Antioxidant Defence Response in Non-Target Fishes Following Pharmaceutical Wastewater Effluent Exposure

by

Nicole Laurette Gauvreau

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Author's declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Statement of contributions

Dr. Paul Craig was the primary investigator on the Natural Sciences and Engineering Research Council (NSERC) Discovery Grant (RGPIN-2015-05643) and the Ontario Early Research Award (ER18-14-254) grants which supported this work. This research was conducted at the University of Waterloo by Nicole Gauvreau under the supervision of Dr. Paul Craig.

Nicole Gauvreau conducted the experimental design, sample collection and analysis, data analysis, and manuscript writing. Ladees Al Hafi assisted with cell culture experimental design, sample, and data analysis. Dr. Paul Craig assisted with experimental design, manuscript edits and provided funding. Leslie Bragg assisted with water chemistry analysis.

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Abstract

The Grand River watershed extends throughout the majority of Southern Ontario with its final outlet at Lake Erie and accommodates thirty wastewater treatment plants (WWTP) with varying types and degrees of treatment. Many WWTPs are currently unable to effectively eliminate several contaminants of concern (CECs) from their final effluent, leading to measurable concentrations in surface waters and ultimately chronically exposing aquatic species to mixtures of CECs. Exposures to CECs have reported impacts on oxidative stress, measurable through reactive oxygen species (ROS) and the antioxidant defense response which helps reduce the toxicity of ROS generated molecules. This thesis aims to investigate the effects of WWTP effluent on four *Etheostoma* (Darter) species endemic to the Grand River. Objectives were to examine if increased antioxidative response markers are present in the brains of darters downstream from the effluent outfall compared to clean reference site upstream relative to the Waterloo, ON WWTP between two separate years (Fall 2020 and Fall 2021). This was assessed using transcriptional analysis and enzymatic analysis of antioxidant enzymes (SOD, GPX, CAT) and an enzyme involved in serotonin synthesis (TPH). In fall 2020, significant differences in transcript expression of markers were found among sites and sexes in greenside darters (GSD) with SOD and CAT showing increased expression downstream. Changes in transcript expression aligned with antioxidative enzyme activity where interactive effects with sex-related differences were observed in fish collected the Fall of 2020. In contrast, transcription markers measured in Fall 2021 were increased upstream compared to species below the effluent outfall.

Field research is essential to understand subtle effects of wastewater effluent on non-target species, meaning a species that is not intentionally targeted by CECs. Using *in vitro* studies to supplement *in vivo* studies provide a better understanding in the mechanisms for any observed

phenotypic response. Therefore, this thesis also aimed to investigate the mechanism in alterations in antioxidant response by exposing isolated brain primary cell culture collected from zebrafish and darters to environmentally relevant concentrations of venlafaxine. Antioxidant response of the cells was assessed through cell viability, and antioxidant enzyme activity. Antioxidant enzyme activity of SOD and CAT were both increased in zebrafish, RBD, and GSD isolated brain cultures exposed to 0.01-1 ug/L of venlafaxine. This response supplements the observed changes in antioxidant response of darters in the Grand River as they are chronically exposed to contaminated effluent containing notably high concentrations of pharmaceuticals.

Overall, this thesis demonstrates how yearly varying abiotic factors such as observed temperature increases in Fall 2021, species-specific differences, sex-differences, and venlafaxine have roles in increased antioxidant response observed in non-target species. Continued investigation on the impacts of pharmaceutical exposures in non-target organisms is crucial to further the knowledge of WWTP effluent impacts.

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APX – ascorbate peroxidase
BOD – biochemical oxygen demands
CAT – catalase
CBZ – carbamazepine
CECs – contaminant(s) of emerging concern
CYP – cytochrome P450
CYTO – cytosolic fraction
DCF – diclofenac
EDC – endocrine disrupting compounds
EIT – Economical Insurance Trailway; immediately downstream of Waterloo WWTP
FTD – fantail darter (*Etheostoma flabellare*)
GPX – glutathione peroxidase
GSD – greenside darter (*Etheostoma blennioides*)
GSH – glutathione
GSI – gonadosomatic index
HSI – hepatosomatic index
JD – johnny darter (*Etheostoma nigrum*)
K – Fulton's condition factor
KIW – Kiwanis; upstream of Waterloo WWTP
LPO – lipid peroxidation
MDA – malondialdehyde
MITO – light mitochondrial fraction
O-VEN – O-desmethyl venlafaxine
PPCP – pharmaceutical and personal care product(s)
PUFA – polyunsaturated fatty acids
RBD – rainbow darter (*Etheostoma caeruleum*)
ROS – reactive oxygen species
SNRI – selective norepinephrine reuptake inhibitor
SSRI – serotonin re-uptake inhibitors
SOD – superoxide dismutase
TPH – tryptophan hydroxylase
TSS – total suspended solids
VEN – venlafaxine
WWTPs – wastewater treatment plants

