female rainbow darter from the downstream site compared to fish collected upstream. No sex-linked differences were found between sexes within sites.

3.4 Exp. 3: Enzyme activities

Activities of six muscle metabolic enzymes were measured and data is presented in Table 3. PK activity was 1.3x higher in males from the upstream site compared to females from the same site, and 1.2x higher than males from the downstream site. HOAD activity was ~1.2x higher in males from the downstream site than any other group. LDH activity was only significantly different between males from the upstream site and females from the downstream site (1.1x). CS activity was ~1.2x higher in males than females within each site. There were no significant differences in COX activity between any of the groups. CAT activity was 1.4x higher in males than females from the downstream site.

4. Discussion

Rainbow darter are a sentinel model organism in the Grand River watershed. Our current and ongoing knowledge about their morphology, behaviour, reproduction, physiology, and population dynamics provide us with strong tools in understanding the effects of anthropogenic disturbances on fish health and well-being. The aim of this study was to assess the effects of multiple stressors associated with WWTP effluent on various stress and metabolic physiology endpoints in the rainbow darter under natural field conditions in the Grand River watershed.

4.1 Morphological measurements

There were some significant variations in morphological indices between sites in fish collected in April, 2017. Males from the downstream site were shorter, had reduced body mass, and in poorer body condition than males from the upstream site. Also, GSI was higher in both males and females in the downstream site, indicating higher investment in reproductive tissue. The relative GSI increase in fish collected from the downstream site was against our expectations, as numerous studies have previously reported a reduction in GSI in response to WWTP effluent

exposure (Hecker et al., 2002; Vajda et al., 2008; Tetreault et al., 2011). However, WWTP effluent is composed of a complex mixture of chemicals that varies spatially and temporally, and as a result, its effect on fishes varies as well (Alvarez et al., 2009). HSI was higher in males from the downstream site, which is consistent with previous studies that indicated higher energy storage in darters (as indicated by a higher HSI) collected from downstream sites compared to reference sites (Tetreault et al., 2011). These results suggest that fish are assimilating the additional resources towards energy storage and reproductive output, however, we are unable to conclude if these additional resources are attributed to WWTP effluent. Only a subset of males collected in April, 2017 displayed poorer body condition than males collected from the upstream site. Since this was not seen in any of the other groups, we believe this could be reflective of fishing conditions and sampling of that day. It has been reported that lower body condition is attributed to exposure to higher concentrations of estrogenic compounds or the physiological state of a fish found in poorer environmental conditions has influenced this gross health parameter (Solé et al., 2002). Further research is required to investigate the distribution of different fish sizes along the riffles in the Grand River. This will lead to a wider size-distribution of collected fish, thereby, improving the representation of heterogeneity of different fish sizes.

4.2 Exp. 1: Stress response

Male and female rainbow darter from both the upstream and downstream sites were able to successfully mount a stress response after a 4-min air-exposure stressor, as indicated by the difference between stress-induced and baseline cortisol levels (Fig. 2). However, females found downstream of WWTP had higher baseline cortisol levels than all other baseline groups. The cortisol stress response is a key adaptive mechanism that allows fish to cope with disturbances in their environment and maintain or regain homeostasis (Wingfield, 2013). It has been suggested that chronic exposure to WWTP effluent can impair this adaptive mechanism and attenuate the magnitude of the stress response (Ings et al., 2012; Ings et al., 2011a; Pottinger et al., 2016). Studies examining rainbow trout (*Oncoryhnchus mykiss*) exposed to WWTP effluent demonstrated diminished cortisol levels following an acute stressor compared to unexposed

fish (Ings et al., 2012). Similar studies have indicated modulation of the corticosteroidgenic stress mechanism in response to WWTP effluent exposure in three-spined sticklebacks (*Gasterosteus aculeatus;* Pottinger et al., 2016, 2013; Pottinger and Matthiessen, 2016). Contaminants associated with WWTP effluent are known to increase ethoxyresorfin-O-deethylase (EROD) activity. This in turn dampens the cortisol stress response via the production of cytochrome P450 enzymes (Aluru and Vijayan, 2006).

Our results indicated that the cortisol response was not significantly impaired, which differed from previous studies that have used different fish species and stress-initiating techniques (Ings et al., 2012, 2011a; Pottinger et al., 2016), although, females found downstream of the WWTP had higher baseline cortisol. This could possibly indicate that female rainbow darter are more responsive to WWTP effluent contamination and are under chronic stress, especially during their spawning period (Wendelaar Bonga, 1997). Further, female plasma glucocorticoids could be transferred in ovo as a form of communication with the offspring (Ghio et al., 2016). Therefore, elevated baseline cortisol levels in females found in poor environments may be a mechanism by which stressed females alter the phenotype of their offspring through maternal stress hormones (Rhees and Fleming, 1981; Sloman, 2010). In a similar study, round goby living in highly contaminated areas did not show stress response impairment (Marentette et al., 2012). This suggests that rainbow dater and round goby may be more resilient in regards to their stress response than other fishes. It could also provide a possible explanation as to why similar results to studies using rainbow trout or sticklebacks were not observed. Further, rainbow darter have high site fidelity, meaning that populations found in contaminated sites are likely to have been there their entire lives, and previous generations before (Hicks and Servos, 2017). Therefore, it is possible that over many generations, rainbow dater have developed tolerance to these contaminants allowing them to demonstrate an unperturbed stress response (Weis, 2002; Wendelaar Bonga, 1997). Further studies could shed more light on the area by examining variations in the stress response across different species exposed to similar conditions.

The majority of these results suggest that sex was a stronger factor in explaining differences in the cortisol stress response. Females from both sites had higher stress-induced cortisol levels

than their male counterparts. This was expected, as mammals display sex-related differences in response to stress (Aloisi et al., 1994; Heuser et al., 1994.; Spinedi et al., 1994). Males often have a reduced response to stress due to elevated androgen levels causing suppression of the HPA axis. This in turn reduces the impact of stress on reproductive success (Handa et al., 1994). Females, on the other hand, may have evolved enhanced stress responsiveness as a mechanism to forego reproduction when exposed to unfavourable environmental conditions (Viau and Meaney, 1991). Fish respond in a very similar way. Male rainbow trout and brook trout (*Salvelinus fontinalis*) in the presence of elevated gonadal steroids associated with sexual maturation, demonstrate a reduced stress response to acute and chronic stressors (Pottinger et al., 1996, 1995). Moreover, female three-spined sticklebacks display greater stress-induced cortisol levels than their male counterparts (Pottinger and Matthiessen, 2016). These traits are expected to manifest during fish spawning periods, when gonadal steroids are present at higher levels, which coincided with the sampling period of this study (Pottinger et al., 1996).

