Multi-stressor impacts on fish energetics and stress response: a comparison between lab and field studies

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

Aquatic organisms are continuously exposed to multiple environmental stressors that work cumulatively and synergistically to alter ecosystems. The objective of this study was to investigate the impacts of multiple stressors under lab and field conditions using two fish species, zebrafish (*Danio rerio*) and rainbow darter (*Etheostoma caeruleum*). Under lab conditions, the study examined the impacts of chronic exposure to environmentally relevant concentrations of venlafaxine and elevated water temperatures on the energetics and stress response of zebrafish. Venlafaxine is a frequently prescribed antidepressant that is readily detectable in many Canadian waterways that receive discharged wastewater treatment plant effluent. Under field conditions, rainbow darter were collected upstream and downstream of the Waterloo wastewater treatment plant in the Grand River, Ontario, Canada. Enzyme activities of major metabolic and mitochondrial enzymes were measured in muscle tissue of both species. Oxygen consumption was measured to assess the effects of stressors on routine metabolic rate, active metabolic rate, aerobic scope, and critical swimming speed, all of which are significant indicators of fishes performance in ecological frameworks. Finally, the impact of chronic exposure to multiple stressors on the cortisol stress response following confinement and air-exposures stressors was investigated in zebrafish and rainbow darter respectively.

In the lab study, it was found that fish exposed to multiple stressors had higher oxygen consumption rates, resulting in diminished aerobic scope. No impairment in the cortisol stress response was observed in response to either stressors, singularly or cumulatively. Exposure to multiple stressors also had an impact on the enzyme activities in the muscle tissue of zebrafish. Fish exposed to multiple stressors had higher pyruvate kinase activity, reduced 3-hydroxyacyl

CoA dehydrogenase temperature sensitivity, and lower catalase activity in response to the higher exposure temperature.

In the field study, it was found that male and female rainbow darter collected downstream of the Waterloo wastewater treatment plant had higher oxygen consumption rates. No impairment in the cortisol stress response between the 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. Elevated cortisol levels were also higher in female fish from both sites when compared to their male counterparts. Differences in enzyme activities measured in the muscle tissue of rainbow darter were detected, however, differences were mostly associated with sex rather than collection site.

The results suggest that multi-stressor research can yield unexpected data that might not have been predicted using conventional single-stressor approach. Aligning field and lab results will highlight the importance of including multi-stressor approach assessments in making predictions regarding the impact of environmental stressors on non-target organisms.

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Chapter 1

General introduction

1.1 Introduction

The need for better understanding of the effects of multiple stressors is rapidly becoming one of the most important and challenging areas of research in applied biology (Zeidberg and Robison, 2007). The challenge of studying multiple stressors stems from the complex nature of how different stressors interact in various ways with each other and the environment, resulting in "ecological surprises". The effects that multiple stressors exhibit on organisms can be additive or non-additive, i.e., synergistic or antagonistic (Folt et al., 1999; U.S. Environmental Protection Agency, 2003). Therefore, in order to understand the complex effects of multiple environmental, multi-stressor approach assessments are required, as single stressor research does not fall into the category of cumulative risk assessments. However, single stressor research is also useful, especially in identifying potential areas of concern or likely interactions of multiple stressors (U.S. Environmental Protection Agency, 2003). The aim of this thesis is to assess the effects of multiple stressors that aquatic organisms continuously face in their environments. This thesis uses lab and field assessments to further understand the interactions between environmental stressors. The results of this thesis will contribute to the recognized importance of including multi-stressor approach assessments to make predictions regarding the impact of stressors on fish health and abundance.

Stress is most commonly defined amongst fish biologists as "a state of threatened homeostasis that is reestablished by a complex suite of adaptive responses" (Chrousos, 1998). Fish found in disturbed ecosystems face a wide variety of stressors; some natural and other anthropogenically-induced. Examples of common stressors that fish and other aquatic organisms face are: predation, competition, chemical pollution, and changes in abiotic factors such as

temperature, pH, and dissolved oxygen levels. The physiological responses invoked by these environmental stressors are generally grouped into three categories: primary, secondary, and tertiary responses (Fig. 1.1). Primary responses involve alterations in neuroendocrine activity, resulting in the stimulation of the hypothalamus-pituitary-interrenal (HPI) axis (Reid et al., 1998). Secondary responses involve changes in plasma and tissue metabolite and hematological levels, resulting in broader physiological responses, such as changes in cellular activity, metabolism, and immune function (Iwama et al., 1997, 1998; Mommsen et al., 1999; Pickering, 1981). Finally, tertiary responses are involved in whole-organism performance, such as growth, reproduction, condition, and metabolic scope (Wedemeyer et al., 1990). Physiological responses that fish carry out in response to stressors are considered adaptive, allowing fish to regain or maintain homeostasis (Barton, 2002). However, if fishes are chronically exposed to stressors, then these responses themselves can become maladaptive and impact the health and well-being of fishes (Barton and Iwama, 1991; Selye, 1976). This thesis aims to examine the impacts of wastewater treatment plant (WWTP) effluent exposure on the physiological responses of fish under field conditions. It also examines the individual and cumulative effects of chronic exposure to a pharmaceutical that is readily detectable in WWTP effluents and surface waters, venlafaxine (VFX), in combination to elevated water temperature on fish under controlled lab conditions.

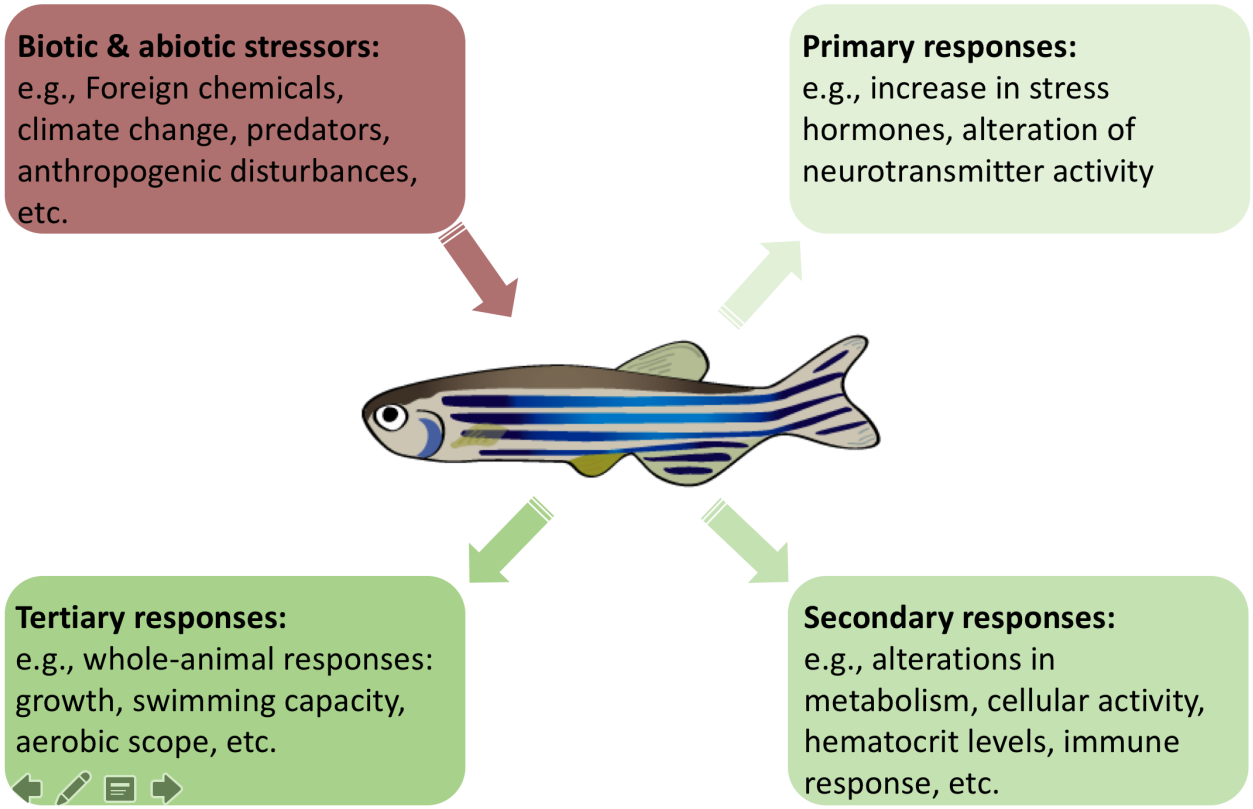


Figure 1.1: Environmental stressors evoke nonspecific physiological responses in fish, which are grouped into primary, secondary, and tertiary responses. (Figure modified from Barton, 2002).

1.2 Lab Study

Pharmaceuticals in the ng/L to µg/L have been detected in WWTP effluents discharged into the Grand River watershed as well as its surface waters (Arlos et al., 2015; Lissemore et al., 2006; Metcalfe et al., 2010). Antidepressants are a major class of pharmaceuticals detected in the Grand River watershed. VFX is a frequently prescribed antidepressant drug that is readily detectable in watersheds receiving discharged WWTP effluents (Horst and Preskorn, 1998; Metcalfe et al., 2010). The full extent of impacts of VFX on non-target species remain largely unknown. However, VFX has been demonstrated to impact reproduction, development, behaviour, survival, stress response, and metabolic capacity in fish (Best et al., 2014; Bisesi et al., 2016; Galus et al., 2013; Ings et al., 2011c; Melnyk-Lamont et al., 2014; Schultz et al., 2011). Our understanding of the impacts of PPCPs on non-target organisms continues to evolve. However, our knowledge regarding the cumulative effects of PPCPs in the presence of other stressors, such as those introduced by climate change is largely limited. Since the majority of fishes are thermal conformers, changes in ambient water temperatures can significantly affect their biochemical and physiological functions (Black et al., 1991). Increasing water temperatures can increase ventilation rates in fishes, resulting in higher a uptake rate of contaminants, such as PPCPs, thereby altering the impacts of these chemicals (Ford et al., 2004; Patra et al., 2009). Therefore, environmental temperature should be included as a factor when assessing the toxicity of chemicals, such as those associated with WWTP effluents. Environmental protection research involving environmental temperatures in addition to other stressors will become more relevant, especially with the ongoing concerns regarding climate change.

The study aims to understand the individual and cumulative effects of chronic exposure to an environmentally relevant concentration of VFX in addition to a 5°C increase in water temperature on the metabolic and stress physiology of fish, using zebrafish as a model species. The strong molecular genetics and genomic data surrounding zebrafish make them an ideal model organism to investigate additional ecotoxicological questions using molecular and genetic frameworks.

1.3 Field study

Environments impacted by discharged WWTP effluents are a typical example of multi-stressed ecosystems. WWTP effluents contain a wide variety of environmental contaminants, including but not limited to fertilizers, pesticides, metals, organic pollutants, and pharmaceuticals and personal care products (PPCPs). Contaminants associated with WWTP effluents pose serious threats on the organisms that interact with them, such as fish and other non-target aquatic organisms. Such associated threats impact fish on all levels of biological organization, ranging from molecular to community-based changes (Fuzzen et al., 2016; Ings et al., 2012; Ings et al., 2011b; Tetreault et al., 2013, 2011). The complexity of contaminants that are found in WWTP effluents as well as the broad to specific impacts of these compounds on aquatic species make effluent-receiving environments ideal, multi-stressed systems to be studied. Fortunately, such environment was in close proximity to the University of Waterloo with study sites located in the Grand River watershed, in southern Ontario, Canada. The ~300-km river drains the largest watershed that is found exclusively in the boundaries of southern Ontario. Over the past 100 years, population growth, urbanization, and agricultural activity have drastically changed the characteristics of the watershed. Approximately one million people live in the watershed, with

the majority of them living in one of the five urban areas in the central portion of the river (Waterloo, Kitchener, Guelph, Cambridge, and Brantford; Fig. 1.2). As a result of such population growth, 30 WWTPs have been installed in the Grand River basin (Fig. 1.2; Region of Waterloo, 2011). Heavy urbanization in the central region of the watershed have placed the river under many pressures and stressors, such as high nutrient input, low dissolved oxygen levels, and WWTP effluent contamination (Jamieson et al., 2013; Venkiteswaran et al., 2015). My study focuses mostly on the impacts of PPCPs on aquatic organisms. PPCPs have been detected in the effluent of WWTPs, effluent-receiving surface waters, as well as in the tissue of fishes residing nearby WWTPs (Arlos et al., 2015; Metcalfe et al., 2010; Wang et al., 2011).

This thesis was aimed at examining the effects of WWTP effluent on the metabolic and stress physiology of fish, using rainbow darter as a model organism. The rainbow darter is a small-bodied, sexually-dimorphic, short-lived (<5 years), and site loyal fish species that is native to North America (Fig. 1.3; Fuzzen et al., 2016; Hicks and Servos, 2017; Scott and Crossman, 1998). Field-based experiments are an essential part of multi-stressor research, as the dynamic and unpredictable nature of stressors in field conditions are often difficult to replicate in laboratories. However, laboratory research provides additional information that may not be acquirable from field-based experiments solely. Therefore, it is believed that only through the combination of both techniques, will we be able fully comprehend the impacts of multiple stressors.

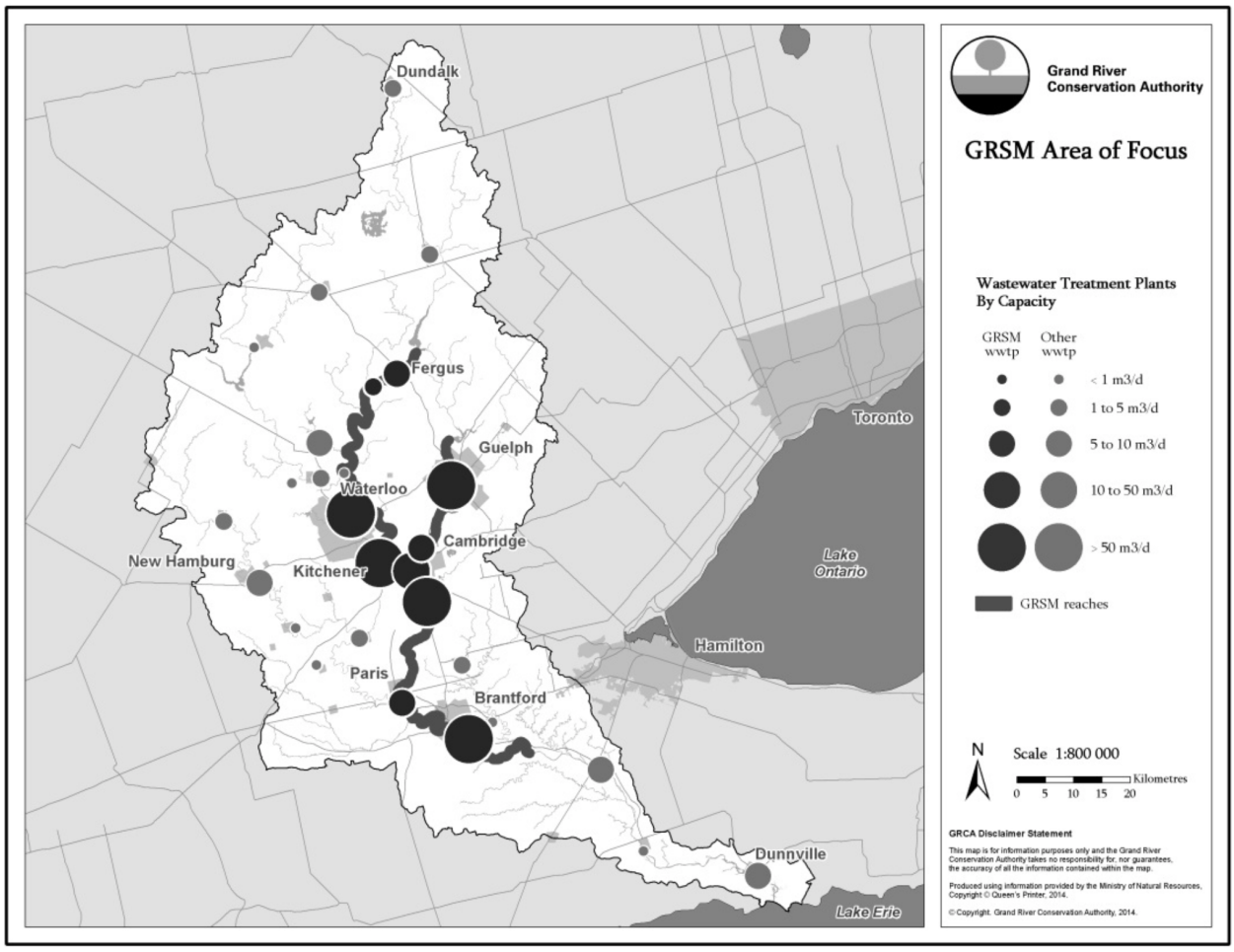


Figure 1.2: Map of the Grand River watershed displaying the urban areas in the watershed as well as the municipal wastewater treatment plants and their capacities (map obtained from Grand River Conservation Authority; GRCA).