Chapter 1

General Introduction

1.0.0 General Introduction

1.1.0 Grand River Watershed

The Grand River watershed is the largest watershed in southern Ontario, covering an area of roughly 6965 km^2. Located primarily in several major municipalities, the watershed is drained by the Grand River and four major tributaries, the Nith, Speed, Eramosa, and Conestogo Rivers, which supports a population of over one million in 39 municipalities and two First Nations. The Grand River receives wastewater effluent from thirty different wastewater treatment plants (WWTPs) all with varying degrees of sewage treatment, and its additionally influenced by agricultural areas and urban development (Cooke, 2006; Srikanthan, 2019). The watershed found upstream in the Grand River (North of Westmontrose, Waterloo) is mostly of good water quality due to the minimal urban influences and the reduced magnitude of agricultural practices (Cooke, 2006). However, descending downstream of the Grand River (South of Kiwanis Park, Waterloo) there is a decrease in the water quality due to the increased influence of urban development and agriculture (Cooke, 2006; Srikanthan, 2019). The increased urban influence, in combination with climate change, water quality downstream of WWTPs are at risk for negative impacts to its ecology and wildlife (Hodgson et al., 2020).

1.1.1 Municipal Wastewater Treatment Plants

WWTPs are generally designed to remove and reduce contaminants such as total suspended solids (TSS), ammonia, phosphorus, and other contaminants such as pharmaceutical compounds (Srikanthan, 2019). If pharmaceuticals and contaminants aren't efficiently removed by WWTPs then treated effluent released into environments can contain the bioactive ingredient of consumed pharmaceuticals, the excreted metabolites or its altered product following human waste excretion (Lishman et al., 2006). The degree of contaminant removal by WWTPs depend on the type of

treatment and operating systems present in each plant (Lishman et al., 2006). In general, WWTPs receives liquids containing solid, organic, and inorganic waste, which are directed to primary treatment that settles solids and organic matter to then be filtered and removed by physical clarification and/or chemical processes (Government of Canada, 2020). The waste is then treated by a secondary treatment which introduces biological treatments and processes to further remove organic matter and suspended solids via sludge systems. Lastly, tertiary treatments may involve filtration, biological oxidation and disinfection processes implemented to remove specific contaminants of concern through physical, chemical, and biological treatments. Once this final step is completed, treated effluent is then released into the environment. Unfortunately, roughly 150 billion litres of untreated and undertreated wastewater containing several contaminants are still released into aquatic environments every year in Canada (Government of Canada, 2017).

1.1.2 Kitchener-Waterloo Wastewater Treatment Plants

Two of the largest WWTPs along the Grand River are located in Kitchener and Waterloo which serve a population of over 600,000. Within the last decade between 2009-2018, both WWTPs have undergone major upgrades to their infrastructures and treatment processes such as added aeration which increases nitrification, increased solid retention time and UV disinfection (Bicudo et al., 2016; Hicks et al., 2017; Srikanthan, 2019). These upgrades have been observed to improve traditional water quality parameters such as biochemical oxygen demands (BOD), total suspended solids (TSS), chlorine and ammonia levels (Hicks et al., 2017; Bicudo et al., 2016; Srikanthan, 2019), as well as decrease concentrations of certain pharmaceuticals in the final effluent (Hicks et al., 2017; Srikanthan, 2019). However, despite these WWTPs having been upgraded to decrease the presence of contaminants being introduced into aquatic environments, several unregulated contaminants of emerging concern (CECs) are still released from the final

effluent and found in low concentrations (ng/L – μg/L; Tran et al., 2018; Srikanthan, 2019), ultimately leading to major effects on gonad development, hormone production and morphological measurements in aquatic populations (Fuzzen et al., 2016).