4.3 Exp. 2: Metabolic rate

Male and female rainbow darter from the downstream site had higher routine metabolic rates than fish from the upstream site, which confirms the initial hypothesis of this study. The increase in metabolic rate can be attributed to the increased metabolic costs associated with higher contaminant exposure from WWTP effluent. The specific mechanisms by which toxicants affect metabolic rate remain unclear. However, exposure to environmental stressors often increases catabolic processes. As a result of higher energy utilization, metabolic and ventilation rates are often elevated to supply oxygen for further metabolic processes (Scott and Sloman, 2004). Previous research has demonstrated an increase in oxidative stress, and liver and muscle glycogen depletion in response to WWTP effluent exposure in *Prochilodus lineatus* and rainbow trout (Carney Almroth et al., 2008; Cazenave et al., 2013). Further research using empire gudgeons (*Hypseleotris compressa*) exposed to different concentrations of WWTP effluent found that fish exposed to higher concentrations had significantly reduced whole-body lipid content (Melvin, 2015). These findings suggest that due to metabolic trade-offs caused by

contaminants exposure, energy reserves would be reduced and oxidative damage would be more apparent (Melvin, 2015; Smolders et al., 2003).

4.4 Exp. 3: Enzyme activities

When fish are exposed to a wide variety of contaminants, such as those found in WWTP effluents, it is important to understand how various metabolic pathways are being affected in response to such stressors. Enzyme activities can be used as indicators of glycolytic rates (PK and LDH), lipid metabolism (HOAD), mitochondrial abundance (CS), the electron transport chain (COX), and oxidative stress (CAT) (McClelland et al., 2006).

Carbohydrates are the first energy reserves used in cases of stress, and are often accompanied by an increase in glycolytic enzyme activities and a reduction in both muscular and hepatic glycogen (Hori et al., 2006). However, the results obtained in this study did not completely support this hypothesis, as PK activity was higher only in female rainbow darter from the downstream site, indicating that this could be a sex-specific response. This suggests that female rainbow darter are more sensitive to WWTP toxicants, causing fish to be energy deficient, as more substrate is being depleted and converted into energy. LDH on the other hand, an enzyme involved in anaerobic glycolysis had lower activity in fish collected downstream of the effluent. Similar results have been observed in fish exposed to pesticides (Mishra and Shukra, 1997) and fertilizers (Yadav et al., 2007). LDH plays an important role in the regulation of glycolysis through its action of catalyzing the reversible reduction of pyruvate into lactate (Yadav et al., 2007). The decrease of LDH activity could potentially have deleterious metabolic outcomes. Its decrease in activity could indicate a reduction in its catalytic efficiency in fish located downstream of WWTP effluents.

HOAD on the other hand, an enzyme involved in β-oxidation of lipids was only higher in males from the downstream site than all other groups of fish. This increase is indicative of a higher lipolytic activity in males (Londraville and Duvall, 2002; Rajotte and Couture, 2002). Higher HOAD activity in males from the downstream site suggests a compensatory mechanism for fish to rely on lipid stores to compensate for the metabolic costs associated with living I

contaminated sites (Goertzen et al., 2012). Further research is required to understand why this mechanism was not seen in female rainbow darter from the downstream site.

CS activity was also measured, as it is a key enzyme in the citric acid cycle and is often used as an indicator of aerobic capacity and mitochondrial abundance in muscle tissue (Lemos et al., 2003; Rajotte and Couture, 2002). While there were no differences in CS activity between sites, female rainbow darter had significantly lower CS activity than males within the same sites. Lower CS activity in females suggests lower ability to produce ATP through aerobic substrate metabolism. Similar results have been observed in spottail shiner (*Notropis hudsonius*) collected downstream of a uranium mill effluent. Shiners in the contaminated site had significantly higher HOAD activity, however, CS activity was the same in both sites (Goertzen et al., 2012). However, these results were observed in sexually immature fish, whereas the fish used in our study were all sexually mature and collected during their breeding season.

COX activity was also measured, also known as complex IV in the electron transport chain. It is responsible for the reduction of oxygen to water and is often used as a measure of overall aerobic capacity in the muscle tissue (Berg et al., 2002). No differences were found in COX activity, indicating that neither site or sex differences have a significant effect on the electron transport chain oxidation capacity.

Finally, CAT activity was measured, which demonstrated that males from the downstream site had significantly higher activity than females from the same site. CAT is an enzyme that is involved in the antioxidant defence mechanism, which protects tissues from oxidative stress by reducing hydrogen peroxide to water and oxygen. CAT activity usually changes in response to cellular damage, environmental stress, or disease (Atli et al., 2006; Kessabi et al., 2013). Our results suggest that in the presence of stressors associated with WWTP effluents, male rainbow darter are more resilient to oxidative stress. The elevation of CAT activity minimizes the oxidative damage caused by reactive oxygen species (ROS) (Kong et al., 2012). Alternatively, males could be producing more ROS, therefore CAT activity would be elevated as a protective response. The increase in CAT activity might be in response to the increase in oxygen consumption (Fig. 3), which typically induces increased ROS production (Ritola et al., 2002). Oxygen consumption was higher in both sexes from the downstream site, however, in the

downstream site, only males had higher CAT activity. This sexual dimorphism in the response to oxidative stress in rainbow darter could be linked to the lower aerobic capacity measured via CS in females compared to males. However, oxygen consumption and production of ROS are not always positively correlated. A study measuring *in vivo* H_2O_2 in brown trout (*Salmo trutta*) reported that individuals with higher standard metabolic rates had lower levels of H_2O_2 (Salin et al., 2015). This suggests that the link between oxygen consumption and ROS production is far from being resolved. Another possible explanation for the sexual dimorphism observed is possibly due to the increased reproductive investment that females often encounter during breeding season. This can result in female rainbow darter experiencing a state of energy deficit, thus leaving them more vulnerable to oxidative damage (Treberg et al., 2016). More research would be required to understand the link between oxygen consumption and ROS production in rainbow darter in response to WWTP effluent exposure.

4.5 Conclusions

To conclude, rainbow darter collected upstream and downstream of the Waterloo WWTP had significant differences in their somatic indices, cortisol stress response, and whole-body and tissue-specific metabolism. Sites impacted by WWTP effluent provide a unique environment for aquatic organisms as they are exposed to a wide variety of stressors simultaneously. WWTP effluents are an emerging source of contaminants of concern, and only recently has research focused its lens on its impacts on aquatic ecosystems. The use of stress and metabolic physiology as sublethal endpoints can further our understanding on how fish and other aquatic organisms can cope with such environmental stressors. This study highlights the energetic costs that animals encounter when exposed WWTP effluents, and how they can be mediated by the stress axis. The use of energetics and stress physiology as biomarkers provides better insight on broader ecological concerns, as these endpoints are often linked to higher biological processes, such as survival and reproduction, thereby impacting overall fitness (Groh et al., 2015; Sárria et al., 2011).