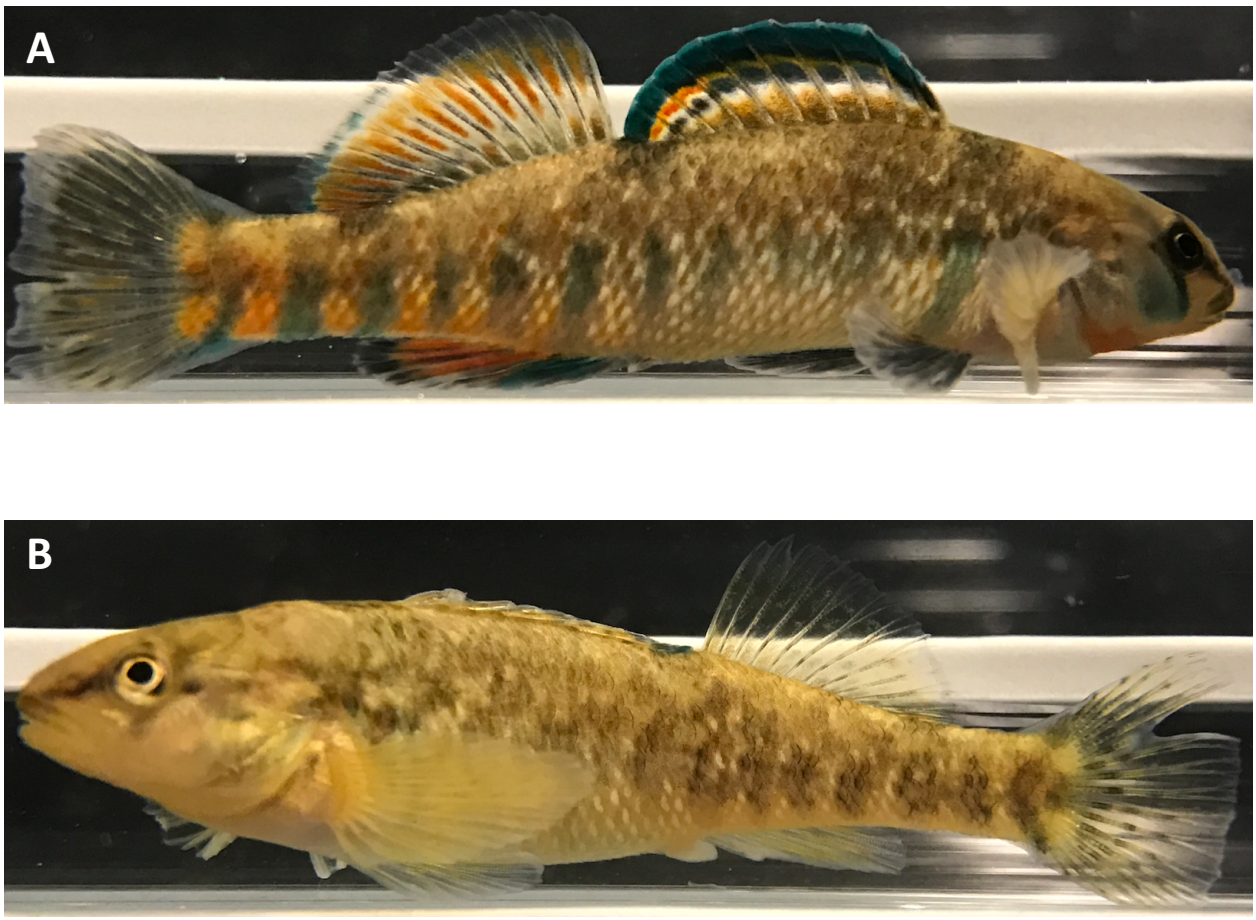


Figure 1.3: Rainbow dater (*Etheostoma caeruleum*) from the Grand River; (A) male; (B) female.

1.4 Study objectives

The general aim of this thesis was to investigate the multiple stressor effects associated with WWTP effluent contamination and climate change on the physiological responses of fish using lab and field studies. The objectives for the lab and field studies were as follows:

1. Assess the individual and combined effects of chronic exposure to an environmentally-relevant concentration of VFX in addition to elevated water temperature on the metabolic capacity, swimming performance, and stress response of zebrafish.
2. Assess the cumulative effects of WWTP effluent on the metabolic capacity and stress response of rainbow darter in the Grand River watershed.
3. Explore the similarities and differences in phenotypic responses between lab and field-based model organisms. Investigate the possibility of developing comparable aspects between lab and field studies.
4. Establish the initial phenotypic responses that fish exhibit to multiple stressors in laboratory and natural field settings, thereby, allowing us to ask more specific questions using molecular and epigenetic frameworks in the future.

1.5 General predictions

I predict that zebrafish will respond differently to multiple stressors than to individual stressors. VFX and elevated water temperature will have an additive effect, thereby putting fish in an energy demanding state to compensate the onset of multiple stressors. I also predict that rainbow darter found downstream of the WWTP will have similar responses to zebrafish exposed to multiple stressors. Fish facing energy deficits from stressor exposures are going to be more vulnerable to additional stressors. This will be further demonstrated by an increase in oxygen

consumption rates, decreased swimming capacity, attenuated stress response, and higher enzyme activities to make up for the elevated energy demands put on by multiple stressors. There will be differences in the physiological responses to stressors between zebrafish and rainbow darter, as these fish are different phylogenetically and have different life history strategies. However, I predict that the responses will be different in severity not kind, as rainbow darter are exposed to a wider range of stressors in their environments compared to zebrafish in the lab.

Chapter 2

Impacts of chronic exposure to environmentally-relevant concentrations of venlafaxine and elevated water temperature on zebrafish (*Danio rerio*) energetics and stress response

2.1 Introduction

Living organisms in heavily disturbed ecosystems are continuously exposed to a variety of different environmental stressors, the resulting effects of such diverse exposures is referred to as multiple stressor effects (Ng et al., 2013). Understanding the cumulative effects of multiple stressors is of crucial importance, as physiological and ecological responses to multiple stressors differ and are more complex than responses to individual stressors (Ng et al., 2013; Piggott et al., 2012). This study is interested in examining the effects of venlafaxine (VFX) found in wastewater treatment plant (WWTP) effluent (Arlos et al., 2015; Daughton and Ternes, 1999; Metcalfe et al., 2010), alone and in combination to elevated water temperatures on the energetics and stress response of zebrafish (*Danio rerio*).

2.1.1 Pharmaceuticals and personal care products (PPCPs)

Pharmaceuticals and personal care products (PPCPs) are frequently being introduced and detected in local aquatic ecosystems (Arlos et al., 2015, 2014; Daughton and Ternes, 1999; Küster and Adler, 2014; Metcalfe et al., 2010). PPCPs find their way into aquatic environments from a variety of sources, including but not limited to, treated domestic and hospital wastewater, agricultural runoff, and pharmaceutical factory effluent (Bottoni et al., 2010). The concentrations of most pharmaceuticals in the surface waters of aquatic environments are generally in the ng/L to low µg/L range (Fent et al., 2006; Kümmerer, 2010). However, despite the relatively low concentrations of these chemicals, their impact on aquatic organisms can be significant. Pharmaceuticals are intended to elicit physiological and biochemical processes in target species, however, they can have adverse effects on non-target organisms because of the highly conserved homology across vertebrates (Arnold et al., 2014; Fick et al., 2010; Margiotta-Casaluci et al.,

2014). The main focus of this study is to understand the effects of VFX, a heavily prescribed and readily detectable antidepressant found in many Canadian waterways that receive discharged effluent from WWTPs (Arlos et al., 2014; Metcalfe et al., 2010). VFX is a selective serotonin-norepinephrine reuptake inhibitor (SNRI) prescribed for anxiety, pain, lack of energy, psychomotor problems, and other symptoms of major depressive disorders (Saltiel and Silvershein, 2015; Vaswani et al., 2003). VFX and its active metabolite, O-desmethyl venlafaxine (O-VFX) are mainly introduced into streams via human excretion; approximately 5% of the average human daily dose is excreted in urine as the unchanged parent form and 29% in the active metabolite form (Metcalfe et al., 2010). VFX and O-VFX are often detected at higher concentrations than any other antidepressant drug and their active metabolite in WWTP effluents and receiving surface waters (Metcalfe et al., 2010; Schultz and Furlong, 2008). In discharged WWTP effluent, VFX and O-VFX have been detected at concentrations ranging from 808 to 2,050 ng/L and 1,637 to 1,927 ng/L, respectively (Arlos et al., 2015; Metcalfe et al., 2010). Whereas in surface waters downstream of WWTP effluents in the Grand River watershed, southern Ontario, Canada, VFX and O-VFX have been detected at concentrations ranging from 61 to 901 ng/L and 167 to 1,472 ng/L, respectively (Metcalfe et al., 2010). Despite these relatively high concentrations, very little is known about the impact of this drug on aquatic organisms (Best et al., 2014). Fish and aquatic organisms have been shown to accumulate VFX in tissues, though at lower concentrations than what is detected in the environment (Metcalfe et al., 2010; Schultz et al., 2011, 2010). Previous studies have demonstrated effects of VFX exposure in fish, both chronically and acutely in laboratory experiments. Reduced survival has been observed in fathead minnow (*Pimephales promelas*) exposed to 0.3 and 1.1 µg/L for 21 days (Schultz et al.,

2011). Egg production has been demonstrated to be reduced in response to VFX exposure in zebrafish (Galus et al., 2013). VFX can also affect transcriptional profiles in zebrafish in the presence of other stressors (hypoxia and elevated water temperature; Ikert and Craig, unpublished data). Exposure to environmentally-relevant concentrations of VFX has also been shown to impact biochemical regulation, neurodevelopment, and behaviour in aquatic vertebrates and invertebrates (Bidel et al., 2016; Fong et al., 2015; Fong and Molnar, 2013; Ings et al., 2012; Painter et al., 2009).

Serotonin and norepinephrine are very important neurotransmitters in vertebrates, playing a key role in food appetite, regulation of feeding and breeding behaviour, energy expenditure, and metabolism (Lam and Heisler, 2007; Mennigen et al., 2010, 2011; Smith et al., 2010). Production of catecholamines, especially epinephrine and norepinephrine, is important in regulating the adaptive stress response, and by extension, modifying metabolic demands and processes in response to stressors. Therefore, pharmaceuticals and other contaminants that disrupt serotonin, epinephrine, and/or norepinephrine reuptake and production mechanisms may have deleterious effects on energy production and expenditure and the stress response in fish and other non-target organisms. Our study is the first to look at the effects of VFX on the energetics and stress response of zebrafish, where its impacts are assessed individually and cumulatively in the presence of another stressor, elevated water temperature.

2.1.2 Temperature

Environmental temperature, known as the 'ecological master factor' is a key determinant of many behavioural and physiological processes in fish such as metabolic function, energy production and expenditure, development, survival, and growth (Farrell et al., 2008; Harig and

Fausch, 2002; Lee, 2003; Schultz and Bertrand, 2011). Temperature has an overarching control over many chemical and biological reactions. Responses to temperature occur at various levels, ranging from molecular to community-based responses. The focus of this study will be on the physiological responses to an elevated water temperature and VFX exposure. Elevated environmental temperatures have especially profound effects on metabolism, which is often accompanied by elevation in organismal overall oxygen consumption rate and oxidative stress in response to the added metabolic demands (Akhtar et al., 2013; Angilletta, 2009.; Bagnyukova et al., 2007). Other responses linked to increased water temperature include changes in aerobic scope, swimming speed, appetite, reproductive condition, and overall cardiovascular work (Black et al., 1991; Brett, 1971; Cunjak et al., 1987; Linton et al., 1998; Newsome and Leduc, 1975). Understanding these responses to changing environmental temperatures is especially important to fishes, since the majority of fishes are thermal conformers, i.e., ectothermic poikilotherms (Brett, 1971). The significance of research regarding the impact of environmental temperature on fishes is rapidly growing, especially with the onset of climate change. In North America, scientists are predicting that air temperatures will rise by as much as 6°C in the next 50-100 years, and with that, water temperatures are expected to rise as well (Brown et al., 2015; Houghton et al., 2011).

2.1.3 Combined stressors

The effects of temperature on the toxicity of chemicals have been well examined in the past under laboratory settings, using a wide variety of fish species and chemical compounds (Nussey et al., 1996). A study using mosquito fish (*Gambusia affinis*) exposed to dichlorodiphenyltrichloroethane (DDT) found a positive correlation between the accumulation

of the chemical and exposure temperature (Murphy and Murphy, 1971). Similar correlations have been established between methylmercury accumulation and water temperatures in rainbow trout (*Oncoryhnchus mykiss*). Generally, the toxicity of chemical pollutants, like organophosphates and heavy metals increases at higher temperatures (Murty, 1986). This phenomenon can be attributed to the increased production of toxic secondary products via metabolizing the parent compound upon uptake (Nemcsók et al., 1987). Another possibility can be attributed to the increase in uptake rate of the chemical compounds due to increased gill ventilation at higher temperatures (Black et al., 1991; Murty, 1986). Two major caveats in the majority of experiments performed in the past are the use of higher concentrations of chemicals than what is found in natural ecosystems and exposures to chemicals over a short period of time; scenarios that might not accurately predict interaction of fishes with these chemicals as observed in field conditions (Ficke et al., 2007).

We have a good understanding of the physiological responses in fishes to temperature change or contaminant exposures, however, these responses can be further amplified or differed when fish are exposed to both of these stressors simultaneously. Fish in natural environments often encounter acute or chronic changes in environmental temperatures, which can have significant impacts on their metabolic physiology. Round goby (*Neogobius melanostomus*) experience changes in ventilation rate and behaviour at higher temperatures, and such physiological and behavioural responses should be taken into account when assessing the toxicity of contaminants that fish could encounter in their environments (Patra et al., 2009). Very little is known about the effects of VFX, an antidepressant found abundantly in many North American water bodies on aquatic organisms especially when combined with other stressors, like elevated

water temperature. The objective of this study was to examine the effects of chronic exposure to environmentally-relevant concentrations of VFX (1.0 µg/L) as well as a 5°C increase in water temperature on the energetics and stress response of non-target aquatic animals, using zebrafish as a model organism. This was tested by measuring whole-organism metabolic capacity using swim-tunnel respirometry to measure routine metabolic rate, active metabolic rate, aerobic scope, and critical swimming speed (RMR, MMR, AS, and U_{crit}, respectively). Excreted plasma cortisol was also measured from holding water samples under a confinement stressor to assess the effects of VFX and temperature on the stress response of exposed fish. Muscle metabolic function and capacity was assessed by measuring the activities of metabolic and mitochondrial enzymes to better understand how various metabolic pathways are affected by exposure to multiple stressors. Understanding the effects of multiple stressors on metabolic and stress physiology of fishes will improve the understanding of how different environmental stressors interact with aquatic organisms causing adverse effects that may have not been predicted using single-stressor research.

2.2 Materials and methods

2.2.1 Animals

Adult zebrafish were acquired from a local pet store (PetSmart, Waterloo, ON, Canada) and maintained in acrylic tanks (density of <5 fish/L) in a Habitats® Z-Hab System (Pentair Aquatic Eco-Systems Inc., Apopka, FL, USA). Water supplying the system underwent reverse osmosis, deionization, aeration, biological and chemical filtration, and UV sterilization. Water in the system was maintained at 27°C, pH of 7.5, and conductivity of 670 µS. Fish were kept under a 12h:12h light-dark cycle and fed twice daily. Food consisted of a mixture of ground commercial

fish food (TetraMin, Tropical Flakes, Blacksburg, VA, USA) and live brine shrimp. This feeding schedule was maintained until start of exposure experiment. All experimental protocols were approved by the animal care committee at the University of Waterloo (AUPP #15-03).

2.2.2 Chronic exposure (21 days)

12-L glass aquarium tanks were used for static exposures. Tanks were supplied with the same system water that fish had previously been housed in before, with sufficient aeration and heating. Fish were exposed to one of four treatments: 1) 0 µg/L venlafaxine (VFX) at 27°C (*Control*); 1.0 µg/L VFX at 27°C (*VFX*); 0 µg/L VFX at 32°C (*Temp*); 1.0 µg/L VFX at 32°C (*VFX & Temp*). Each treatment had 3 tank replicates, with 20 fish per tank. VFX (Sigma-Aldrich, Oakville, ON, Canada) aliquots dissolved in water were made in advance and stored at -20°C prior to daily dosing. Only male zebrafish with similar size and weight were selected for this experiment to avoid potential sex, size, and age linked differences. Fish sex was determined using known morphological characteristics and later confirmed upon dissection, only confirmed males were used in data analyses. Fish were slowly acclimated to the temperature conditions over a period of 7 days prior to the start of the exposure.

Fish were fed ground flakes once daily until satiety, and 50% daily water changes were performed 1 hour after feeding to remove waste and buildup of nitrogenous products. Once a week, 100 mL water samples were collected from each tank and frozen at -20°C for later extraction and analysis of VFX concentration using mass spectrometry. Ammonia, nitrate, and nitrites were monitored weekly, and at no point in the exposure did nitrogenous waste product pose an issue for fish health (data not shown). After the 21-day exposure, fish were subsampled in one of three experiments: (i) respirometry and swimming performance, (ii) stress test and

cortisol analysis, and (iii) enzyme analysis. Feeding was ceased on the last day of the exposure to ensure they were in a fasted state before the start of experiments.