Prior to the upgrades, CECs such as estrogen, ibuprofen and naproxen were cause for concern due to their high concentrations found within effluent (Arlos et al., 2015; Fuzzen et al., 2016; Hicks et al., 2017). Fortunately, following the upgrades, these contaminants have been significantly reduced associated with the increase in nitrification processes (Srikanthan, 2019). In contrast, several pharmaceuticals such as venlafaxine (VEN), carbamazepine (CBZ) and diclofenac (DCF) have remained untreated as they are unsusceptible to biological treatment and are therefore still found in relevant concentrations within the effluent of the WWTPs on the Grand River (Srikanthan, 2019; Hodgson et al., 2020).

1.2.1 Pharmaceuticals in Effluent

Pharmaceutical compounds are referred to as chemicals or contaminants of emerging concerns as they have been observed to have impacts across all levels of biological organizations (Mehdi et al., 2018). For instance, common problems caused by CECs are increased eutrophication and decreased dissolved oxygen levels (Carney Almroth et al., 2008; Carey and Migliaccio, 2009; Mehdi et al., 2018). In addition, a major concern about CECs is that several are disruptors of the endocrine and neuroendocrine systems, as well as of metabolic processes (Lange et al., 2009; Mennigen et al., 2011) via the stimulation of the hypothalamus-pituitary-interrenal (HPI) axis (Vijayan et al., 2010; Mehdi et al., 2018). Many pharmaceuticals are psychotropic drugs due to their ability to alter endocrine and neurological pathways (Simmons et al., 2017a). Therefore, exposure to these pharmaceuticals often result in changes in behaviour most likely associated to alterations in neurological pathways (Vijayan et al., 2010; Simmons et al., 2017a). Thus, the

inefficient removal of certain persistent pharmaceuticals such as VEN and CBZ may leave persistent negative effects on aquatic life found near these WWTPs.

Venlafaxine ($C_{17}H_{27}NO_2$) is a selective norepinephrine reuptake inhibitor (SNRI) compound commonly prescribed to treat depression, anxiety, obsessive-compulsive disorder (OCD) and panic disorders as it functions as an antidepressant (Horst and Preskorn, 1998; Saltiel and Silvershein, 2015; Mehdi et al., 2018). The mode of action of VEN and its metabolite, O-desmethyl venlafaxine (O-VEN) is well established in humans whereVEN functions by modulating serotonin reuptake and norepinephrine signaling in the brain (Horst and Preskorn, 1998; Melnyk-Lamont et al., 2014). This mechanistic action plays an important role in teleost physiological responses due to the stimulation of the HPI axis and cortisol synthesis, which in turn causes complications to reproductive abilities, metabolic responses, and survival rate of aquatic organisms (DiBattista et al., 2005; Melnyk-Lamont et al., 2014; Mehdi et al., 2018).

Carbamazepine ($C_{15}H_{12}N_2O$) is a synthetic compound efficiently used to treat epilepsy, bipolar disorder, attention deficit hyperactive disorder (ADHD), schizophrenia and many other neurological impairments as it functions as an anticonvulsant and mood stabilizing drug (Tolou-Ghamari et al., 2013; Qiang et al., 2016). The average annual usage of CBZ is approximated to be 919.89 tonne globally (Zhang et al., 2008; Qiang et al., 2016). In humans, the mode of action of CBZ functions by decreasing neuronal excitability or by increasing inhibition through alterations of sodium, potassium or calcium channels and other neurotransmitters involved in seizure activity, thus ultimately decreasing synaptic function (Tolou-Ghamari et al., 2013). Due to the long half life of CBZ being around 82 +/- 11 days (Brandão et al., 2013; Qiang et al., 2016) and a reported removal rate of roughly 7 - 10% by WWTPs (Ternes, 1998; Nielsen et al., 2015; Wang and Zhou, 2016), CBZ remains predominant within wastewater and leads to detrimental effects on aquatic

organisms. Studies have demonstrated that concentrations of CBZ ranging from 62.5 – 1000 μg/L can cause problems related to oxidative stress, increased reactive oxygen species (ROS) generations (Brandão et al., 2013), failed hatching (van Wodenberg et al., 2014) and other impacts relating to physiology such as growth, larval development, light sensitivity and more (Qiang et al., 2016).