Acknowledgments. H Mehdi was responsible for designing and conducting the experiments as well as writing the manuscript. FH Dickson provided help with respirometry measurements. LM Bragg provided help in field collection and organization as well as providing many of the necessary equipment for this study. PM Craig and MR Servos provided extensive experimental design and manuscript editorial input. Funding was provided through Natural Sciences and Engineering Council of Canada (NSERC) as well as the Canadian Foundation for Innovation (CFI).

References

Alanärä, A., Winberg, S., Brännäs, E., Kiessling, A., Höglund, E., Elofsson, U., 1998. Feeding behaviour, brain serotonergic activity levels, and energy reserves of Arctic char (*Salvelinus alpinus*) within a dominance hierarchy. Can. J. Zool. 76, 212–220.

Aloisi, A.M., Steenbergen, H.L., van de Poll, N.E., Farabollini, F., 1994. Sex-dependent effects of restraint on nociception and pituitary-adrenal hormones in the rat. Physiol. Behav. 55, 789–93.

Aluru, N., Vijayan, M.M., 2006. Aryl hydrocarbon receptor activation impairs cortisol response to stress in rainbow trout by disrupting the rate-limiting Steps in steroidogenesis. Endocrinology 147, 1895–1903.

Alvarez, D.A., Cranor, W.L., Perkins, S.D., Schroeder, V.L., Iwanowicz, L.R., Clark, R.C., Guy, C.P., Pinkey, A.E., Blazer, V.S., Mullican, J.E., 2009. Reproductive health of bass in the Potomac, USA, Drainage: Part 2: Seasonal occurrence of persistent and emerging organic contaminants. Environ. Toxicol. Chem, 28, 1084-1095.

Arlos, M.J., Bragg, L.M., Parker, W.J., Servos, M.R., 2015. Distribution of selected antiandrogens and pharmaceuticals in a highly impacted watershed. Water Res. 72, 40–50.

Atli, G., Alptekin, Ö., Tükel, S., Canli, M., 2006. Response of catalase activity to Ag+, Cd2+, Cr6+, Cu2+ and Zn2+ in five tissues of freshwater fish *Oreochromis niloticus*. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 143, 218–224.

Bahamonde, P.A., McMaster, M.E., Servos, M.R., Martyniuk, C.J., Munkittrick, K.R., 2015. Molecular pathways associated with the intersex condition in rainbow darter (*Etheostoma caeruleum*) following exposures to municipal wastewater in the Grand River basin, ON, Canada. Part B. Aquat. Toxicol. 159, 302–316.

Barton, B.A., Iwama, G.K., 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Annu. Rev. Fish Dis. 1, 3–26.

Berg, J.M., Tymoczko, J.L., Stryer, L., 2002. Biochemistry. 5th ed. W.H. Freeman, New York.

Biro, P.A., Stamps, J.A., 2010. Do consistent individual differences in metabolic rate promote consistent individual differences in behavior? Trends Ecol. Evol. 25, 653–659.

Boxall, A.B.A., Rudd, M.A., Brooks, B.W., Caldwell, D.J., Choi, K., Hickmann, S., Innes, E., Ostapyk, K., Staveley, J.P., Verslycke, T., Ankley, G.T., Beazley, K.F., Belanger, S.E., Berninger, J.P., Carriquiriborde, P., Coors, A., DeLeo, P.C., Dyer, S.D., Ericson, J.F., Gagné, F., Giesy, J.P., Gouin, T., Hallstrom, L., Karlsson, M. V., Larsson, D.G.J., Lazorchak, J.M., Mastrocco, F., McLaughlin, A., McMaster, M.E., Meyerhoff, R.D., Moore, R., Parrott, J.L., Snape, J.R., Murray-Smith, R., Servos, M.R., Sibley, P.K., Straub, J.O., Szabo, N.D., Topp, E., Tetreault, G.R., Trudeau, V.L., Van Der Kraak, G., 2012. Pharmaceuticals and Personal Care Products in the Environment: What Are the Big Questions? Environ. Health Perspect. 120, 1221–1229.

Brodin, T., Piovano, S., Fick, J., Klaminder, J., Heynen, M., Jonsson, M., 2014. Ecological effects of pharmaceuticals in aquatic systems--impacts through behavioural alterations. Philos. Trans. R. Soc. B Biol. Sci. 369, 20130580.

Brown, A.R., Gunnarsson, L., Kristiansson, E., Tyler, C.R., 2014. Assessing variation in the potential susceptibility of fish to pharmaceuticals, considering evolutionary differences in their physiology and ecology. Philos. Trans. R. Soc. London B Biol. Sci. 369.

Carey, R.O., Migliaccio, K.W., 2009. Contribution of wastewater treatment plant effluents to nutrient dynamics in aquatic Systems: a review. Environ. Manage. 44, 205–217.

Carney Almroth, B., Albertsson, E., Sturve, J., Förlin, L., 2008. Oxidative stress, evident in antioxidant defences and damage products, in rainbow trout caged outside a sewage treatment plant. Ecotoxicol. Environ. Saf. 70, 370–378.

Cazenave, J., Bacchetta, C., Rossi, A., Ale, A., Campana, M., Parma, M.J., 2013. Deleterious effects of wastewater on the health status of fish: A field caging study. Ecol. Indic. 38, 104–112.

Chen, T.H., Chou, S.M., Tang, C.H., Chen, C.Y., Meng, P.J., Ko, F.C., Cheng, J.O., 2016. Endocrine disrupting effects of domestic wastewater on reproduction, sexual behavior, and gene expression in the brackish medaka *Oryzias melastigma*. Chemosphere 150, 566–575.

Cleuvers, M., 2004. Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid. Ecotoxicol. Environ. Saf. 59, 309–315.

Cleuvers, M., 2003. Aquatic ecotoxicity of pharmaceuticals including the assessment of

combination effects. Toxicol. Lett. 142, 185–194.

Daughton, C.G., Ternes, T. a., 1999. Pharmaceuticals and personal care products in the environment: Agents of subtle change? Environ. Health Perspect. 107, 907–938.

Diamond, S.R., Sultana, T., Servos, M.R., Metcalfe, C.D., 2016. Biological responses to contaminants in darters (*Etheostoma spp*.) collected from rural and urban regions of the Grand River , ON , Canada. Comp. Biochem. Physiol. Part B 199, 126–135.