2.2.3 (i) Respirometry and swimming performance

A 170-mL glass swim tunnel respirometer equipped with a polymer optical fiber oxygen dipping probe, DAQ oxygen data acquisition system, Witrox oxygen reader, and Autoresp respirometry software (Loligo System, Tjele, Denmark) was used to measure oxygen uptake and critical swimming speed (U_{crit}) in this experiment. A water bath circulator equipped with a submersible water heater and a return pump controlled the temperature in the swim tunnel which was kept at 27°C or 32°C to match the exposure conditions. Throughout the experiment, the swim tunnel was programmed to automatically cycle through 3 phases over 5 minutes: 60 seconds of flushing, 20 seconds of waiting, and 220 seconds of measuring oxygen concentration. Individual zebrafish were introduced into the swim tunnel and allowed to acclimate for 1 hour at a slow velocity (5 cm/s) to mimic normal activity in the exposure tanks and reduce spontaneous activity that would otherwise be observed in static water flow. Following the acclimation period, the velocity of the swim tunnel was gradually increased 5 cm/s every 5 minutes until fish were not able to maintain position and were pushed against the mesh in the back of the swim tunnel due to fatigue. Upon this, U_{crit} was calculated using the standard equation (Brett, 1964).

Minimum aerobic metabolic rate ($M_{O2,min}$) and maximum aerobic metabolic rate ($M_{O2,max}$) were measured to best estimate routine metabolic rate (RMR) and active metabolic rate (AMR) respectively. $M_{O2,min}$ was obtained from the last 6 measurements during the acclimation period, where only the lowest, most frequent M_{O2} measurements were used, while outliers were presumed to resemble spontaneous activity. $M_{O2,max}$ was obtained from M_{O2} measurements

taken after fish fatigued. These two measurements were then used to calculate aerobic scope (AS) in absolute terms (i.e., $M_{O,max}$ - $M_{O2,min}$) (Clark et al., 2013).

2.2.4 (ii) Stress test and cortisol analysis

All experiments were conducted in the morning (between 09:00-10:00) to avoid diurnal effects on steroid variation. Holding water samples were collected using 100-mL beakers filled with 50 mL of reverse osmosis, deionized, unused aquarium water. Prior to introduction of fish into beakers, beakers were placed in a water bath set at 27°C or 32°C to match the exposure temperature. Confinement stress was initiated by placing one fish in 50-mL water at the same temperature the fish had been exposed to, and left for 1 hour. At the end of the confinement stressor, fish were removed from beakers, euthanized, and the water was transferred into separate 125-mL amber glass bottles (Fisherbrand®, Fisher Scientific, Mississauga, ON, Canada) and stored at -20°C until cortisol extraction.

Water samples were thawed at room temperature prior to extraction. Solid phase extraction (SPE) was used to extract free steroid hormones that are released into the water via passive diffusion across the gills over the 1 hour confinement stressor period. Steroid hormones from each water sample were collected using a LC-18 SPE cartridge (Sigma-Aldrich) attached to a 12-port vacuum manifold (Sigma-Aldrich). Each cartridge was primed by flushing 5 mL of 100% methanol followed by 5 mL Millipore ultrapure water through the cartridge. Water samples were then drawn into separate SPE cartridges using SPE tubes to collect free steroid hormones. Cartridges were eluted with 2 x 5 mL ethyl acetate into 13 x 100 mm glass tubes. The eluted samples were completely dried under a gentle steam of nitrogen gas. The dried samples were then resuspended in 300 µL ELISA buffer (provided in kit) for enzyme-linked immunosorbent

cortisol assay (Cayman Chemicals, Ann Arbor, Michigan, USA). Each resuspended sample was assayed in triplicate following the explicit instructions of the manufacturer. Numerous studies have previously verified the reliability of this method of steroid extraction from holding water samples (Friesen et al., 2012; Kidd et al., 2010; Pottinger et al., 2016; Pottinger and Matthiessen, 2016a).

2.2.5 (iii) Enzyme analysis

Frozen muscle tissue collected were 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 27°C and 32°C 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 fish, 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.

2.2.6 Water chemistry

100-mL water samples were taken every 7 days from each tank and later analyzed to ensure VFX concentrations were maintained within environmentally-reported levels throughout the exposure experiment. Water samples were collected 1 hour after dosing and stored immediately at -20°C until extraction. VFX samples were quantified following (Rahman et al., 2010). Briefly, 100-mL samples were spiked with 100 µL [100 µg/L] deuterated VFX. Samples were then extracted using solid-phase extraction (SPE) in Oasis HLB cartridges (6 cc, 500 mg, Waters Corporation, Milliford, MA, USA). The eluents were collected in glass tubes and evaporated under a gentle stream of nitrogen gas, and then reconstituted with 500 µL of methanol and stored at -20°C until analysis. Samples were then quantified using a Sciex API 3200 QTRAP LC-MS/MS system. The method detection limit (MDL) in a 500-mL sample was 1 ng/L. Since 100-mL samples were extracted in this experiment, the detection limit was calculated to be 5 ng/L based on the original MDL.

2.2.7 Statistical analysis

Data was analyzed using SigmaPlot 13.0 software (Systat Software Inc., San Jose, CA, USA). Data is presented as means ± 1 standard error of the mean (SEM). A one-way ANOVA and

Tukey's post-hoc test were performed to determine significant differences between treatments in metabolic rate, swimming performance, and cortisol analysis. A two-way ANOVA and Tukey's post-hoc test were used to determine significant differences between treatments and assay temperatures for enzyme analysis. Finally, a repeated-measures ANOVA was used to test significant differences between tank replicates in VFX concentration. Non-parametric statistics (ANOVA on ranks) were only used if data was not normally distributed. The alpha value was set at 0.05 (i.e., $P<0.05$) for all tests.

2.3 Results

2.3.1 Water chemistry

VFX concentrations were below detection limit for the Control treatment, 980.6 (± 27.0) ng/L for the VFX treatment, below detection limit for the Temp treatment, and 965.2 (± 59.3) ng/L for the VFX & Temp treatment during the exposure period (Fig. 2.1). During the acclimation period, no VFX was detected in any of the tanks. Using a repeated measures ANOVA, no significant differences were observed between any of the tank replicates within each treatment. As there were no significant differences in VFX concentration and water temperature between replicates within treatments, data was pooled from all three tank replicates for each treatment for statistical analysis.

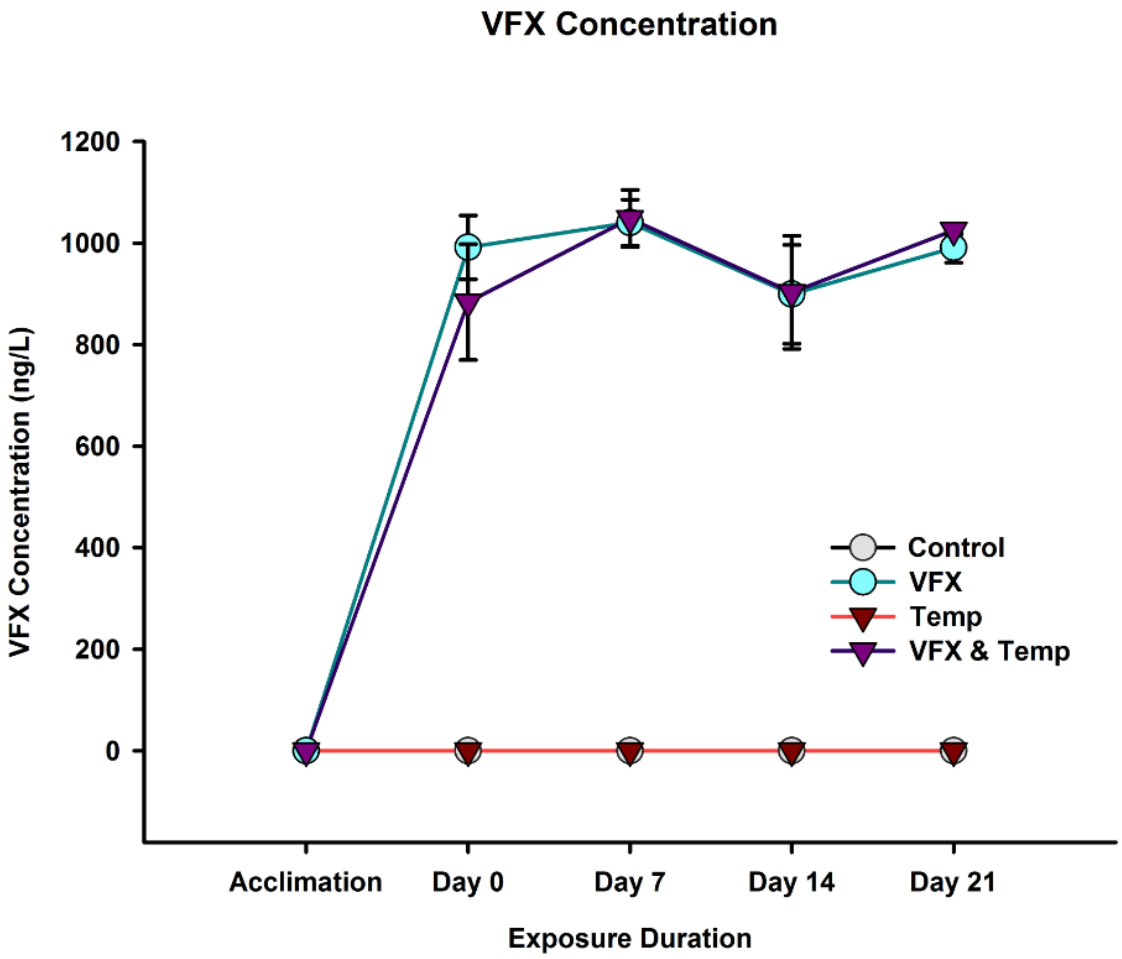


Figure 2.1: Mean VFX concentrations across treatments during acclimation and exposure periods. Repeated-measures ANOVA was used to identify any significant differences between tank replicates and treatments, (n=3 tank replicates per treatment).

2.3.2 Respirometry and swimming performance

Changes in routine metabolic rate (RMR), active metabolic rate (AMR), aerobic scope (AS), and critical swimming speed (U_{crit}) were determined in response to chronic exposure to VFX and elevated water temperature (Fig. 2.2). Analysis revealed that RMR was significantly higher in the VFX & Temp group compared to the Control. No significant differences were observed in AMR. However, both Temp and VFX & Temp groups had significantly diminished AS compared to the Control group. Critical swimming speed (U_{crit}) was slightly lower in the treatment groups compared to the Control, but no significant differences were detected (Fig. 2.2).

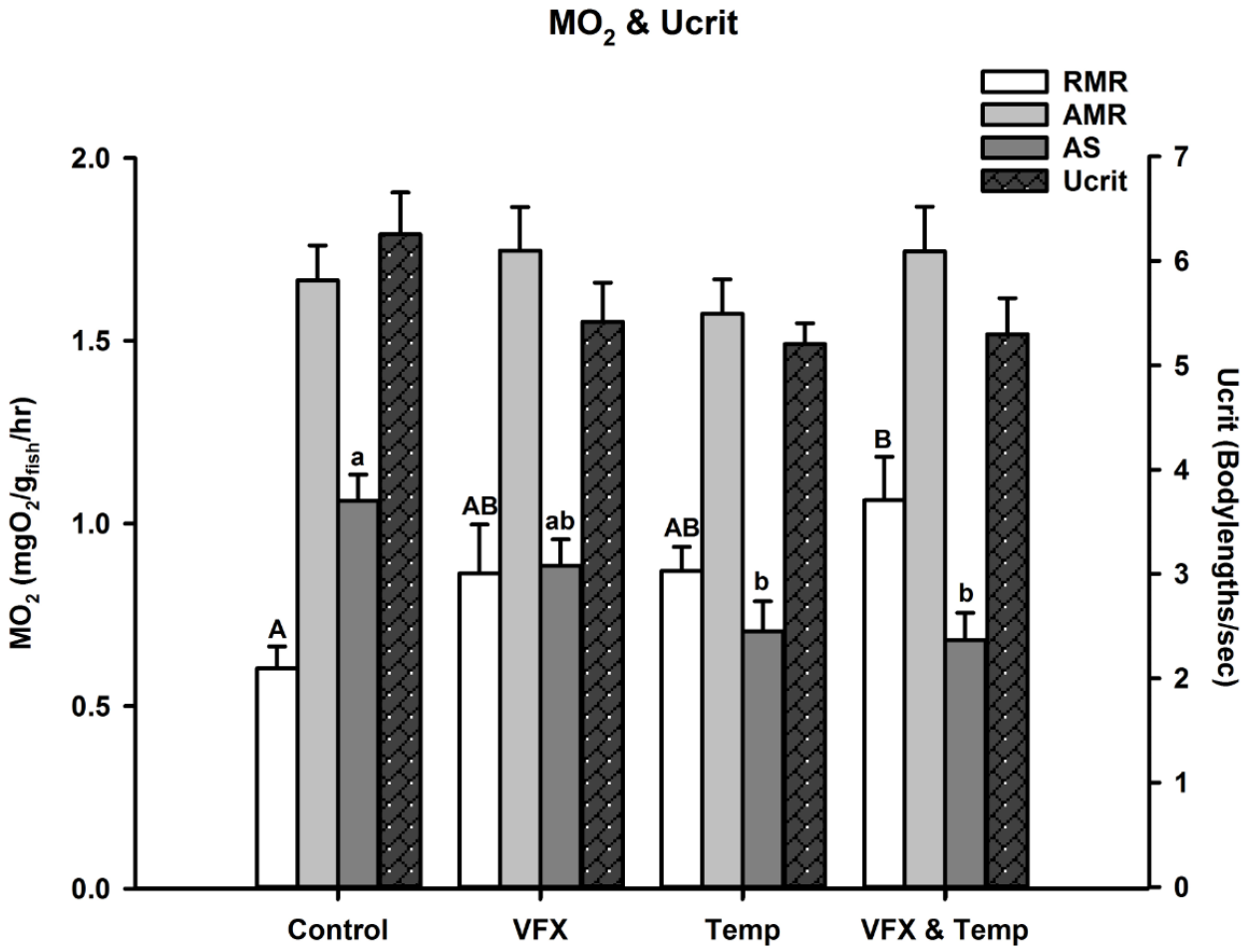


Figure 2.2: Routine metabolic rate (RMR), active metabolic rate (AMR), aerobic scope (AS), and critical swimming speed (U_{crit}) measured in adult male zebrafish 24 hrs post exposure. Data are presented as mean ± SEM of n=11 individuals per treatment. Bars that do not share letters indicate significant difference using a one-way ANOVA followed by a Tukey's post-hoc test ($P<0.05$). As there were no significant differences in AMR and U_{crit}, uppercase letters were used for RMR and lowercase letters for AS.

2.3.3 Stress response

Extracted cortisol concentrations from holding water following a confinement stressor are presented in Fig. 2.3. Analysis revealed no significant differences in elevated cortisol levels between groups. Some samples from each treatment group had to be omitted due to low cortisol concentrations falling outside the detection range, which resulted in a decreased sample size in each treatment group than what was initially intended.