Within the Grand River watershed, the concentrations of CBZ in the effluent of the WWTPs have been reported to be relatively the same as pre-upgrade concentrations (Srikanthan, 2019). The pre-upgrade concentration of CBZ in Kitchener effluent is around 619 ng/L and 560 ng/L in the Waterloo effluent, and remained relatively stable following upgrades (Srikanthan, 2019). A similar pattern is observed for concentrations of VEN which have been also reported to be relatively the same as pre-upgrade concentrations (Srikanthan, 2019). In the Grand River watershed, concentrations of VEN and O-VEN have been reported to range from 61 to 901 ng/L and 167 to 1472 ng/L, respectively (Metcalfe et al., 2010; Arlos et al., 2015; Mehdi et al., 2018). The pre-upgrade concentration of VEN in Kitchener effluent was around 1631 ng/L and 678 ng/L in the Waterloo effluent, both demonstrating significantly high concentrations, which remained stable following upgrades (Srikanthan, 2019). It is important to note that seasonality does lead to fluctuations of spatial and temporal pharmaceutical concentrations found in the effluent (Alvarez et al., 2009; Mehdi et al., 2018). With the concentrations of neuroactive pharmaceuticals remaining stable in the Grand River following recent upgrades, there arises questions concerning their impact on non-target fish species found downstream of WWTPs. Non-target fish are very sensitive vertebrates that are easily impacted by effluent exposure and are often used for toxicological studies to assess effects of contaminants (Porter and Janz, 2003).

1.2.2 Pharmaceutical Impacts in Non-Target Fishes

Pharmaceuticals and contaminants have been shown to have numerous behavioural impairment effects in fishes. In hybrid striped bass (*Morone saxatilis x Morone chrysops*), exposure to increased concentrations of VEN (1-500 μg/L) resulted in increased predation time, as well as decreased brain serotonin levels (Bisesi et al., 2014). Moreover, decreased growth rate, slowed reaction times, and anti-anxiety behaviour was exhibited in juvenile and adult brown trout (*Salmo trutta*) after exposure to 10 μg/L VEN (Ziegler et al., 2021). Embryonic and larval fathead minnows (*Pimephales promelas*) also demonstrated reduced predator avoidance measured through delayed latency period when exposed to VEN at environmentally relevant concentrations of 0.5 μg/L for embryos and 2 μg/L in larval minnows (Painter et al., 2009). Additionally, VEN exposures from 0.001 to 3 μg/L during critical developmental windows in fathead minnows (Thompson and Vijayan, 2020a) and larval zebrafish (*Danio rerio*) (Thompson et al., 2017) demonstrated significant impacts on behavioural responses such as reduced travelling distance and reduced activity in light with hyperactivity in darkness. These behavioural changes are likely attributed to disruption in brain serontonergic levels due to antidepressant exposure (Thompson and Vijayan, 2020b). At 10 μg/L, CBZ exposure in zebrafish has demonstrated reduced feeding times and reproductive impairment (da Silva Santos et al., 2018). Moreover, DCF, a non-steroidal anti-inflammatory drug that inhibits cyclooxygenase enzymes in mammals, has been observed to increase aggressive behaviour in juvenile brown trout at concentrations as low of 10 μg/L (Schwarz et al., 2017). These studies suggest that waterborne exposure to pharmaceuticals have significant neurological impairment with major behaviour disruptions, which can lead to population level impacts on fish species. These effects can be supported with few studies that have demonstrated the measurable presence of antidepressants such as fluoxetine and sertraline within brain tissue of native fish populations in USA streams and the Niagara River (Brooks et al., 2005; Schultz et al.,

2010; Arnnok et al., 2017). Currently, mechanisms for behavioural impairment following wastewater exposure are poorly understood, however reactive oxygen species (ROS) biomarkers and related damage may play a role in understanding these changed behaviours.