Filby, A.L., Neuparth, T., Thorpe, K.L., Owen, R., Galloway, T.S., Tyler, C.R., 2007. Health impacts of estrogens in the environment, considering complex mixture effects. Environ. Health Perspect. 115, 1704–1710.

Fuzzen, M.L.M., Bennett, C.J., Tetreault, G.R., McMaster, M.E., Servos, M.R., 2015. Severe intersex is predictive of poor fertilization success in populations of rainbow darter (*Etheostoma caeruleum*). Aquat. Toxicol. 160, 106–116.

Fuzzen, M.L.M., Bragg, L.M., Tetreault, G.R., Bahamonde, P.A., Tanna, R.N., Bennett, C.J., McMaster, M.E., Servos, M.R., 2016. An assessment of the spatial and temporal variability of biological responses to municipal wastewater effluent in rainbow darter (*Etheostoma caeruleum*) collected along an urban gradient. PLoS One 11, e0164879.

Galus, M., Jeyaranjaan, J., Smith, E., Li, H., Metcalfe, C., Wilson, J.Y., 2013. Chronic effects of exposure to a pharmaceutical mixture and municipal wastewater in zebrafish. Aquat. Toxicol. 132–133, 212–222.

Ghio, S.C., Leblanc, A.B., Audet, C., Aubin-Horth, N., 2016. Effects of maternal stress and cortisol exposure at the egg stage on learning, boldness and neophobia in brook trout. Behaviour, 153, 1639-1663.

Goede, R.W., and Barton, B.A., 1990. Organismic indices and an autopsy-based assessment as indicators of health and condition of fish. Am. Fish. Soc. Symp, 8, 93-108.

Goertzen, M.M., Hauck, D.W., Phibbs, J., Weber, L.P., Janz, D.M., 2012. Swim performance and energy homeostasis in spottail shiner (*Notropis hudsonius*) collected downstream of a uranium mill. Ecotoxicol. Environ. Saf. 75, 142–150.

Groh, K.J., Carvalho, R.N., Chipman, J.K., Denslow, N.D., Halder, M., Murphy, C.A., Roelofs, D., Rolaki, A., Schirmer, K., Watanabe, K.H., 2015. Development and application of the adverse

outcome pathway framework for understanding and predicting chronic toxicity: II. A focus on growth impairment in fish. Chemosphere 120, 778–792.

Guillette, L.J., Gunderson, M.P., 2001. Alterations in development of reproductive and endocrine systems of wildlife populations exposed to endocrine-disrupting contaminants. Reproduction 122, 857–64.

Gunnarsson, L., Jauhiainen, A., Kristiansson, E., Nerman, O., Larsson, D.G.J., 2008. Evolutionary conservation of human drug targets in organisms used for environmental risk assessments. Environ. Sci. Technol. 42, 5807–5813.

Handa, R.J., Nunley, K.M., Lorens, S.A., Louie, J.P., McGivern, R.F., Bollnow, M.R., 1994. Androgen regulation of adrenocorticotropin and corticosterone secretion in the male rat following novelty and foot shock stressors. Physiol. Behav. 55, 117–24.

Handy, R.., Sims, D.., Giles, A., Campbell, H.., Musonda, M.., 1999. Metabolic trade-off between locomotion and detoxification for maintenance of blood chemistry and growth parameters by rainbow trout (*Oncorhynchus mykiss*) during chronic dietary exposure to copper. Aquat. Toxicol. 47, 23–41.

Hecker, M., Tyler, C.R., Hoffmann, M., Maddix, S., Karbe, L., 2002. Plasma biomarkers in fish provide evidence for endocrine modulation in the Elbe River, Germany. Environ. Sci. Technol 36, 2311-2321.

Heuser, I.J., Gotthardt, U., Schweiger, U., Schmider, J., Lammers, C.H., Dettling, M., Holsboer, F., 1994. Age-associated changes of pituitary-adrenocortical hormone regulation in humans: importance of gender. Neurobiol. Aging 15, 227–31.

Hicks, K.A., Servos, M.R., 2017. Site fidelity and movement of a small-bodied fish species, the rainbow darter (*Etheostoma caeruleum*): Implications for environmental effects assessment. River Res. Appl. 1–10.

Hori, T.S.F., Avilez, I.M., Inoue, L.K., Moraes, G., 2006. Metabolical changes induced by chronic phenol exposure in matrinxã *Brycon cephalus* (teleostei: Characidae) juveniles. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 143, 67–72.

Hotchkiss, A.K., Rider, C. V, Blystone, C.R., Wilson, V.S., Hartig, P.C., Ankley, G.T., Foster, P.M., Gray, C.L., Gray, L.E., 2008. Fifteen years after ‘‘wingspread’’—environmental endocrine

disrupters and human and wildlife health: where we are today and where we need. Toxicol. Sci. 105, 235–59.

Huggett, D.B., Cook, J.C., Ericson, J.F., Williams, R.T., 2003. A theoretical model for utilizing mammalian pharmacology and safety data to prioritize potential impacts of human pharmaceuticals to fish. Hum. Ecol. Risk Assess. An Int. J. 9, 1789–1799.

Ings, J.S., Oakes, K.D., Vijayan, M.M., Servos, M.R., 2012. Temporal changes in stress and tissue-specific metabolic responses to municipal wastewater effluent exposure in rainbow trout. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 156, 67–74.

Ings, J.S., Servos, M.R., Vijayan, M.M., 2011a. Exposure to municipal wastewater effluent impacts stress performance in rainbow trout. Environ. Sci. Technol. 45, 2368–2376.

Ings, J.S., Vijayan, M.M., Servos, M.R., 2011b. Tissue-specific metabolic changes in response to an acute handling disturbance in juvenile rainbow trout exposed to municipal wastewater effluent. Aquat. Toxicol. 108, 53–59.

Kaplin, S., 2013. Review: pharmacological pollution water. Crit. Rev. Environ. Sci. Technol. 43, 1-44.

Kelly, N.I., Azaid, A., Nash, G.W., Gamperl, A.K., 2014. Metabolic depression in cunner (Tautogolabrus adspersus) is influenced by ontogeny, and enhances thermal tolerance. PloS One, 9, e114765.

Kessabi, K., Said, K., Messaoudi, I., 2013. Comparitive study of longevity, growth, and biomarkers of metal detoxification and oxidative stress between normal and deformed *Aphanius fasciatus* (Pisces, Cyprinodontidae). J. Toxicol. Environ. Health, Part A, 76, 1269-1281.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999−2000: a national reconnaissance. Environ. Sci. Technol. 36, 1202–1211.