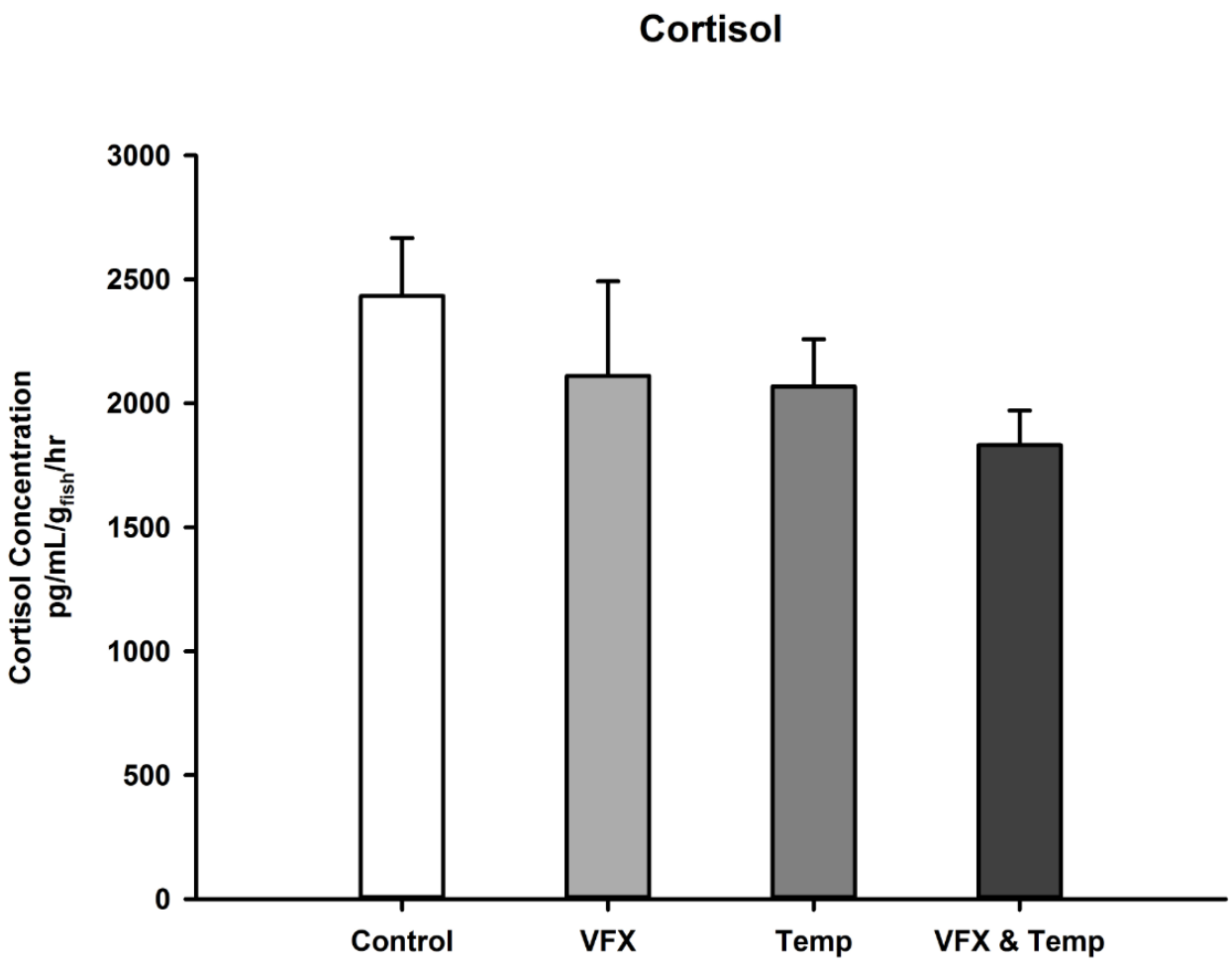
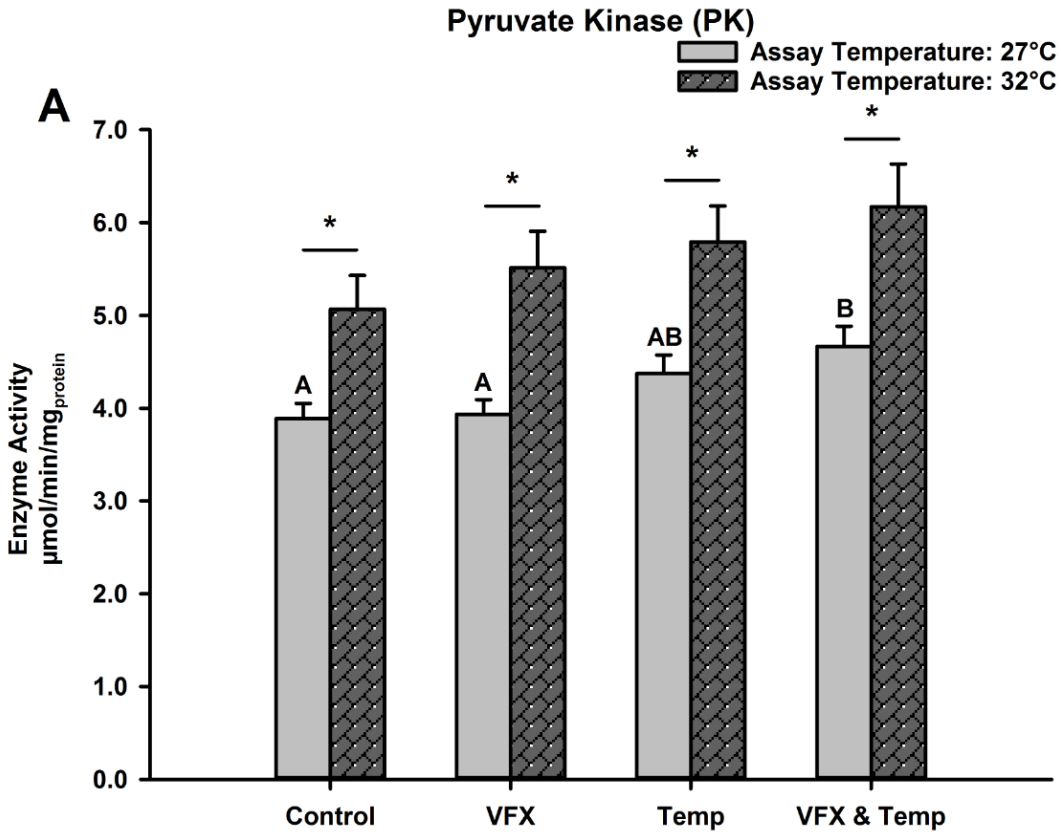
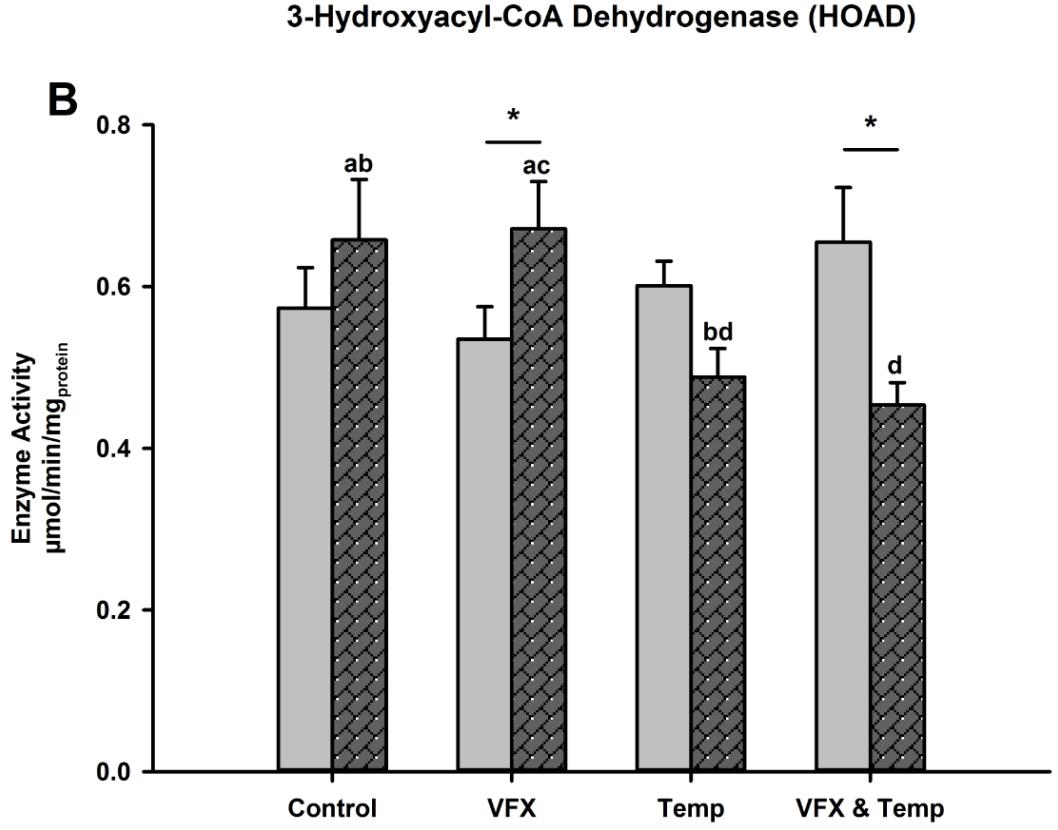


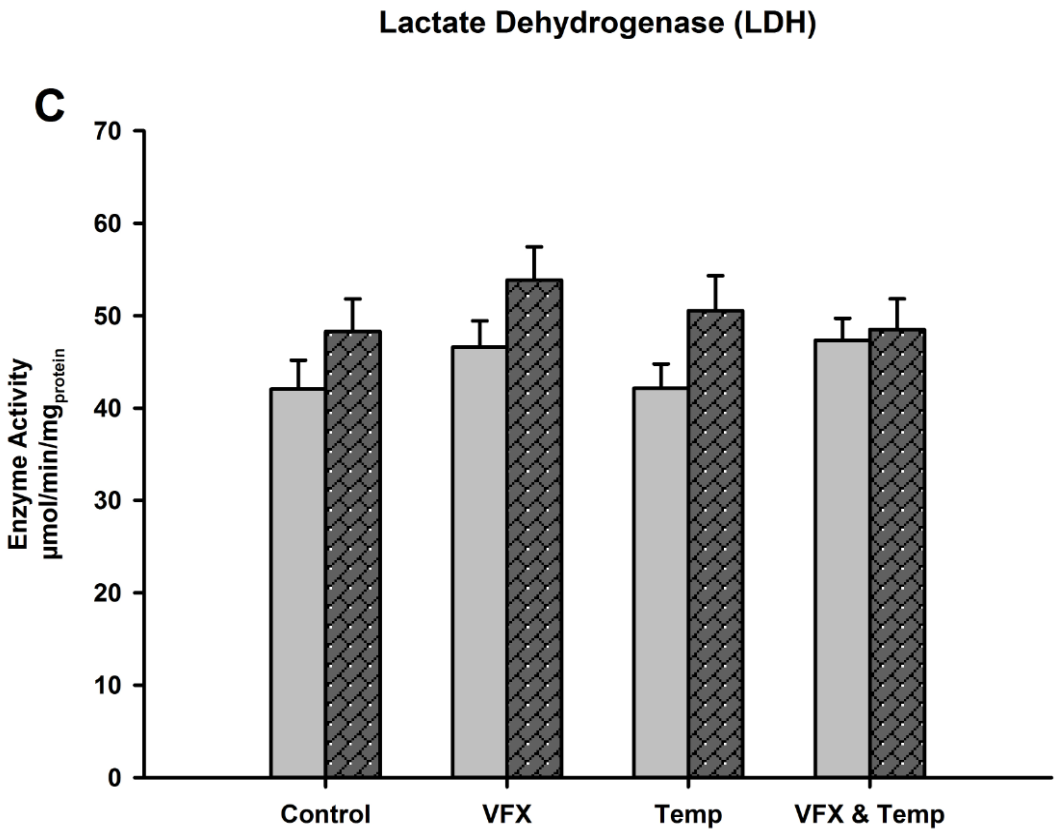
Figure 2.3: Extracted water cortisol concentrations from individual holding water samples in treatment groups corrected to individual zebrafish body mass. Individuals were placed in a 100-mL beaker for 1 hour to elicit a confinement stress response. Data is presented as mean ± SEM of Control (n=7), VFX (n=3), Temp (n=4), and VFX & Temp (n=8). Differences in group means were determined using a one-way ANOVA followed by a Tukey's post-hoc test ($P<0.05$).

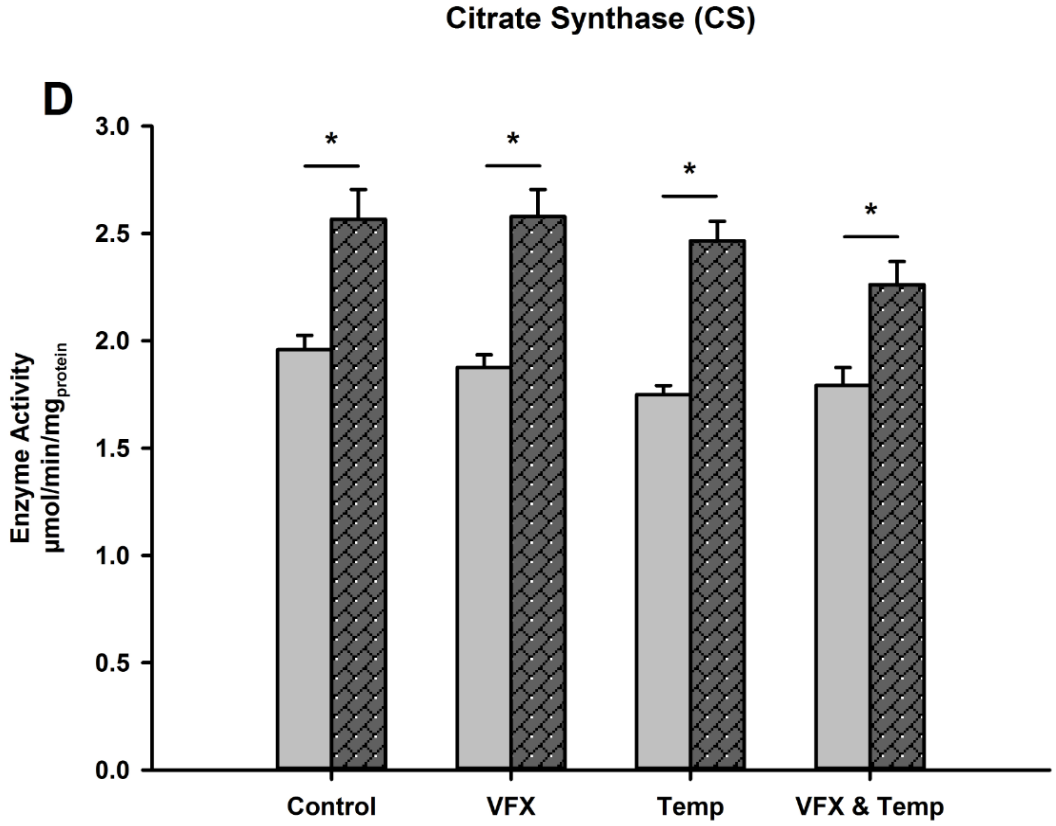
2.3.4 Enzyme activities

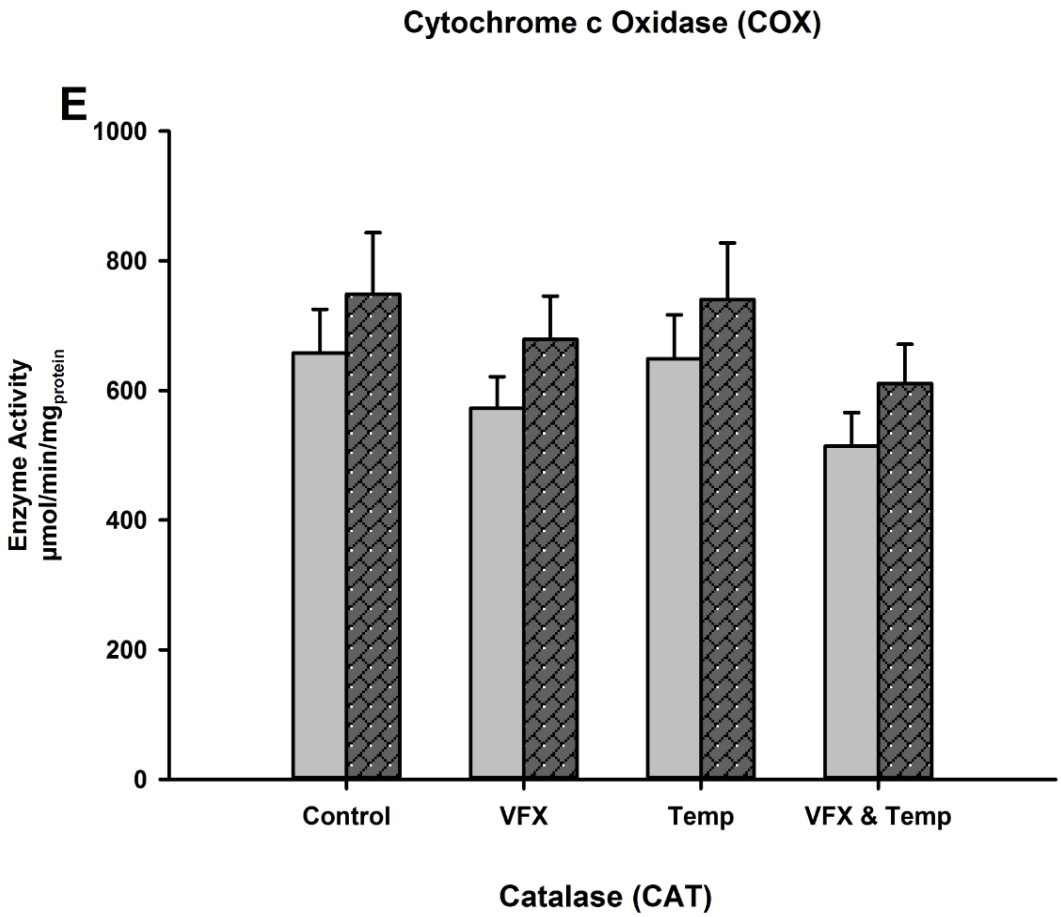
Activities of six muscle metabolic enzymes were measured at 27°C and 32°C (Fig. 2.4 and Table 2.1). PK activity was highest in the VFX & Temp group when measured at both assay temperatures, representing fold change values of 1.20x and 1.27x, respectively, in comparison to the Control group. However, a statistical difference was only detected at 27°C between VFX & Temp and Control and VFX, a 1.20x and 1.19x increase respectively. PK activity was significantly higher when measured at 32°C than 27°C in all groups. HOAD activity was not significantly different among treatment groups when measured at 27°C. However, when measured at 32°C, HOAD activity was significantly lower in the VFX & Temp group than Control and VFX, representing 0.68x decrease in both. Also, when measured at 32°C, the Temp group was significantly lower than the VFX group, representing a 0.73x decrease. HOAD activity only significantly changed between the two assay temperatures in the VFX and VFX & Temp group, representing Q_{10} values of 1.57 and 0.48, respectively. LDH activity was not different among treatment groups at 27°C or 32°C, and activities were not affected by the increase in assay temperature. CS was also not different between treatment groups, however, the increase in assay temperature resulted in a higher enzyme activity in all treatments. COX activity was not different between treatment groups and assay temperatures. CAT activity was highest in the Temp group when compared to Control and VFX & Temp, representing a 1.41x and 1.38x increase respectively at 27°C, and a respective 1.24x and 1.38x increase at 32°C.