1.3.1 Reactive Oxygen Species

ROS is often used to characterize an array of molecules and free radicals found within a chemical species with one unpaired electron originating from molecular oxygen (Turrens, 2003; Beckhauser et al., 2016). In the ground state triplet, molecular oxygen is found as a bi-radical, consisting of two outer unpaired electrons on separate orbits with parallel spins, meaning oxygen can only react with one electron at a time in a chemical bond (Turrens, 2003; Apel and Hirt, 2004). ROS can be generated by energy transfer which results in the formation of a singlet oxygen, meaning one of the two electrons is excited and experiences a change in its spin, ultimately becoming a powerful oxidant allowing for quick reactions with other pairs of electrons (Turrens, 2003; Apel and Hirt, 2004). It can also be generated through electron transfer reactions which results in sequential reduction of reactive by-products such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (·OH) (Klotz, 2002; Apel and Hirt, 2004; Beckhauser et al., 2016).

A precursor to majority of ROS and mediator in oxidative chain reactions is O_2^-, the product of the univalent reduction of oxygen (Turrens, 2003). As a primary antioxidant defense mechanism, O_2^- can be spontaneously dismutated or catalyzed by superoxide dismutases (SOD), resulting in the production of H_2O_2 which can then be further reduced to water via the enzyme catalase (CAT), thus protecting cells from oxidative stress (Turrens, 2003; Apel and Hirt, 2004). However, it can also be partially reduced to ·OH in the presence of transition metal ions which in turn increases toxicity and oxidative stress (Bandyopadhyay et al., 1999; Turrens, 2003; Apel and Hirt, 2004). In addition to SOD, there are secondary enzymatic antioxidants in ROS defense which

include ascorbate peroxidase (APX) and glutathione peroxidase (GPX) (Apel and Hirt, 2004). GPX which uses co-factor glutathione (GSH) is expressed in low concentrations throughout the cell, but it has anti-apoptotic properties which help reduce oxidative injury (Savaskan et al., 2007; Malandrakis et al., 2014). These enzymes have the ability to detoxify H_2O_2 via oxidation with the help of reducing agents, resulting in the production of water (Apel and Hirt, 2004).

Antioxidant enzyme activity are present in different areas throughout the cell, but their functionality relies on the balance of compartmentalized enzymes in organelles (Figure 1.1; Benáková et al., 2021). Mitochondria are a major source of ROS production and are important in redox signaling from the organelle to the outside of the cell (Murphy, 2009). Mitochondria possess their own isoform of SOD, known as manganese superoxide dismutase, MnSOD or SOD2, coded by the gene *sod2* (Murhpy, 2009; Fukui and Zhu, 2010). Studies have shown that oxidative damage to mitochondria increases the release of membrane proteins such as cytochrome *c* to the cytosol and in turn, initiating the cell's apoptotic process (Murphy, 2009; Ribas et al., 2014). However, SOD has two isoenzymes found in the cytosol, known as copper/zinc superoxide dismutase, Cu,ZnSOD or SOD1, coded by the gene *sod1* (Fukui and Zhu, 2010). Cytosolic SOD is the predominant form of the enzyme in most tissues and cells, accounting for 70-80% of the cellular activity (Fukui and Zhu, 2010). Interestingly, CAT is mainly only present in peroxisomes but can be found in low concentrations in the mitochondria (Du et al., 2016; Benáková et al., 2021). Like mitochondria, peroxisomes are a significant source of intracellular ROS production and thus, they can produce downstream effects on cellular functions (Walton and Pizzitelli, 2012). In fact, there has been a link observed between peroxisomal produced ROS and inhibited CAT activity leading to upstream inhibition of mitochondria function as they are metabolically associated (Walton and Pizzitelli, 2012). Lastly, GPX has several enzyme homologs, however only GPX-1 and GPX-4

isoforms have been characterized in fish (Malandrakis et al., 2014). GPX-4 is coded by the gene *gpx4* and synthesized in the cytosol, but it can be compartmentalized in the mitochondria has been shown to demonstrate the most protection agaisnt oxidative injury (Savaskan et al., 2007; Ribas et al., 2014; Malandrakis et al., 2014). Although, antioxidative enzymes can be found both in the cytosol and mitochondria, studies have shown that mitochondrial damage is at the heart of oxidative induced neuronal cell death and is increased when exposed to pharmaceuticals (Fukui and Zhu, 2010; Ribas et al., 2014). Therefore, investigating ROS scavenging enzyme activity by isolating mitochondria from the cytosol may provide insightful knowledge of the fate of cells and tissues following effluent exposure.