Kong, X., Wang, S., Jiang, H., Nie, G., Li, X., 2012. Responses of acid/alkaline phosphatase, lysozyme, and catalase activities and lipid peroxidation to mercury exposure during the embryonic development of goldfish *Carassius auratus*. Aquat. Toxicol. 120–121, 119–125.

Lange, A., Paull, G.C., Coe, T.S., Katsu, Y., Urushitani, H., Iguchi, T., Tyler, C.R., 2009. Sexual

reprogramming and estrogenic sensitization in wild fish exposed to ethinylestradiol. Environ. Sci. Technol. 43, 1219–1225.

Lemos, D., Salomon, M., Gomes, V., Phan, V.N., Buchholz, F., 2003. Citrate synthase and pyruvate kinase activities during early life stages of the shrimp *Farfantepenaeus paulensis* (Crustacea, Decapoda, Penaeida*e*): effects of development and temperature. Comp. Biochem. Physiol. Part C Biochem. Mol. Biol. 135, 707–719.

Londraville, R.L., Duvall, C.S., 2002. Murine leptin injections increase intracellular fatty acid-binding protein in green sunfish (*Lepomis cyanellus*). Gen. Comp. Endocrinol. 129, 56–62.

Luo, Y., Guo, W., Hao, H., Duc, L., Ibney, F., Zhang, J., Liang, S., Wang, X.C., 2014. A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. Sci. Total Environ. 473–474, 619–641.

Mandic, M., Todgham, A.E., Richards, J.G., 2009. Mechanisms and evolution of hypoxia tolerance in fish. Proc. R. Soc. London B Biol. Sci. 276.

Marentette, J.R., Tong, S., Balshine, S., 2012. The cortisol stress response in male round goby (*Neogobius melanostomus*): Effects of living in polluted environments? Environ. Biol. Fishes 96, 723–733.

Marjan, P., Martyniuk, C., Fuzzen, M.L.M., Maclatchy, D.L., McMaster, M.E., Servos, M.R., 2017. Returning to normal? Assessing transcriptome recovery over time in male rainbow darter (*Etheostoma caeruleum*) liver in response to wastewater-treatment plant upgrades. Environ. Toxicol. Chem. 36, 2108-2122.

McClelland, G.B., Craig, P.M., Dhekney, K., Dipardo, S., 2006. Temperature- and exercise-induced gene expression and metabolic enzyme changes in skeletal muscle of adult zebrafish (*Danio rerio*). J. Physiol. 577, 739–751.

Melvin, S.D., 2015. Short-term exposure to municipal wastewater influences energy, growth, and swimming performance in juvenile Empire Gudgeons (*Hypseleotris compressa*) 170, 271–278.

Mennigen, J. a, Stroud, P., Zamora, J.M., Moon, T.W., Trudeau, V.L., 2011. Pharmaceuticals as neuroendocrine disruptors: lessons learned from fish on prozac. J. Toxicol. Environ. Health. B. Crit. Rev. 14, 387–412.

Metcalfe, C.D., Chu, S., Judt, C., Li, H., Oakes, K.D., Servos, M.R., Andrews, D.M., 2010. Antidepressants and their metabolites in municipal wastewater, and downstream exposure in an urban watershed. Environ. Toxicol. Chem. 29, 79–89.

Mills, L.J., Chichester, C., 2005. Review of evidence: Are endocrine disrupting chemicals in the aquatic environment impacting fish populations? Sci. Total Environ, 343, 1-34.

Mishra, R., Shukla, S.P., 1997. Impact of endosulfan on lactate dehydrogenase from the freshwater catfish *Clarias batrachus*. Pest. Biochem. Phsyiol, 57, 220-234.

Mommsen, T.P., Vijayan, M.M., Moon, T.W., 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Rev. Fish Biol. Fish. 9, 211–268.

Niemuth, N.J., Klaper, R.D., 2015. Emerging wastewater contaminant metformin causes intersex and reduced fecundity in fish. Chemosphere 135, 38–45.

Overturf, M.D., Anderson, J.C., Pandelides, Z., Beyger, L., Holdway, D.A., 2015. Pharmaceuticals and personal care products: A critical review of the impacts on fish reproduction. Crit. Rev. Toxicol. 45, 469–491.

Pickering, A.D., Pottinger, T.G., 1989. Stress responses and disease resistance in salmonid fish: Effects of chronic elevation of plasma cortisol. Fish Physiol. Biochem. 7, 253–258.

Pickering, A.D., Pottinger, T.G., 1987. Poor water quality suppresses the cortisol response of salmonid fish to handling and confinement. J. Fish Biol. 30, 363–374.

Pottinger, T.G., Balm, P., Pickering, A., 1995. Sexual maturity modifies the responsiveness of the pituitary-interrenal axis to stress in male rainbow trout. Gen. Comp. Endocrinol, 98, 311-320.

Pottinger, T.G., Carrick, T.R., Hughes, S.E., Balm, P.H., 1996. Testosterone, 11-ketotestosterone, and estradiol-17 beta modify baseline and stress-induced interrenal and corticotropic activity in trout. Gen. Comp. Endocrinol. 104, 284–295.

Pottinger, T.G., Henrys, P.A., Williams, R.J., Matthiessen, P., 2013. The stress response of three-spined sticklebacks is modified in proportion to effluent exposure downstream of wastewater treatment works. Aquat. Toxicol. 126, 382–392.

Pottinger, T.G., Matthiessen, P., 2016. Long-term water quality data explain interpopulation variation in responsiveness to stress in sticklebacks at both wastewater effluent-

contaminated and uncontaminated sites. Environ. Toxicol. Chem. 35, 3014–3022.

Pottinger, T.G., Williams, R.J., Matthiessen, P., 2016. A comparison of two methods for the assessment of stress axis activity in wild fish in relation to wastewater effluent exposure. Gen. Comp. Endocrinol. 230–231, 29–37.

Rajotte, J.W., Couture, P., 2002. Effects of environmental metal contamination on the condition, swimming performance, and tissue metabolic capacities of wild yellow perch (*Perca flavescens*). Can. J. Fish. Aquat. Sci. 59, 1296–1304.

Rhees, R.W., Fleming, D.E., 1981. Effects of malnutrition, maternal stress, or ACTH injections during pregnancy on sexual behavior of male offspring. Physiol. Behav. 27, 879–882.

Ritola, O., Livingstone, D.., Peters, L.., Lindström-Seppä, P., 2002. Antioxidant processes are affected in juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to ozone and oxygen-supersaturated water. Aquaculture 210, 1–19.

Rudd, M.A., Ankley, G.T., Boxall, A.B., Brooks, B.W., 2014. International scientists' priorities for research on pharmaceutical and personal care products in the environment. Integr. Environ. Assess. Manag. 10, 576–587.