Pyruvate Kinase (PK)
Assay Temperature: 27°C
Assay Temperature: 32°C
A
Enzyme Activity
µmol/min/mg protein
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
*
*
*
*
A
A
AB
B
Control
VFX
Temp
VFX & Temp


3-Hydroxyacyl-CoA Dehydrogenase (HOAD)
B
Enzyme Activity
µmol/min/mg protein
0.8
0.6
0.4
0.2
0.0
*
*
ab
ac
bd
d
Control
VFX
Temp
VFX & Temp


Lactate Dehydrogenase (LDH)
C
Enzyme Activity
µmol/min/mg protein
70
60
50
40
30
20
10
0
Control
VFX
Temp
VFX & Temp


Citrate Synthase (CS)
D
Enzyme Activity
µmol/min/mg protein
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
*
Control
VFX
Temp
VFX & Temp


Cytochrome c Oxidase (COX)
E
Enzyme Activity
µmol/min/mg protein
1000
800
600
400
200
0
Control
VFX
Temp
VFX & Temp

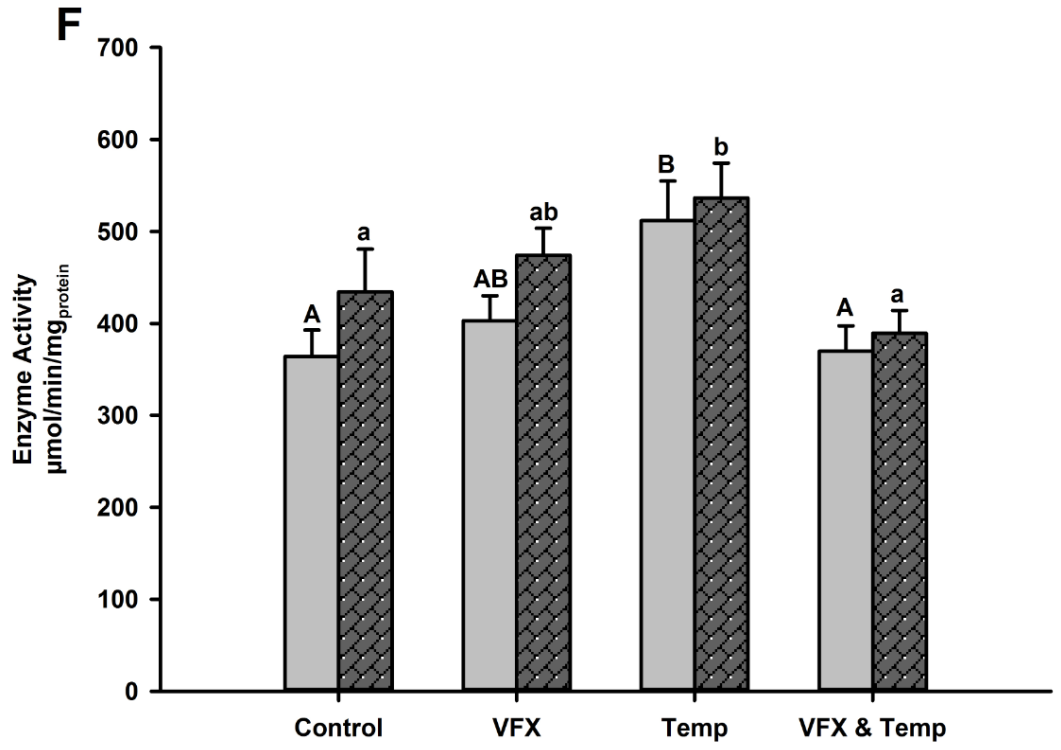

Catalase (CAT)
F
Enzyme Activity
µmol/min/mg protein
700
600
500
400
300
200
100
0
A
a
AB
ab
B
b
A
a
Control
VFX
Temp
VFX & Temp

Figure 2.4: Muscle enzyme activity of (A) pyruvate kinase (PK), (B) 3-hydroxyacyl CoA dehydrogenase (HOAD), (C) lactate dehydrogenase (LDH), (D) citrate synthase (CS), (E) cytochrome c oxidase (COX), and (F) catalase (CAT) measured in adult male zebrafish following exposure. Data is presented as mean ± SEM of n=17-24 individuals per treatment. Bars that do not share letters indicate significant difference using a two-way ANOVA followed by a Tukey's post-hoc test ($P<0.05$). Uppercase letters were reserved to show differences in enzyme activities when measured at 27°C and lowercase letters when measured at 32°C, * indicates a significant difference within treatment's enzyme activity measured at 27°C and 32°C.

Table 2.1: Fold changes (FC) in muscle enzyme activity among treatment groups relative to Control measured at two assay temperatures (Ta=27°C and 32°C). Temperature coefficient (Q_{10}) values represent changes in enzyme activity measured at two different assay temperatures calculated using this formula: $Q_{10} = (R_2/R_1)^{(10/(T2-T1))}$, where R_2 and R_1 are the enzyme activity values at 32°C and 27°C respectively, and T_2 and T_1 are the different assay temperatures, 32°C and 27°C respectively.

		Control	VFX	Temp	VFX & Temp
PK	*FC (Ta=27°C)*	-	1.01	1.12	1.2
	FC (Ta=32°C)	-	1.14	1.19	1.27
	Q_{10}	***1.56***	***1.96***	***1.75***	***1.75***
HOAD	*FC (Ta=27°C)*	-	0.93	1.05	1.14
	FC (Ta=32°C)	-	1.02	0.74	0.69
	Q_{10}	***1.32***	***1.57***	***0.66***	***0.48***
LDH	*FC (Ta=27°C)*	-	1.11	1	1.13
	FC (Ta=32°C)	-	1.12	1.05	1
	Q_{10}	***1.32***	***1.34***	***1.44***	***1.05***
CS	*FC (Ta=27°C)*	-	0.96	0.89	0.92
	FC (Ta=32°C)	-	1	0.96	0.88
	Q_{10}	***1.72***	***1.89***	***1.99***	***1.59***
COX	*FC (Ta=27°C)*	-	0.87	0.99	0.78
	FC (Ta=32°C)	-	0.91	0.99	0.82
	Q_{10}	***1.29***	***1.41***	***1.3***	***1.41***
CAT	*FC (Ta=27°C)*	-	1.11	1.41	1.02
	FC (Ta=32°C)	-	1.09	1.24	0.9
	Q_{10}	***1.42***	***1.38***	***1.09***	***1.11***

2.4 Discussion

This study aimed to assess the impacts of chronic exposure to an environmentally-relevant concentration of VFX and elevated water temperature on non-target species, using zebrafish as a model organism. Studies often look at the effects of environmental perturbations using a single stressor approach. However, this is the first study of its kind to look at the effects of both of these stressors (VFX and elevated temperature) individually and cumulatively using metabolic and stress physiology as sublethal endpoints under controlled lab conditions.

2.4.1 Respirometry and swimming performance

Routine and active metabolic rates were measured (RMR and AMR respectively), in addition to aerobic scope (AS) and swimming capacity (U_{crit}) in adult male zebrafish after a 21-day exposure period in four different treatments: Control, VFX, Temp, and VFX & Temp. These measurements are often used as basic indices of fish bioenergetics costs (Fangue et al., 2014). The results suggest that differences in metabolic rate are mostly driven by temperature rather than VFX exposure. Although, VFX did have an additive effect as observed in the RMR measurements, where it was only elevated in the fish exposed to both stressors. However, when looking at the difference between RMR and AMR, defined as AS, we saw that both groups that were exposed to elevated water temperature had a significantly reduced AS. These results were in line with our initial hypotheses, as temperature can heavily influence physiological responses, like metabolic rates, in an attempt to mediate effects on fishes (Chown and Gaston, 2015; Schulte, 2015; Somero, 2010). Changes in AS are often used as indicators of stress and ecological performance, as AS represents the energetic capacity that an organism has to perform critical processes such as growth, locomotion, and reproduction (Claireaux and Lefrancois, 2007; Farrell

et al., 2008; Pörtner, 2002, 2001; Pörtner and Knust, 2007). Therefore, the reduction in AS that was observed in our study suggests reduced ability for fish to function in an ecological framework.

No differences were observed in swimming performance amongst our treatment groups. There was a slight reduction in U_{crit} in the groups exposed to the higher water temperature (Temp and VFX & Temp), however, these differences were not significant. Swimming performance tests are considered more ecologically-relevant indices, as they are related to higher-level biological processes (Groh et al., 2015; Sárria et al., 2011). Therefore, our findings suggest that the stressors we exposed fish to in our study are not strong enough to cause ecologically-relevant perturbations in regards to swimming capacity. This observed resilience in swimming capacity can be explained by the wide thermal tolerance that zebrafish exhibit (6.7-41.7°C), making them one of the most eurythermal fish species on record (Cortemeglia and Beitinger, 2005). It is also possible that zebrafish have evolved a wide range of temperature flexibility because they are often found in environments where they experience such extreme temperature swings (Spence et al., 2006). Thereby, a 5°C increase in water temperature might not be significant enough to register such an ecologically-relevant response as swimming performance.

2.4.2 Stress response

Individuals from all four treatments (Control, VFX, Temp, and VFX & Temp) were subjected to a confinement stressor. Our results indicated that neither a 1.0 µg/L concentration of VFX nor a 5°C increase in water temperature have a significant impact on the cortisol stress response of zebrafish in response to a confinement stressor, singularly or in combination. The mode of action by which VFX can disrupt the hypothalamus-pituitary-interrenal (HPI) axis in non-target organism remains largely unknown (Ings et al., 2012). However, VFX has been previously

labeled as a neuroendocrine disruptor to non-target organisms in aquatic environments. It has been demonstrated in rainbow trout that exposure to environmentally-realistic concentrations of VFX affects brain monoamine levels, which can further impact downstream processes, thereby disturbing homeostasis. Additionally, VFX exposure elevates plasma cortisol levels in socially-stressed fish (Melnyk-Lamont et al., 2014). Furthermore, in rainbow trout hepatocyte cell cultures, it has been demonstrated that VFX is able to disrupt glucose production capacity involved in the stress response but only at higher concentrations that what is detected in aquatic environments (Ings et al., 2012). Our results suggest that cortisol levels of the VFX group is not different than the Control, further demonstrating that at environmentally-relevant concentrations, VFX does not impair the cortisol stress response.

The second stressor used in our study was elevated temperature. Changes in ambient water temperatures can alter the stress response in fish. It has been reported that fish can tolerate handling stress better at lower water temperatures. Striped bass (*Morone saxatilis*) were found to have significantly higher plasma cortisol levels following a confinement stressor when housed at higher temperatures (Davis and Parker, 1990). Other studies using sunshine bass (*Morone chrysops* x *Morone saxatilis*) and channel catfish (*Ictalurus puncatus*) also reported that temperature has a significant effect on plasma cortisol levels (Davis, 2004; Davis et al., 1984; Strange, 1980). It is suggested lower levels of cortisol measured at lower temperatures are caused by lower rates of secretion and synthesis of cortisol due to lower metabolic activity (Davis, 2004). In our study, we did not observe any significant changes in the Temp group compared to the Control. It is worth mentioning though, that the studies demonstrating an effect of temperature on the stress response often observed this effect only when the difference between

temperatures compared was large enough. Whereas, in our study, we only looked at the effect of a modest increase in water temperature relative to other studies. Another explanation is attributed to exposure or acclimation time. Studies demonstrating a temperature effect on the stress response only exposed fish to the experimental temperature regimen for 5-8 days (Davis, 2004; Davis et al., 1984; Davis and Parker, 1990). Whereas in our study, fish were allowed to acclimate to the exposure temperature over a 7-day period and were then exposed to that temperature for an additional 21 days. Therefore, the response that we measured is representative of an acclimated response compared to other studies, where the exposure periods were significantly shorter.

Fish exposed to both VFX and elevated water temperature did have a slightly lower cortisol levels after the confinement stressor, however, that difference was not statistically significant. This was against the study's initial hypothesis as it was expected that exposure to multiple stressors would result in an impairment of the cortisol stress response. This would have been attributed to the exhaustion of the stress response mechanism as a result of the hyperactivity of the HPI axis due to exposure to multiple stressors simultaneously (Wendelaar Bonga, 1997). Chronic exposure to stressors, such as chemical pollutants, can impair the ability to mount a successful stress response by elevating plasma cortisol levels in response to an acute stressor (Hontela et al., 1995). Further research is required to see the effects of VFX and elevated water temperature on other endpoints of the stress response, as this study only examined cortisol as a singular marker of stress. Further research could examine other markers such as protein transcript levels and metabolites associated with the stress response.

2.4.2 Enzyme activities

Enzyme activities are often indicators of the metabolic response to stressors (McClelland et al., 2006). They were used as indices of how aerobic and anaerobic glycolysis (PK and LDH), lipid metabolism (HOAD), mitochondrial abundance and aerobic capacity (CS), the electron transport chain (COX), and oxidative stress (CAT) are affected in response to VFX and elevated water temperature exposure. We saw that carbohydrate metabolism was affected by increasing water temperature and VFX exposure, as indicated by the increase seen in PK activity (Fig. 2.4A). This was expected, as energy demand is often higher at elevated environmental temperatures, and carbohydrates are the first energy reserve to be used (Hori et al., 2006; Pörtner and Knust, 2007). It was observed that PK activity was higher in all groups when the assay temperature was set at 32°C instead of 27°C. This was expected as according to the Arrhenius equation, enzyme activities often increase exponentially at higher assay temperatures (Schulte et al., 2011). When measuring HOAD, an enzyme involved in β-oxidation of lipids, there were no differences in activity measured at 27°C between all treatment groups. However, there was clear reduction in activity when the assay temperature was set at 32°C in the groups that were exposed to the higher temperature, although only the VFX & Temp group was significantly different from the Control. The decrease in sensitivity in HOAD at higher temperatures could be an acclimatization response, where the enzyme is more suited to work at higher temperatures (Schnurr et al., 2014). These results are comparable to zebrafish reared at low and high temperatures, where it was found that HOAD activity was higher in zebrafish reared at lower temperatures (Schnurr et al., 2014). The acclimatization response that we observe in groups exposed to higher temperatures could be maladaptive in fishes that experience frequent temperature fluctuations. It is known

that metabolic demand increases in response to increasing water temperatures especially in ectotherms (Englund et al., 2011; Gillooly et al., 2001; Lemoine and Burkepile, 2012). Therefore, fish that are not able to plastically respond to these changes may be at a disadvantage, as they are unable to increase their lipolytic activity to make up for the increased metabolic costs of higher temperatures. We also measured the activity of CS, a rate-limiting enzyme in the citric acid cycle, which is often used as an indicator of aerobic capacity and mitochondrial abundance in the muscle (Lemos et al., 2003; Rajotte and Couture, 2002). We found no differences in CS activity between treatments, however, higher assay temperature resulted in increased activity in all groups. This indicates that CS follows the Arrhenius equation and its thermal sensitivity is not affected by moderate increases in temperature and VFX exposure. Similar results were observed in the muscle tissue of American alligators (*Alligator mississippiensis*) undergoing seasonal acclimatization, where CS activity was higher at increasing assay temperatures (Seebacher, 2003). We also measured the activity of COX, also known as complex IV in the electron transport chain, responsible for the reduction of oxygen to water and is a measure of overall aerobic capacity (Berg et al., 2002). There were no differences in COX activity across treatments and assay temperatures, suggesting that neither environmentally-relevant concentrations of VFX or a 5°C increase in water temperature has an effect on the oxidation capacity of the electron transport chain. Finally, we assessed the effects of VFX and temperature on oxidative stress in the muscle tissue. We measured the activity of CAT, an enzyme involved in the defence against oxidative stress from reactive oxygen species (ROS) production, and is often used as an indicator of cellular damage and environmental stress (Atli et al., 2006; Kessabi et al., 2013). In our study, we observed increased CAT activity in the group exposed to elevated water temperature. This was

expected, as oxygen consumption was also elevated in that group suggesting higher antioxidant enzyme activities in response to the predicted higher levels of ROS produced through mitochondrial respiration (Bagnyukova et al., 2007; Davidson and Schiestl, 2001; Lushchak and Bagnyukova, 2006; Rocha et al., 2003). Similar results were observed in marble trout (*Salmo marmoratus*) larvae exposed to increasing water temperatures, where oxygen consumption was positively correlated with CAT activity (Simčič et al., 2015). The increase in CAT activity in response to elevated water temperature is considered an adaptive response, indicating higher resistance to oxidative stress (Rudneva, 1999). However, this response was not present in fish exposed to both VFX and elevated water temperature, an indication that VFX could have a deleterious effect on the antioxidant defence mechanism, especially since oxygen consumption was elevated in the group of fish exposed to both stressors similar to the group exposed to elevated water temperature alone. This suggests that ROS production is elevated in the multi-stressed group but is not being combated by antioxidant enzymes, leading to oxidative damage (Abele et al., 2002; Akhtar et al., 2013; Bagnyukova et al., 2007). Further research is required to investigate the levels of ROS build-up in the muscle tissue of fish exposed to VFX and elevated temperatures.

2.4.3 Conclusions

Male zebrafish are sensitive to environmentally relevant concentrations of VFX and a 5°C increase in water temperature. Zebrafish exposed to both VFX and high water temperature had elevated RMR, resulting in a reduced AS. Chronic exposure to the stressors used in this study did not result in impairment of the cortisol stress response. It was also evident that VFX and temperature have additive and antagonistic effects on various metabolic pathways in zebrafish.