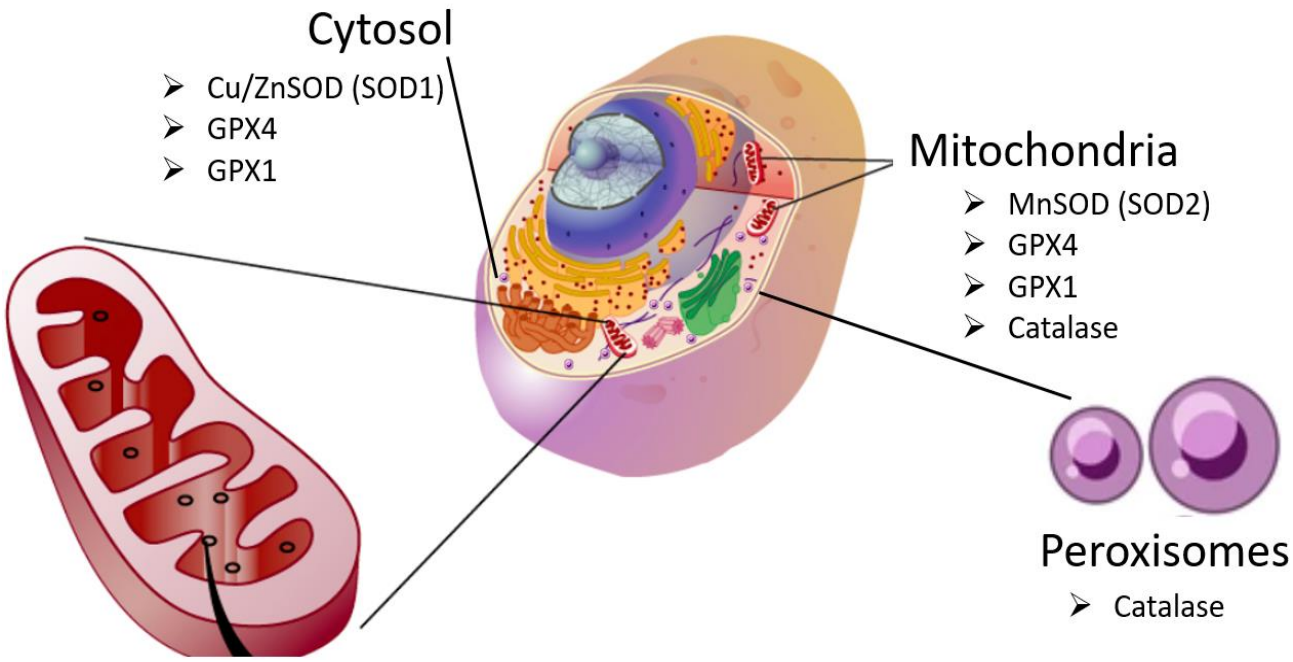

Figure 1.1 Representative compartmentalization of antioxidant superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) isoforms in the cytosol and mitochondria adapted from Benáková et al. (2021) and NIH Talking Glossary of Genetic Terms, NHGRI (public domain)

1.3.2 Effects of Reactive Oxygen Species on Fishes

A common problem with ROS is its ability to cause oxidative damage to proteins, DNA, and lipids as the production of O_2^- , H_2O_2 and ·OH are toxic for cells (Apel and Hirt, 2004; Fedorova et al., 2014). ROS reactions affect membrane properties such as fluidity, ion transport, enzyme activity, protein synthesis and DNA damage which all lead to cell death (Bandyopadhyay et al., 1999). A major issue with free radicals is the ability to initiate lipid peroxidation (LPO), the process where free radical oxidants attack the double bond of a methylene group in polyunsaturated fatty acids (PUFA) (Turrens and Boveris, 1980; Bandyopadhyay et al., 1999). This process can propagate throughout cells and thus, may severely damage membrane fluidity, permeability, and other cellular functions (Bandyopadhyay et al., 1999). In addition, ROS may lead to DNA damage on a nuclear and/or mitochondrial level through various free radical generated products which can cause mutagenic alterations (Bandyopadhyay et al., 1999). This damage can further induce signaling cascades which can ultimately lead to changes in gene expression by targeting and modifying transcription factors as a response from oxidative stress (Turrens, 2003; Apel and Hirt, 2004). Oxidation of lipids, proteins and nucleic acids produce reactive carbonyl groups such as aldehydes, ketones and lactams (Fedorova et al., 2014). The increased production of carbonyl groups may lead to biomolecule impairments which can then result in apoptotic cell death (Fedorova et al., 2014). Protein carbonyl content is often used as an index of metal-catalyzed oxidation of proteins which is a universal marker of oxidative stress (Bandyopadhyay et al., 1999; Craig et al., 2007; Fedorova et al., 2014)