Salin, K., Auer, S.K., Rudolf, A.M., Anderson, G.J., Cairns, A.G., Mullen, W., Hartley, R.C., Selman, C., Metcalfe, N.B., 2015. Individuals with higher metabolic rates have lower reactive oxygen species *in vivo*. Biol. Lett, 11, 20150538.

Sárria, M.P., Soares, J., Vieira, M.N., Filipe C. Castro, L., Santos, M.M., Monteiro, N.M., 2011. Rapid-behaviour responses as a reliable indicator of estrogenic chemical toxicity in zebrafish juveniles. Chemosphere 85, 1543–1547.

Schulte, P.M., Healy, T.M., Fangue, N.A., 2011. Thermal performance curves, phenotypic plasticity, and the time scales of temperature exposure. Integr. Comp. Biol. 51, 691–702.

Scott, G.R., Sloman, K.A., 2004. The effects of environmental pollutants on complex fish behaviour: Integrating behavioural and physiological indicators of toxicity. Aquat. Toxicol. 68, 369–392.

Sloman, K.A., 2010. Exposure of ova to cortisol pre-fertilisation affects subsequent behaviour and physiology of brown trout. Horm. Behav. 58, 433–439.

Sloman, K.A., Gilmour, K.M., Taylor, A.C., Metcalfe, N.B., 2000. Physiological effects of

dominance hierarchies within groups of brown trout, *Salmo trutta*, held under simulated natural conditions. Fish Physiol. Biochem. 22, 11–20.

Smolders, R., De Boeck, G., Blust, R., 2003. Changes in cellular energy budget as a measure of whole effluent toxicity in zebrafish (*Danio rerio*). Environ. Toxicol. Chem. 22, 890–899.

Solé, M., Barceló, D., Porte, C., 2002. Seasonal variation of plasmatic and hepatic vitellogenin and EROD activity in carp, *Cyprinus carpio*, in relation to sewage treatment plants. Aquat. Toxicol. 60, 233–248.

Spinedi, E., Salas, M., Chisari, A., Perone, M., Carino, M., Gaillard, R.C., 1994. Sex differences in the hypothalamo-pituitary-adrenal axis response to inflammatory and neuroendocrine stressors. Evidence for a pituitary defect in the autoimmune disease-susceptible female Lewis rat. Neuroendocrinology 60, 609–17.

Sumpter, J.P., 2005. Endocrine disrupters in the aquatic environment: an overview. Acta Hydrochim. Hydrobiol. 33, 9–16.

Tetreault, G.R., Bennett, C.J., Shires, K., Knight, B., Servos, M.R., McMaster, M.E., 2011. Intersex and reproductive impairment of wild fish exposed to multiple municipal wastewater discharges. Aquat. Toxicol. 104, 278–290.

Treberg, J.R., Killen, S.S., MacCormack, T.J., Lamarre, S.G., Enders, E.C., 2016. Estimates of metabolic rate and major constituents of metabolic demand in fishes under field conditions: methods, proxies, and new perspectives. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 202, 10–22.

Vajda, A.M., Barber, L.B., Gray, J.L., Lopez, E.M., Woodling, J.D., Norris, D.O., 2008. Reproductive disruption in fish downstream from an estrogenic wastewater effluent. Environ. Sci. Technol 42, 3407-3414.

Viau, V., Meaney, M.J., 1991. Variations in the hypothalamic-pituitary-adrenal response to stress during the estrous cycle in the rat. Endocrinology 129, 2503–2511.

Vijayan, M.M., Aluru, N., Leatherland, J.F., 2010. Stress response and role of cortisol. In Fish Diseases and Disorders, Volume 2: non-infectious disorders, 2nd edition (eds Leatherland, J.F., Woo, P.), pp.182-201. Wallinford, UK: CABI.

Vijayan, M.M., Prunet, P., Boone, A.N., 2005. Xenobiotic impact on corticosteroid signaling. In:

Mommsen, T.P., Moon, T.W. (Eds.), Biochemistry and Molecular Biology of Fishes, vol 6. Elsevier, Amsterdam, pp. 87-101.

Vliegenthart, A.D.B., Starkey Lewis, P., Tucker, C.S., Del Pozo, J., Rider, S., Antoine, D.J., Dubost, V., Westphal, M., Moulin, P., Bailey, M. a, Moggs, J.G., Goldring, C.E., Park, B.K., Dear, J.W., 2014. Retro-orbital blood acquisition facilitates circulating microRNA measurement in zebrafish with paracetamol hepatotoxicity. Zebrafish 11, 219–26.

Weis, J., 2002. Tolerance to environmental contaminants in the mummichog, *Fundulus heteroclitus*. Hum. Ecol. Risk Assess. An Int. J. 8, 933–953.

Wendelaar Bonga, S.E., 1997. The stress response in fish. Physiol. Rev. 77, 591.

Wingfield, J.C., 2013. The comparative biology of environmental stress: behavioural endocrinology and variation in ability to cope with novel, changing environments. Anim. Behav. 85, 1127–1133.

Yadav, A., Gopesh, A., Pandey, R.S., 2007. Fertilizer industry effluent induced biochemical changes in fresh water teleost, *Channa striatus* (*Bloch*). Bull. Environ. Contam. Toxicol, 79, 588-595.

Ytrestoyl, T., Finstad, B., McKinley, R.S., 2001. Swimming performance and blood chemistry in Atlantic salmon spawners exposed to acid river water with elevated aluminium concentrations. J. Fish Biol. 58, 1025–1038.

Legends

Figure 1. Map of the central portion of the Grand River watershed illustrating the location of the collection sites and Waterloo Wastewater Treatment Plant. The site GPS coordinates were: WMR (upstream): 43°35'08"N (latitude) 80°28'53"W (longitude); EIT (downstream): 43°28'24"N (latitude) 80°28'22"W (longitude), and Waterloo MWWTP: 43°29'16"N 80°30'25"W (map modified from Marjan et al., 2017).