PK activity was highest in fish exposed to both stressors, however, CAT activity was not positively associated with oxygen consumption in the multi-stressed group. An acclimatization response in lipid metabolism was observed, demonstrated by reduced HOAD activities at the higher assay temperature in the groups that were exposed to elevated water temperature. This is the first study of its kind to demonstrate the effects of both VFX and elevated water individually and cumulatively on the metabolic and stress physiology of zebrafish. VFX and other contaminants are frequently introduced into aquatic ecosystems through treated and untreated wastewater effluents (Arlos et al., 2015; Metcalfe et al., 2010). This study aimed to improve the understanding of how the toxicity of contaminants can change in the presence of other stressors like elevated temperatures. Physiological and ecological responses to multiple stressors can prove to be challenging and complex. However, multi-stressor research continues to be essential, as it aims to bridge the gap between laboratory settings and natural field settings, thereby, providing more accurate assessments than single-stressor research (Ng et al., 2013; Noyes et al., 2009).

Chapter 3

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

3.1 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 including 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 and find their way into surface waters of watersheds receiving WWTP effluent (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), 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 like 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.

3.1.1 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 refocused on 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. Very 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., 2011c; 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). This study aims to investigate how the stress response in rainbow darter 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.

3.1.2 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 like 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 results in energy allocation trade-offs, thereby impeding energy allocation to other basal processes (Handy et al., 1999; Scott and Sloman, 2004; Treberg et al., 2016). Whole-body resting and 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). Higher energy utilization towards detoxification purposes can also constrain fish from properly responding to

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, such as PPCPs, metals, polycyclic aromatic hydrocarbons, pesticides, fertilizers, and others (Abessa et al., 2005; Arlos et al., 2015; Bolong et al., 2009; Daughton and Ternes, 1999; Metcalfe et al., 2010; Ternes et al., 2004). As a result of this complexity, it is essential to understand how the metabolic physiology and other nonlethal endpoints of fish can be affected (Boxall et al., 2012; Rudd et al., 2014). Several studies have examined the impact of wastewater effluent on growth, energy status, and swimming capacity (Adams et al., 1989; Galloway et al., 2003; Goertzen et al., 2011; Smolders et al., 2003). However, more research is needed on whether these endpoints remain affected in fish exposed to treated wastewater effluent under natural conditions.

3.1.3 Study scope

The goals 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 (*Etheostoma caeruleum*) 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 WTTPs that discharge their effluent in the river. The chemical profile of the discharged effluent from WTTPs and surface waters has been well studied and reported in the past (Arlos et al., 2015; Metcalfe et al., 2010). This study hypothesizes that fish exposed to the effluent will have elevated baseline cortisol levels and lowered elevated cortisol levels due to chronic exposure to stress causing an impaired stress response. Additionally, we hypothesize that routine metabolic rate will be higher in fish exposed to the effluent accompanied by elevated enzyme activity in the muscle tissue, likely as a compensatory mechanism for the added metabolic costs of detoxification.

3.2 Materials and methods

3.2.1 Fish collection

In April 2017, male and female rainbow dater were collected from 2 sites in the Grand River, southwestern Ontario, Canada, this collection period coincided with their breeding season (Fuzzen et al. 2016). The sites were located upstream (WMR) and downstream (EIT) of the Waterloo Municipal Wastewater Treatment Plant, serving as the reference and contaminated sites respectively (Fig. 3.1). Fish were collected using a backpack electrofisher (Smith Root, LR-20) and held in aerated buckets. A subset of the collected fish we used for enzyme analysis, the length (±0.1 cm), total, gonadal, and hepatic weight (±0.001 g) were recorded prior to the collection of epaxial muscle tissue. Muscle samples were snap-frozen in liquid nitrogen and stored at -80°C until further analysis. Another subset of the collected fish was brought back to the laboratory for cortisol stress response analysis. 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 14°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. 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, but the water temperature in the lab was maintained at 20°C to mimic the conditions in the river. Fish collected in September were used fish in respirometry experiments to measure whole-body routine metabolic rates. All experimental and handling protocols follow the Canadian Council of Animal Care guidelines and were approved by the Animal Care Committee at the University of Waterloo (AUPP #15-03).

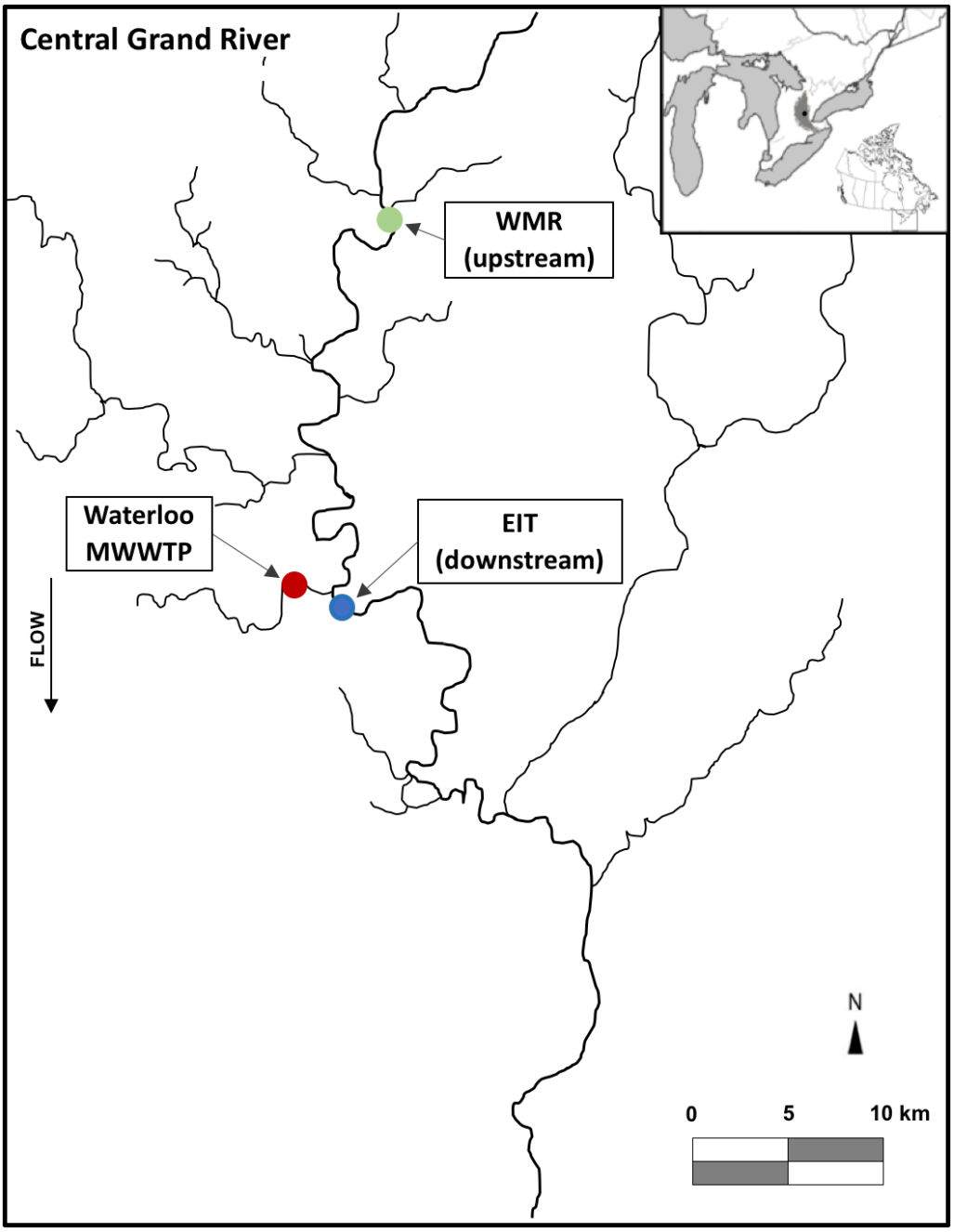


Figure 3.1: Map of the central portion of the Grand River watershed illustrating the location of the collection sites and Waterloo Wastewater Treatment Plant. The site GPS coordinates were: WMR (upstream): 43°35'08"N (latitude) 80°28'53"W (longitude); EIT (downstream): 43°28'24"N (latitude) 80°28'22"W (longitude), and Waterloo MWWTP: 43°29'16"N 80°30'25"W (map modified from Marjan et al., 2017).

3.2.2 Exp. 1: Stress and cortisol analysis

Fish were randomly assigned into 2 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. 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. Blood samples were collected using the retro-orbital technique described in (Vliegenthart et al., 2014). Briefly, following euthanasia, fish were dried gently and 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 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 recording of gonadal weight for GSI calculations. This method of blood collection was appropriate due to the small body size of 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.

3.2.3 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). Following oxygen consumption measurements, fish were weighed (±0.01 g) and returned to their housing tanks.

3.2.4 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 cytochrome oxidase (COX; E.C. 1.9.3.1), citrate synthase (CS; E.C. 2.3.3.1), and catalase (CAT; E.C. 1.11.1.6). Enzyme activity was normalized to the protein concentration of individual fish, which was determined using bicinchoninic acid (BCA) assay with bovine serum being used as a standard. The assays were prepared as described in (Mehdi et al. 2017, unpublished). All chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada).

3.2.5 Statistical analyses

Data were analyzed using SigmaPlot 13.0 software (Systat Software Inc., San Jose, CA, USA). Data is presented as means ± 1 standard error of the mean (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. Non-parametric statistics (ANOVA on ranks) were only used if data was not normally distributed. The alpha value was set at 0.05 (i.e., $P<0.05$) for all tests.

3.3 Results

3.3.1 Morphological measurements

Rainbow darter used in *Exp 1* demonstrated significant morphological differences between sites (Table 3.1). Males collected from the upstream site were significantly longer, heavier, and in better body condition 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 (GSI) than fish from the upstream site, whereas only males collected from the downstream site had higher hepatic investment (HSI) compared to males from the upstream site. No differences were observed in any of the remaining fish, regardless of sex or site.

Table 3.1: Morphological measurements of rainbow darter collected upstream and downstream of the Waterloo Wastewater Treatment Plant in the Grand River. Values are represented 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.*

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

3.3.2 Exp. 1: Cortisol stress response

Baseline plasma cortisol concentrations are presented in Fig. 3.2A. 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, where elevated cortisol levels were always significantly higher than baseline levels (Fig. 3.2A, B). Analysis revealed female cortisol response were similar between upstream and downstream sites, however significantly higher than their male counterparts in the upstream and downstream sites (2.1x and 2.3x respectively).

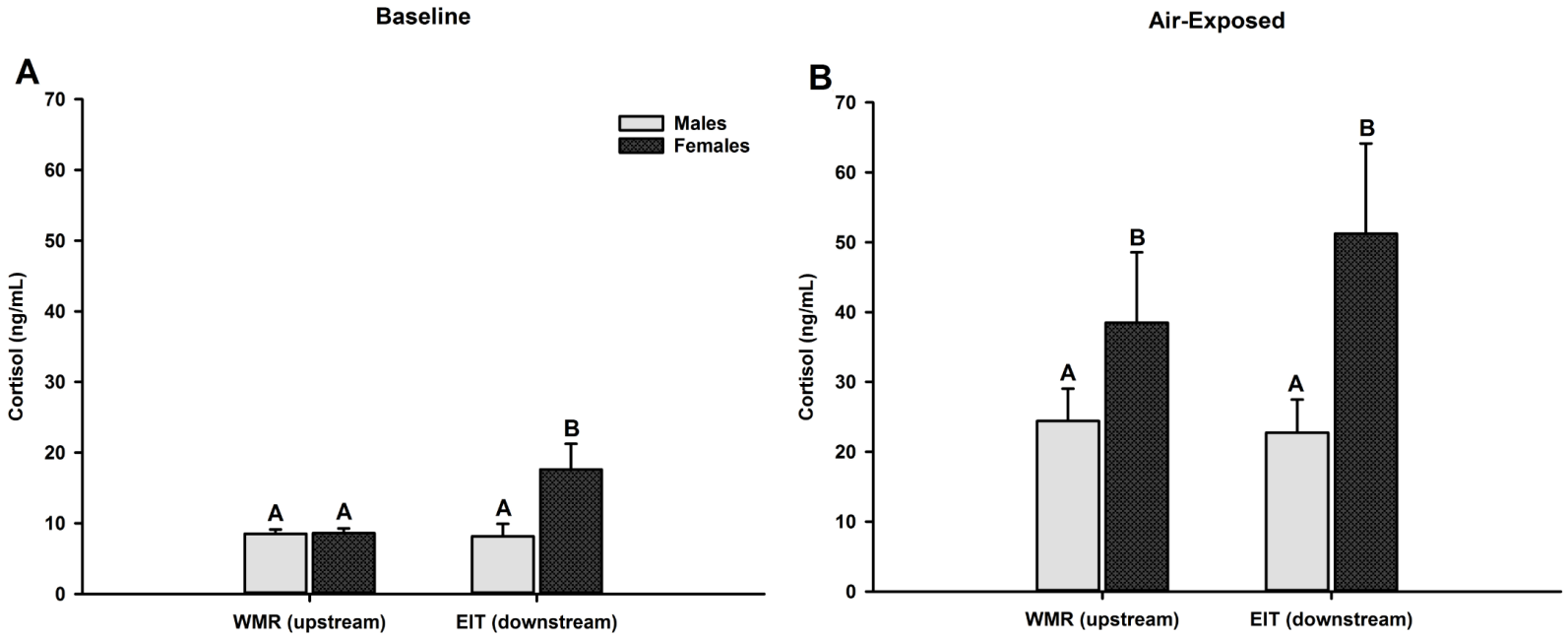


Figure 3.2: Plasma cortisol concentrations separated by sex and site, (A) presents baseline plasma cortisol levels and (B) presents elevated plasma cortisol levels following an air-exposure stressor. Data are represented as mean ± S.E.M of n=9 replicates per site per sex. Bars that do not share letters indicate significant difference using a two-way ANOVA followed by Tukey's post-hoc test ($P<0.05$).

3.3.3 Exp. 2: Metabolic rate

Differences in routine metabolic rate (RMR) were determined in male and female rainbow darter using closed-chamber respirometry (Fig. 3.3). There was a significant, ~2x increase in RMR in both male and female rainbow darter from the downstream site compared to fish collected upstream.