When the balance between antioxidants and ROS production is disrupted, oxidative stress occurs through varying cellular events leading to problems such as cardiovascular dysfunction, neurodegenerative diseases, metabolic dysfunctions, and surrounding tissue damage (Bandyopadhyay et al., 1999; Fedorova et al., 2014). Thus, the severity of oxidative stress in a cell

is established by the amount of O_2^- , H_2O_2 and ·OH that are present in the cells and the proportion of enzymatic and metabolite antioxidants (Bandyopadhyay et al., 1999; Apel and Hirt, 2004; Li et al., 2010). Many pharmaceutical residuals found in effluent wastewater can stimulate the production of ROS resulting in high risk of oxidative stress or damage to aquatic species (Fig 1; Li et al., 2010; Beckhauser et al., 2016).

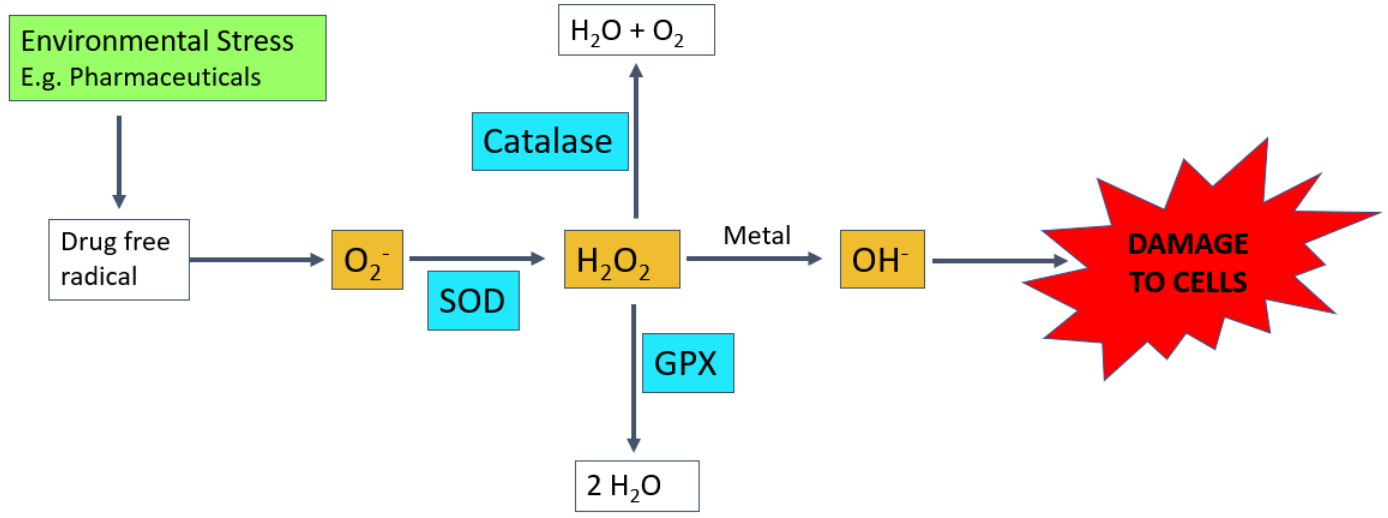

Figure 1.2 Flow diagram of an environmental pharmaceutical radical initiating the reactive oxygen species (ROS) pathway ultimately leading to oxidative stress/damage to cells.