Figure 2. Baseline (A) and stress-induced (B) plasma cortisol levels separated by sex and site. Data are represented as mean ± S.E.M of n=9 replicates per site per sex. Bars that do not share letters indicate significant difference using a two-way ANOVA followed by Tukey's post-hoc test. WMR males had *4/9* and *0/9* samples pooled for baseline cortisol and stress-induced cortisol blood collection, respectively. EIT males had *2/9* and *5/9* samples pooled for baseline cortisol and stress-induced cortisol blood collection, respectively. WMR females had *1/9* samples pooled for both baseline cortisol and stress-induced cortisol blood collection. EIT females had *2/9* samples pooled for both baseline cortisol and stress-induced cortisol blood collection. *(A) effect of site $F_{1,32} = 4.399$, $P = 0.044$; effect of sex $F_{1,32} = 5.327$, $P = 0.028$; interaction of site and sex $F_{1,32} = 5.148$, $P = 0.030$. (B) effect of site $F_{1,32} = 0.393$, $P = 0.535$; effect of sex $F_{1,32} = 5.804$, $P = 0.022$; interaction of site and sex $F_{1,32} = 0.664$, $P = 0.421$.*

Figure 3. Routine metabolic rate (RMR) measured in rainbow darter collected upstream and downstream of the effluent. Data are represented as mean ± S.E.M of n=5-6 fish per site per sex. Bars that do not share letters indicate significant difference using a two-way ANOVA followed by Tukey's post-hoc test. *Effect of site $F_{1,19} = 5.870$, $P = 0.026$; effect of sex $F_{1,19} = 0.052$, $P = 0.822$; interaction of site and sex $F_{1,19} = 0.089$ $P = 0.779$.*

Table 1. Water quality paramaters for the Grand River from both upstream and downstream sites of the Waterloo WWTP during September 2016 and April 2017. Vales are represented as the mean monthly value and were obtained from the Grand River Conservation Authority.

Table 2. Morphological measurements of rainbow darter collected upstream and downstream of the Waterloo Wastewater Treatment Plant in the Grand River. Values are presented as mean ± S.E.M, statistical differences in measurements between sites were determined using a student's t-test. Site differences in total length, total mass, HSI (*hepatosomatic index = [liver mass/body mass] x 100*), GSI (*gonadosomatic index = [gonad mass/body mass] x 100*), and K (*condition factor = [body mass/length^3] x 100)* are presented in the table and separated based on sexes and what experiment the fish were used for. One male collected from the downstream site showed gross intersex, therefore its morphological measurements were omitted from statistical analysis. **= p<0.05; **= p<0.01; ***= p<0.001.*

Table 3. Muscle protein content and enzyme activity of pyruvate kinase (PK), 3-hydroxyacyl CoA dehydrogenase (HOAD), lactate dehydrogenase (LDH), citrate synthase (CS), cytochrome c oxidase (COX), and catalase (CAT) measured in rainbow darter collected from upstream and downstream of the effluent. Muscle protein content measured in mg and enzyme activity measured in μmol/min/mg$_{protein}$. Data are presented as mean ± SEM of n=10-12 fish per site per sex. Means that do not share letters indicate significant difference using a two-way ANOVA followed by Tukey's post-hoc test (P<0.05). Significant effects are bolded.