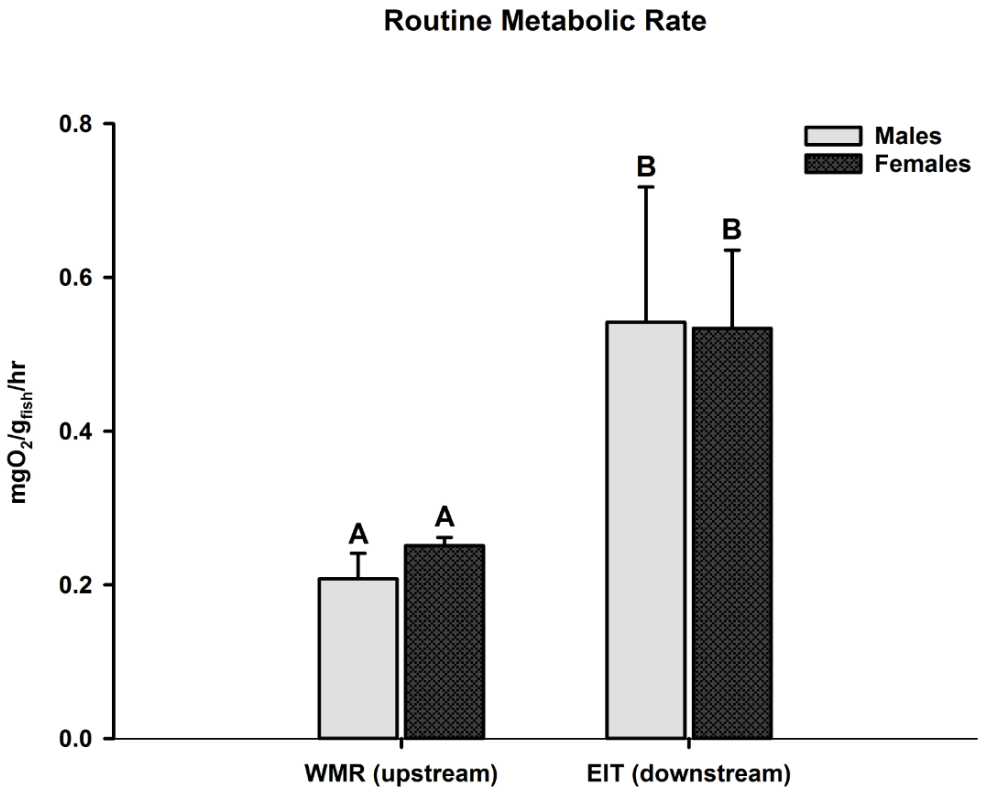
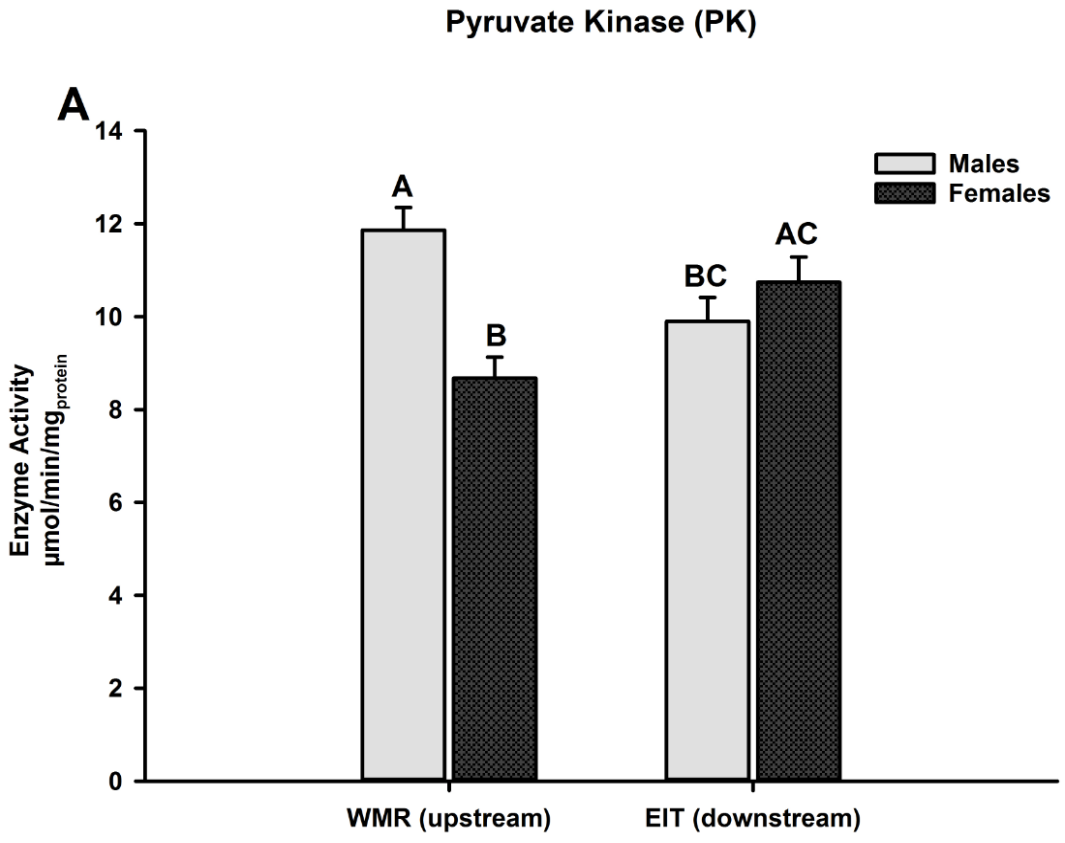
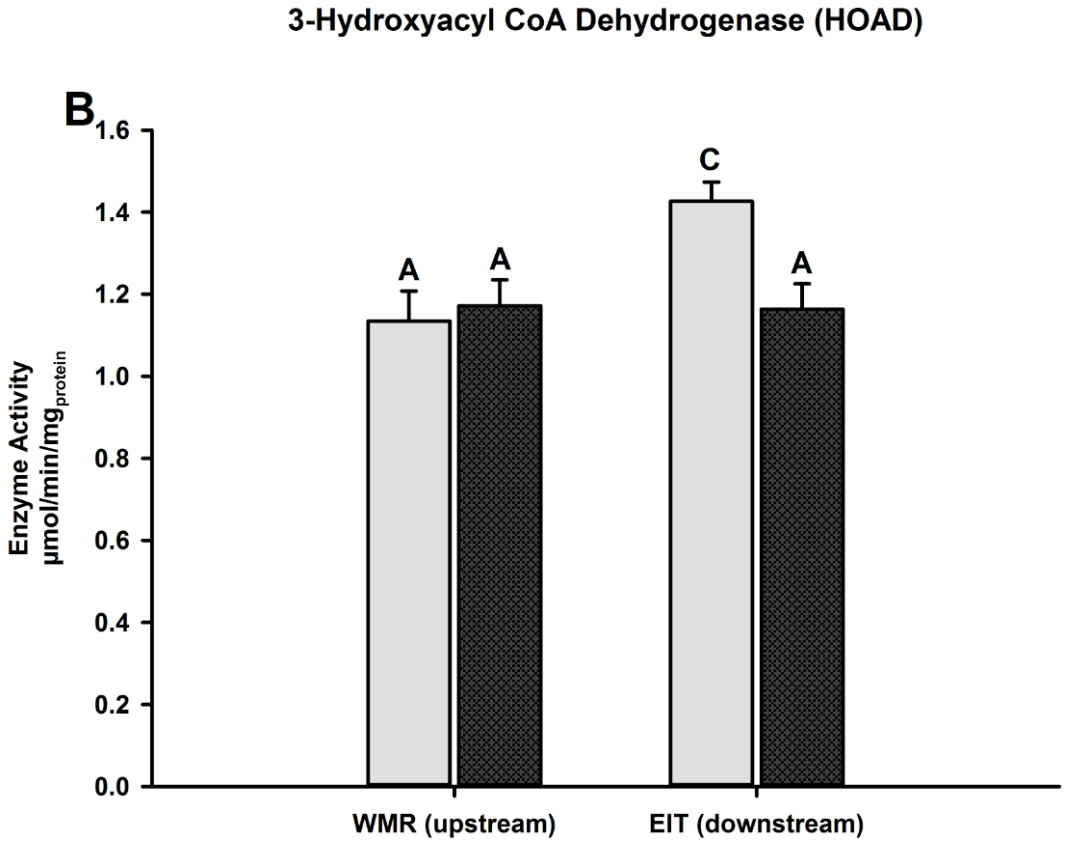


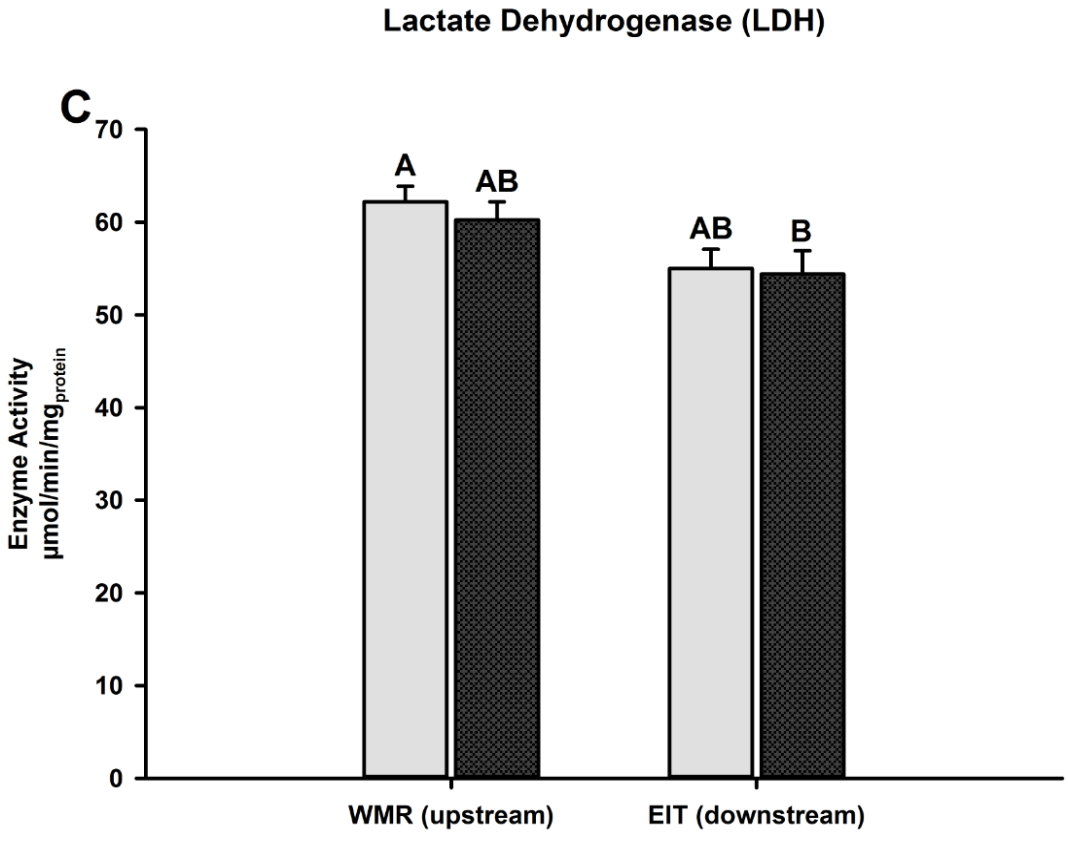
Figure 3.3: Routine metabolic rate (RMR) measured in rainbow darter collected upstream and downstream of the effluent. Data are represented as mean ± S.E.M of n=5-6 fish per site per sex. Bars that do not share letters indicate significant difference using a two-way ANOVA followed by Tukey's post-hoc test ($P<0.05$).

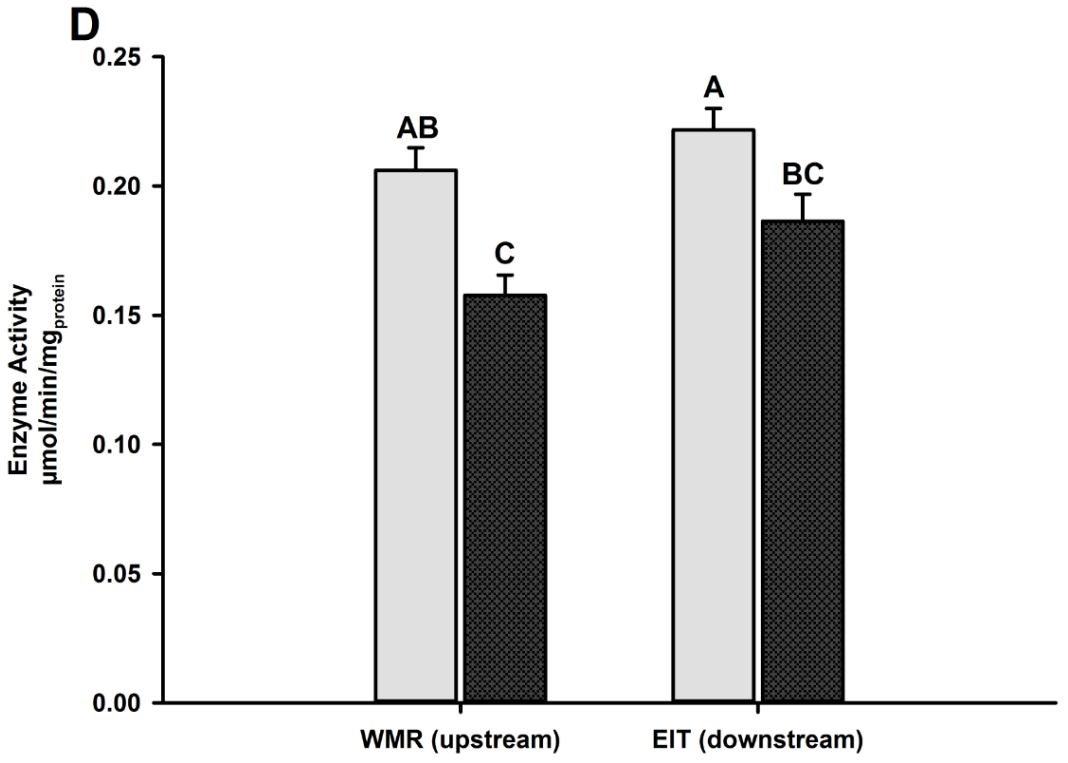
3.3.4 Exp. 3: Enzyme activities

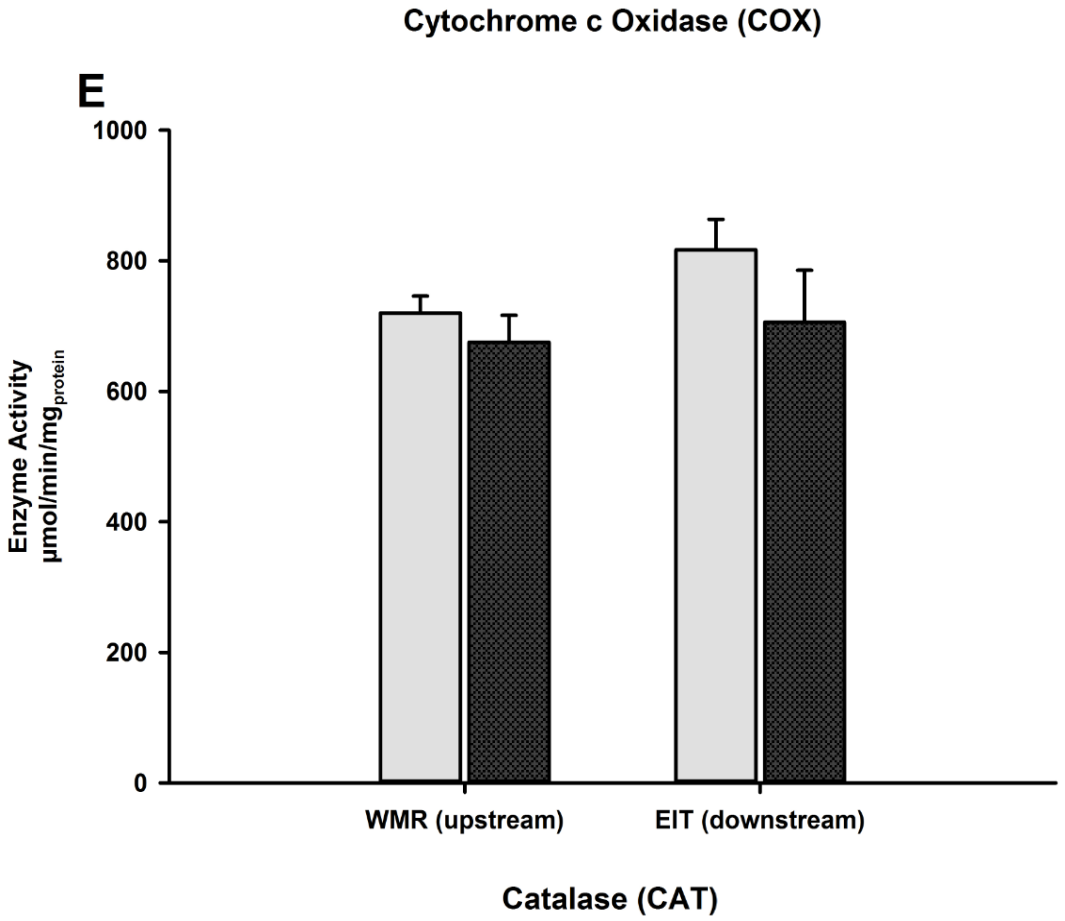
Activities of six muscle metabolic enzymes were measured and data is presented in Fig. 3.4. Pyruvate kinase (PK) activity was 1.3x higher in males from the upstream site compared to females from the same site and 1.2x than males from the downstream site (Fig. 3.4A). 3-hydroxyacyl CoA dehydrogenase (HOAD) activity was ~1.2x higher in males from the downstream site than any other group (Fig. 3.4B). Lactate dehydrogenase (LDH) activity was only significantly different between males from the upstream site and females from the downstream site (1.1x) (Fig. 3.4C). Citrate synthase (CS) activity was ~1.2x higher in males than females within each site (Fig. 3.4D). There were no significant differences in cytochrome c oxidase (COX) activity between any of the groups (Fig. 3.4E). Catalase (CAT) activity was 1.4x higher in males than females in rainbow darter from the downstream site (Fig. 3.4F).


Pyruvate Kinase (PK)
A
Males
Females
Enzyme Activity
µmol/min/mg protein
0
2
4
6
8
10
12
14
A
B
BC
AC
WMR (upstream)
EIT (downstream)


3-Hydroxyacyl CoA Dehydrogenase (HOAD)
B
Enzyme Activity
µmol/min/mg protein
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
A
A
C
A
WMR (upstream)
EIT (downstream)


Lactate Dehydrogenase (LDH)
C
Enzyme Activity
µmol/min/mg protein
70
60
50
40
30
20
10
0
A
AB
AB
B
WMR (upstream)
EIT (downstream)


Citrate Synthase (CS)
D
Enzyme Activity
µmol/min/mg protein
0.25
0.20
0.15
0.10
0.05
0.00
AB
C
A
BC
WMR (upstream)
EIT (downstream)


Cytochrome c Oxidase (COX)
E
1000
800
600
400
200
0
Enzyme Activity
µmol/min/mg protein
WMR (upstream)
EIT (downstream)

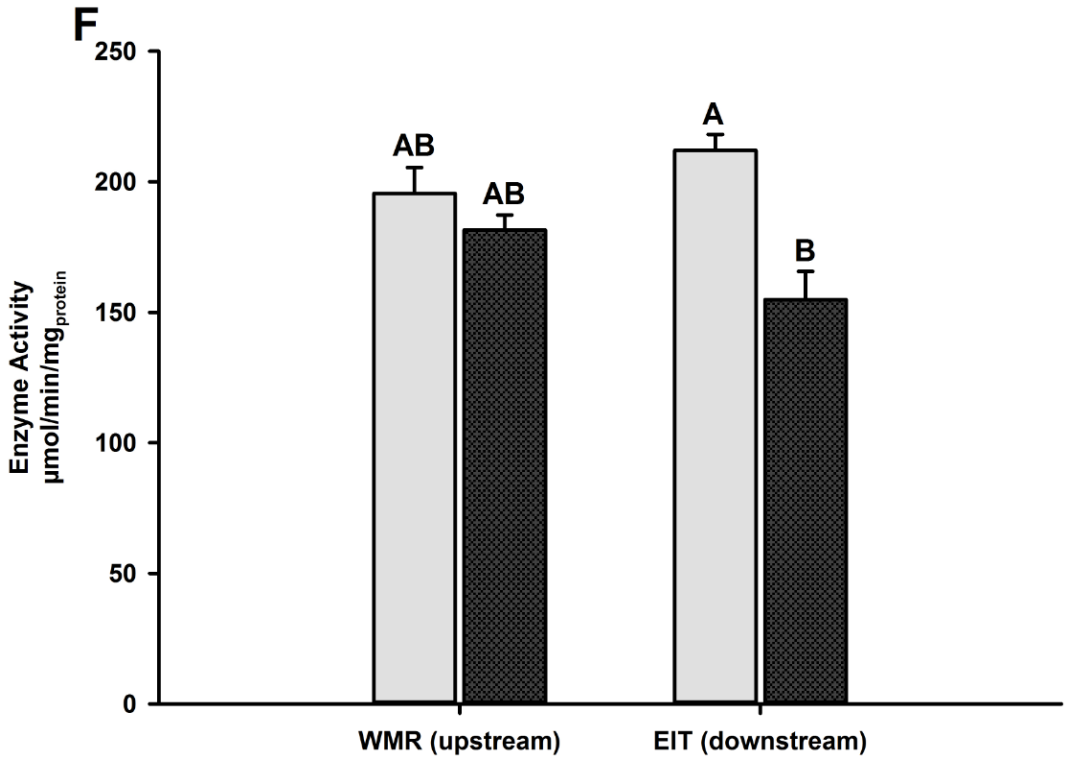

Catalase (CAT)
F
250
200
150
100
50
0
Enzyme Activity
µmol/min/mg protein
AB
AB
A
B
WMR (upstream)
EIT (downstream)

Figure 3.4: Muscle enzyme activity of (A) pyruvate kinase (PK), (B) 3-hydroxyacyl CoA dehydrogenase (HOAD), (C) lactate dehydrogenase (LDH), (D) citrate synthase (CS), (E) cytochrome c oxidase (COX), and (F) catalase (CAT) measured in rainbow darter collected from upstream and downstream of the effluent. Data are presented as mean $\pm$ SEM of n=12 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 ($P<0.05$).

3.4 Discussion

Rainbow darter are a sentinel model organism in the Grand River watershed, and our current and ongoing knowledge about their morphology, behaviour, reproduction, physiology, and population dynamics provide us with strong tools to understand the effects of anthropogenic disturbances on fish health and well-being. This is the first study of its kind to assess sublethal impacts of WWTP effluent on the bioenergetics and stress response in rainbow darter under natural field conditions.

3.4.1 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 poor 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. 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). This study's 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 the fish collected downstream of the effluent displayed poor body condition, which could be reflective of fishing conditions and sampling of that day. It has been reported that fish with 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 to improve our understanding in fish collecting techniques.

3.4.2 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 elevated and baseline cortisol levels, but only females found downstream of WWTP had higher baseline cortisol levels (Fig. 3.2). 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., 2011b; 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, 2016b). Contaminants associated with WWTP effluent are known to increase ethoxyresorfin-O-deethylase (EROD) activity, which 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, 2011b; 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), female plasma glucocorticoids in females could be transferred in ovo as a form of communication with the offspring. Therefore, elevated baseline cortisol levels in females found in poor environments may be a mechanism by which stressed females could alter the phenotype of their offspring through maternal stress hormones (Rhees and Fleming, 1981; Sloman, 2010). It has been shown that manipulation of maternal cortisol levels affects the morphology, behaviour, and physiology of the offspring (Eriksen et al., 2011, 2006; Leatherland et al., 2010; Nesan and Vijayan, 2012; Saino et al., 2005; Sloman, 2010). In a similar study, round goby (*Neogobius melanostomus*) living in highly contaminated areas did not show stress response impairment (Marentette et al., 2012). Rainbow dater and round goby may be more resilient in regards to their stress response than other fish, possibly providing an 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 (J. Weis, 2002; Wendelaar Bonga, 1997). Further studies could examine the variations in the stress response across different species exposed to similar conditions.

The majority of this study's results suggest that sex was the stronger variable in explaining differences in the cortisol stress response. Females from both sites had higher elevated 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 to reduce 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, where both male rainbow trout and brook trout (*Salvelinus fontinalis*) in the presence of elevated gonadal steroids associated with sexual maturation have demonstrated a reduced stress response to acute and chronic stressors (Pottinger et al., 1996, 1995). Moreover, female three-spined sticklebacks display greater levels of cortisol released following an acute stressor (Pottinger and Matthiessen, 2016b). 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).

3.4.3 Exp. 2: Metabolic rate

The oxygen consumption data confirms the initial hypotheses of this study, male and female rainbow darter from the downstream site had higher routine metabolic rates than fish from the upstream site. The increase in metabolic rate can be attributed to the increased metabolic costs associated with higher contaminant exposure from WWTP effluent. Previous research has demonstrated an increase in oxidative stress, 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 higher concentrations had significantly reduced whole-body lipid content (Melvin, 2015). These findings suggest that due to metabolic trade-offs caused by detoxification, energy

reserves would be reduced and oxidative damage would be more apparent (Melvin, 2015; Smolders et al., 2003). Whole-body metabolic rate measurements allow further investigation in the increase in energy demand put on by metabolic costs of detoxification (Scott and Sloman, 2004). The increase in oxygen consumption observed in this study in fish from the downstream site supports this hypothesis.

3.4.4 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). It was predicted that both glycolytic enzymes (PK and LDH) would have higher activities in the fish from the downstream site than those from the upstream site. Carbohydrates are the first energy reserves used in cases of stress, and is 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 in the fish from the downstream site, but only in females, 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. 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 make up for the metabolic costs of detoxification in 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 rate-limiting enzyme in the citric acid cycle and is often used as an indicator of aerobic capacity and mitochondrial abundance in the muscle (Lemos et al., 2003; Rajotte and Couture, 2002). While there were no site differences in CS activity, female rainbow darter had significantly lower CS activity than males in the same sites. Lower CS activity in females than males suggests that females have a lower ability to produce ATP through aerobic substrate metabolism. These results are similar to data from spottail shiner (*Notropis hudsonius*) collected downstream of a uranium mill effluent. Shiners in the exposure site had significantly higher HOAD activity, however CS activity was the same in both sites (Goertzen et al., 2012). However, the fish used in this study were sexually immature, whereas the fish used in our study were all sexually mature and collected during their breeding season. COX (complex IV) activity was also measured, which is found in the electron transport chain and 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 an effect on the electron transport chain oxidation capacity. Finally, CAT activity was measured, which demonstrated that males from the downstream site had a 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). These results suggest that in the presence of stressors associated with WWTP effluents, male rainbow darter are more resilient to oxidative stress through the elevation of CAT activity to minimize the oxidative damage caused by reactive oxygen species (ROS) (Kong et al., 2012). Alternatively, males could be producing more ROS, therefore CAT activity is elevated as a protective response. The increase in CAT activity might be in response to the increase in oxygen consumption (Fig. 3.3), which typically induces increased ROS production (Ritola et al., 2002). Oxygen consumption was higher in both males and females 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. Another explanation to the sexual dimorphism observed is possibly due to the increased reproductive investment that females often encounter during breeding season, females could be experiencing a state of energy deficit, which could leave them vulnerable to oxidative damage (Treberg et al., 2016).

3.4.5 Conclusions

It was found that 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 non-target species as they are exposed to a wide variety of stressors simultaneously. Previously, research had been concentrated on the reproductive responses to WWTP effluents, but recently, research has been refocused on other sublethal endpoints such as metabolic and stress physiology, and behaviour. This is the first study of its kind to look at a wide variety of nonlethal endpoints spanning several levels of biology and biochemistry regarding the

effects of WWTP effluents on rainbow darter in the Grand River watershed. This work has further implications on the protection of aquatic ecosystems from contaminants of emerging concern. 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).

Chapter 4

General Conclusion

The goal of this thesis was to explore the effects of multiple stressors associated with WTTP contamination and climate change on the physiological responses of fish using both lab and field assessments. Understanding the cumulative effects of stressors is important, as fish and other wildlife are simultaneously exposed to a wide variety of stressors in their environments, causing multiple stressor effects. Effects of multiple stressors are different and more complex than the sum effects of individual stressors. This was further highlighted in this thesis, as it was able to explain the effects of multiple stressors using controlled-lab experiments in combination with field-based experiments. These studies advance the understanding of how fishes and other non-target aquatic organisms respond to multiple stressors. In the lab, fish were exposed to known stressors (VFX and high temperature) and their responses to these stressors were assessed individually and cumulatively. Further, field-based assessments were performed on fish exposed to multiple stressors in their natural environments. This allowed for a comparison to be done in the responses measured between zebrafish exposed to stressors in the lab and rainbow darter exposed to WWTP effluent in the field. A summary of the findings from both studies are listed below and in table 4.1:

- **Lab study (chapter 2):** This chapter investigated the individual and cumulative effects of chronic exposure to environmentally-relevant levels of VFX and elevated water temperatures on the energetics and stress response of zebrafish. Fish exposed to multiple stressors had elevated oxygen consumption rates, resulting in diminished aerobic scope. Neither stressor, singularly or cumulatively had a significant impact on the cortisol stress response when fish were subjected to a confinement stressor. Additionally, the enzyme activity results indicated that exposure to multiple stressors poses a higher energy

demand, demonstrated by increased PK activity. However, long-term exposure to stressors can also cause an acclimatization response, indicated by reduced HOAD activity at higher assay temperatures. Finally, VFX was observed to have deleterious effects on the antioxidant defence mechanism.

- **Field study (chapter 3):** This chapter investigated the effects of municipal WWTP effluents on the energetics and stress response of rainbow darter, a native fish species in the Grand River watershed. It was found that fish located downstream of the effluent had elevated oxygen consumption rates. Long-term exposure to WWTP effluent did not seem to impair the stress response of rainbow darter, although female fish caught downstream of the effluent had elevated baseline cortisol levels. Females from both sites had higher elevated cortisol levels than their male counterparts. Finally, differences in enzyme activities were observed, however, differences were more associated with sex rather than collection site.

The similarities in responses measured between lab and field studies could potentially be used as future biomarkers of how organisms found in heavily disturbed ecosystems may respond to multiple stressors. The results presented in this thesis suggest that the use of metabolic rate data could help explain how organisms exposed to multiple stressors may exhibit changes in overall metabolic scope. Much of the stressors that fish are exposed to in their environments can pose additional metabolic costs which may interfere with organismal energy allocation to other basal processes. The use of enzyme activity analyses could allow for further investigation as to which specific metabolic processes are being affected by the onset of stressors. In this thesis, overall metabolic rate was elevated in both fish species exposed to multiple stressors, however, changes in specific enzyme activities were different. This could further highlight the nature of

how different stressors affect specific metabolic processes differently. The similarities and differences between the lab and field studies are further highlighted in Table 4.1.

It is important to realize that there are major differences between laboratory- and field-based research. In the lab study presented in this thesis, zebrafish were only exposed to two stressors (VFX and elevated water temperature), whereas rainbow darter were exposed to a wider variety of stressors simultaneously in their natural habitat. Such stressors are considered novel for an organism that has been reared in a laboratory setting. Thereby, responses exhibited by laboratory-reared organisms may be different than what other organisms exhibit, if exposed to similar stressors under natural field conditions. Rainbow darter have been shown to have high site fidelity (Hicks and Servos, 2017), therefore, it is expected that they have been exposed to stressors found in their environments far longer than what zebrafish were exposed for in the lab study. Such long-term exposure may have allowed rainbow darter to evolve tolerance to chemical contaminants. The responses measured in rainbow darter could have been a result of many years of acclimatization (Weis, 2002). It is believed that tolerance as well as other factors such as life history, behaviour, population variation, and phylogeny in organisms should be considered in future risk assessments, especially when extrapolations are being made from laboratory studies to natural environments. The studies presented in this thesis have further implications on the protection of aquatic ecosystems from contaminants of emerging concern and climate change. The use of nonlethal endpoints, such as metabolic and stress physiology, improve our understanding of how fishes respond to environmental perturbations. Nonlethal endpoints are linked to higher biological processes such as reproduction and survival, and can

therefore act as early indicators of population or community level stress (Brodin et al. 2014; Scott and Sloman, 2004).

To my knowledge, this is the first study to look at the effects of multiple stressors associated with WWTP contamination and climate change on energetics and stress physiology of fishes using both laboratory- and field-based assessments. Previous research has examined the effects of stressors on non-target organisms using a range of assessment techniques, including but not limited to lab exposures, cage exposures, and field collection of exposed fish from wild populations (Ings et al., 2012; Marentette et al. 2012; McCallum et al., 2017). While each technique has its positives and negatives, it is important to integrate all aspects of research when asking scientific questions. Laboratory-based experiments can provide an initial understanding of how organisms will respond to stressors in a straightforward manner, where control for many of the confounding variables is possible. However, findings often do not align perfectly with experiments set out in an organism's natural environment, where control over biotic and abiotic factors is limited (Fig. 4.1). As reviewed in Calisi and Bentley (2009), due to a variety of differences between laboratory and field-based experiments, it is important to be cognizant of the data research yields, and the limitations associated with it. Due to the limitations of laboratory studies, one should be aware of how data is interpreted to what extent it can be extrapolated to the real world. On the other hand, field studies have limitations of their own too, due to noise caused by uncontrolled factors in natural environments, important relationships might be missed. To summarize, neither laboratory or field experiments is more pertinent over the other. Rather, it is only through the combination of both will we be able to fully comprehend the impacts of multi-stressed environments, such as those impacted by WWTP effluents.

Table 4.1: Summary of all endpoints measured in lab and field studies in zebrafish and rainbow darter, respectively. Responses in the lab study are shown relative to the Control treatment. In the field study, responses are separated by sex, where males from the downstream site are directly compared to males from the upstream site and females from the downstream site are directly compared to females from the upstream site.

		Lab study				Field study			
	Endpoint	***Control***	***VFX***	***Temp***	***VFX & Temp***	***Upstream (males)***	***Downstream (males)***	***Upstream (females)***	***Downstream (females)***
Metabolic rate and respirometry	RMR	-	No change	No change	Elevated	-	Elevated	-	Elevated
	AMR	-	No change	No change	No change	-	-	-	-
	AS	-	No change	Reduced	Reduced	-	-	-	-
	Ucrit	-	No change	No change	No change	-	-	-	-
Stress response	Baseline cortisol	-	-	-	-	-	No change	-	Elevated
	Elevated cortisol	-	No change	No change	No change	-	No change	-	No change
Enzyme activities	PK	-	No change	No change	Elevated	-	Reduced	-	Elevated
	HOAD	-	No change	No change	Reduced @32°C	-	Elevated	-	No change
	LDH	-	No change	No change	No change	-	No change	-	No change
	CS	-	No change	No change	No change	-	No change	-	No change
	COX	-	No change	No change	No change	-	No change	-	No change
	CAT	-	No change	Elevated	No change	-	No change	-	No change

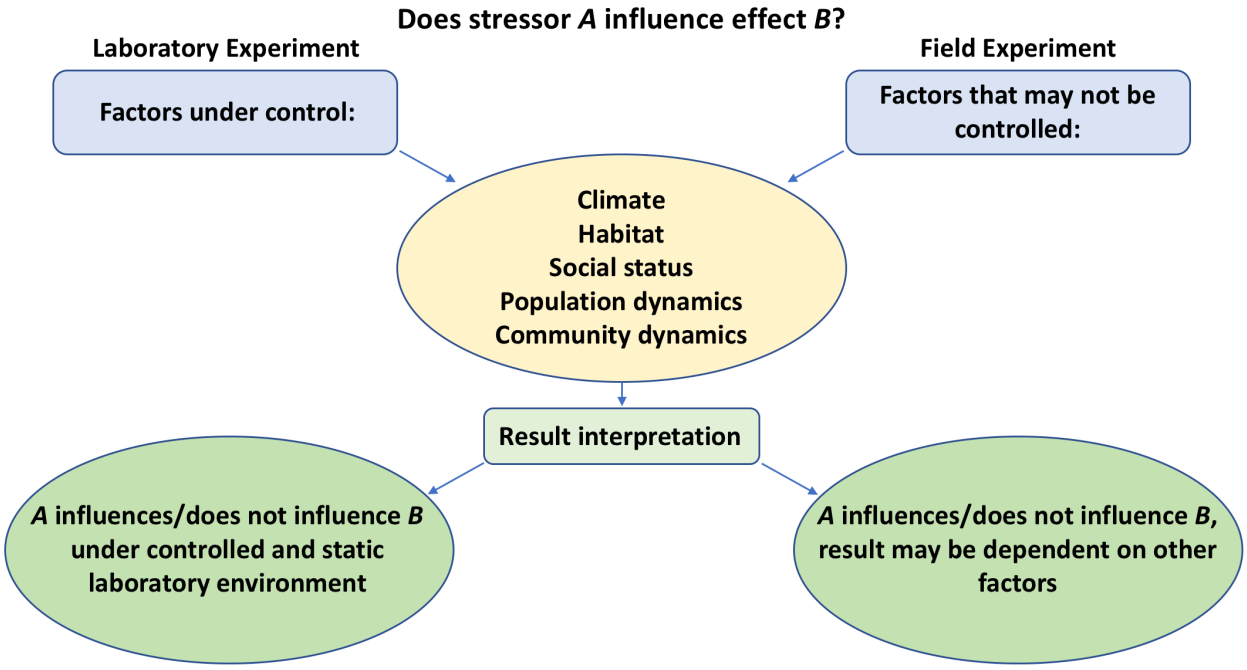


Figure 4.1: Differences in laboratory and field experiments and the factors that can influence each. Both techniques are not sufficient on their own, thus, a combination of the is needed to yield more accurate and holistic data (Figure modified from Calisi and Bentley, 2009).

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