Figure 1

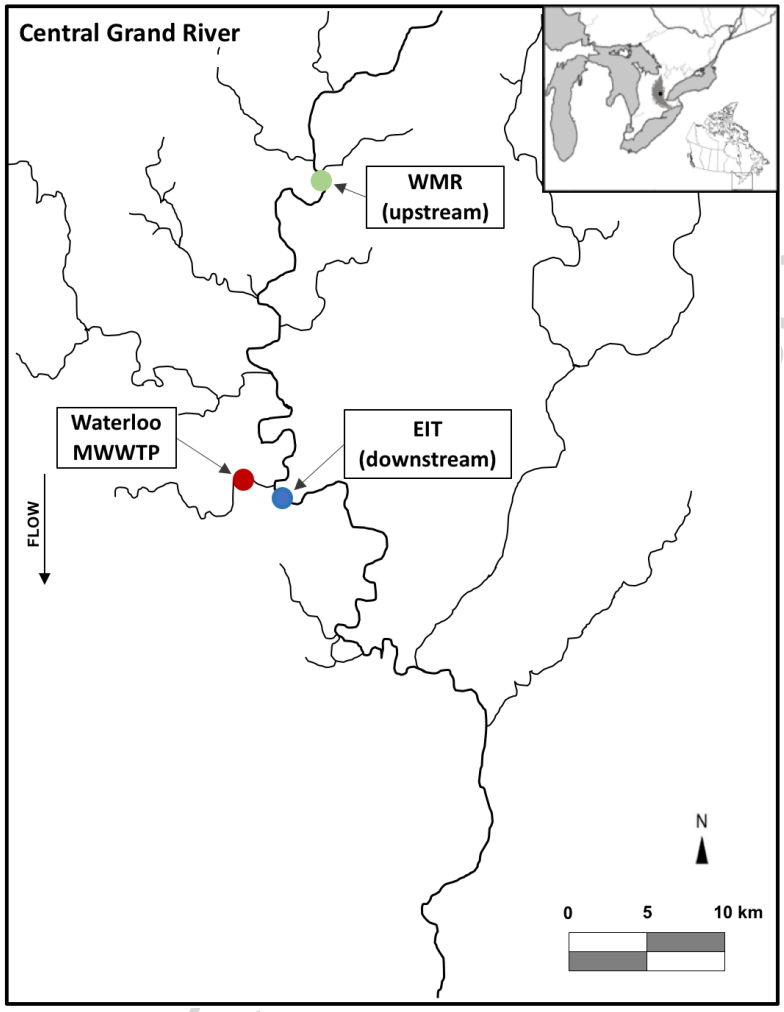

Figure 2

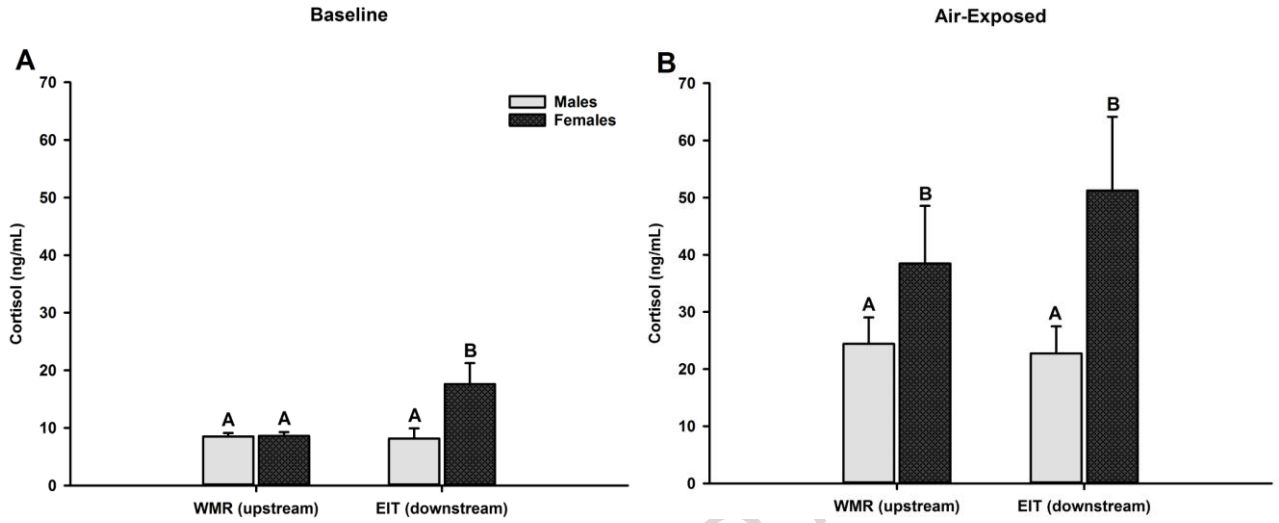

Figure 3

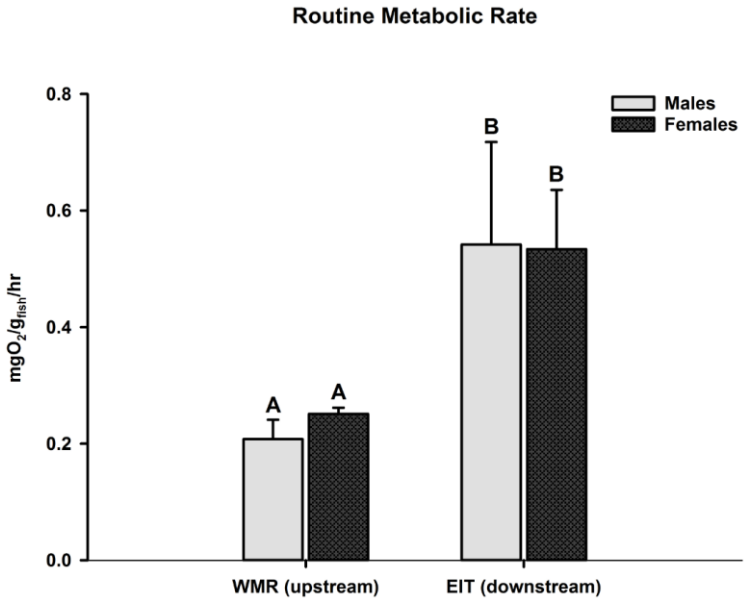

Table 1

Date	Collection Site	Water Temperature (°C)	Dissolved Oxygen (mg/L)	pH	Conductivity (µS/cm)
Sep-16	Upstream	20.7	9.9	8.6	415.5
	Downstream	19.7	9.9	8.4	772.2
Apr-17	Upstream	9.9	9.9	8.5	529.9
	Downstream	8.4	11.5	8.5	680.6

Table 2

Exp. 1: Cortisol analysis (collected April, 2017)

	Males		*Females*	
Measurement	**Upstream (WMR) n=22**	**Downstream (EIT) n=26**	**Upstream (WMR) n=22**	**Downstream (EIT) n=21**
Total length (cm)	5.5±0.164	4.4±0.120***	5.2±0.144	5.2±0.178
Total mass (g)	2.119±0.191	0.927±0.145***	1.803±0.144	1.890±0.226
K	1.17±0.041	1.01±0.028**	1.22±0.022	1.23±0.030

Exp. 2: Metabolic rate (collected September, 2016)

	Males		*Females*	
Measurement	**Upstream (WMR) n=12**	**Downstream (EIT) n=11**	**Upstream (WMR) n=12**	**Downstream (EIT) n=12**
Total mass (g)	2.11±0.235	2.93±0.719	1.61±0.072	2.15±0.301

Exp. 3: Enzyme analysis (collected April, 2017)

	Males		*Females*	
Measurement	**Upstream (WMR) n=12**	**Downstream (EIT) n=11**	**Upstream (WMR) n=12**	**Downstream (EIT) n=12**
Total length (cm)	5.4±0.118	5.0±0.201	5.1±0.091	5.3±0.200
Total mass (g)	2.032±0.174	1.621±0.264	1.846±0.104	2.192±0.267
HSI	1.80±0.108	2.11±0.087*	5.53±0.483	6.35±0.170
GSI	1.59±0.091	1.86±0.063*	14±0.971	17.08±0.951*
K	1.24±0.030	1.17±0.026	1.38±0.022	1.42±0.041

Table 3

Measurement	*Males*		*Females*				
	Upstream (WMR)	**Downstream (EIT)**	**Upstream (WMR)**	**Downstream (EIT)**	**Interaction Site x Sex**	**Factor: site**	**Factor: Sex**
PK	11.86±0.49^{A}	9.90±0.51^{BC}	8.68±0.45^{B}	10.74±0.54^{AC}	***P < 0.001*** ***$F_{1,44}$ = 16.27***	*P = 0.915* *$F_{1,44}$ =0.011*	***P = 0.024*** ***$F_{1,44}$ = 5.495***
HOAD	1.13±0.07^{A}	1.43±0.05^{C}	1.17±0.06^{A}	1.16±0.06^{A}	***P = 0.020*** ***$F_{1,44}$ = 5.865***	***P = 0.027*** ***$F_{1,44}$ = 5.227***	*P = 0.075* *$F_{1,44}$ = 3.328*
LDH	62.18±1.68^{A}	55.02±2.05^{AB}	60.23±1.96^{AB}	54.40±2.52^{B}	*P = 0.735* *$F_{1,42}$ = 0.100*	***P = 0.003*** ***$F_{1,42}$ = 9.607***	*P = 0.543* *$F_{1,42}$ = 0.376*
CS	0.206±0.008^{AB}	0.221±0.008^{A}	0.158±0.008^{C}	0.186±0.010^{BC}	*P = 0.471* *$F_{1,43}$ = 0.530*	***P = 0.017*** ***$F_{1,43}$ = 6.168***	***P < 0.001*** ***$F_{1,43}$ = 21.98***
COX	720.15±25.96	816.97±46.54	675.29±41.32	705.68±80.06	*P = 0.533* *$F_{1,42}$ = 0.395*	*P = 0.235* *$F_{1,42}$ = 1.449*	*P = 0.147* *$F_{1,42}$ = 2.183*
CAT	195.47±10.02^{AB}	212.04±6.07^{A}	181.50±5.80^{AB}	154.81±10.91^{B}	***P = 0.015*** ***$F_{1,44}$ = 6.453***	*P = 0.555* *$F_{1,44}$ = 0.353*	***P < 0.001*** ***$F_{1,44}$ = 17.48***
Total Protein	1.84±0.15	2.66±0.31	2.06±0.41	2.83±0.41	*P = 0.931* *$F_{1,44}$ =0.008*	***P = 0.023*** ***$F_{1,44}$ = 5.569***	*P = 0.562* *$F_{1,44}$ =0.341*

Formatted: Line spacing: 1.5 lines