Furthermore, the brain of aquatic species is a common and easy target for free radicals, as it is composed of large amounts of PUFA and has a lower activity of antioxidant enzymes (Migliore and Coppede, 2009; Li et al., 2010). Several pharmaceuticals and contaminants have been observed to increase oxidative stress in fish (Stancova et al., 2017). For instance, ibuprofen, DCF and CBZ in low concentrations, both singular and combined, were shown to induce antioxidant enzyme activity significantly (Ghelfi et al., 2016; Stancova et al., 2017). DCF as low as 0.2 ug/L demonstrated inducing effects of SOD in juvenile Rhamdia quelen fish (Ghelfi et al., 2016). In addition, a study conducted on rainbow trout, displayed enzymatic activity of SOD, CAT and GPX was significantly increased in the brain following a 7-day CBZ exposure suggesting a significant

effect of CBZ in inducing ROS (Li et al., 2010). Fluoxetine, a selective serotonin reuptake inhibitor (SSRI), was observed to increase SOD and CAT activity in the liver of *Argyrosomus regius* (Duarte et al., 2020). Additionally, in the brain of mice treated with antidepressants such as VEN, there is a significant change in the activity of SOD, further suggesting an influence of pharmaceuticals on ROS production (Abdel-Wahab et al., 2011; Beckhauser et al., 2016). Overall, several studies have demonstrated the relationship between pharmaceuticals and oxidative stress in mammals and fish. Notably, the accumulation of ROS in the brain has been correlated with the onset of neurodegenerative diseases, consequently leading to impacts on neuronal function which can be observed in both mammal and fish through different biomarkers (Beckhauser et al., 2016).

1.3.3 Neurodegeneration Impacts of Pharmaceuticals

The central nervous system (CNS) is responsible for a multitude of tasks involved in receiving and processing sensory information in order to control complicated behaviours allowing for appropriate survival responses (Cannon and Greenamyre, 2011). However, the nervous system varies greatly among organisms across the vertebrate lineage which causes an abundance of different physiological and behavioural responses (Cannon and Greenamyre, 2011). Due to the high complexity of the nervous system, it can be influenced by several factors, one of those being environmental exposures of CECs, both acutely or chronically (Cannon and Greenamyre, 2011). In an acute exposure, typically an exposure lasting from hours to days, generally a neurological phenotype can be observed. As an illustration, exposure of fluoxetine on *Daphnia magna*, increased offspring production which is a known phenotype for SSRI exposure in the brain (Campos et al., 2012; Rivetti et al., 2016). In contrast, in a chronic exposure, typically lasting from weeks to years, more complex problems often occur such as cell dysfunction and/or cell death, ultimately resulting in neurodegeneration, the loss of structure or neuronal function in the brain

(Cannon and Greenamyre, 2011). As an example, catfish that were chronically exposed to xenobiotics displayed structural damage pituitary cells suggesting neurological impairment (Hontela et al., 1997). As such, relevant pharmaceutical concentrations in effluent wastewater may cause similar patterns of damage within brain regions resulting in neurodegeneration. A study by Simmons et al. (2017b) on caged and wild goldfish exposed to pharmaceutical effluent, an ingenuity pathway analysis (IPA core analysis) which uses the expression of plasma proteins and metabolites to determined changes in biological functions, found ~ 47 functions that were significantly altered compared to control fish. For instance, in caged goldfish exposed to effluent, evidence demonstrated increased liver necrosis, activation of metal ion transportation and inhibition of the ATP production (Simmons et al., 2017b). In addition, in the wild goldfish exposed to effluent, the IPA analysis predicted an increase of glutathione levels in the liver, suggesting a response to reduce the accumulation of ROS most likely due to pharmaceutical concentrations (Simmons et al., 2017).

Teleost' are especially vulnerable to oxidative stress, as their brains are composed of a large amount of PUFA, thus increasing the incidence and risk of oxidative damage (Day, 2009; Yan et al., 2013). In the brain, when the ability to detoxify is lost, it leads to neuronal cell loss through oxidative stress (Yan et al., 2013). In vertebrates, there is a large amount of evidence demonstrating that increased oxidative stress to lipids, proteins and nucleic acids is present in the brain and peripheral tissues of individuals with neurodegenerative diseases which can be measured through LPO (Emerit et al., 2004; Migliore and Coppede, 2009). LPO results in several reactive by-products one of which being a common biomarker, malondialdehyde (MDA; Marnett, 1999). These toxic and mutagenic products can interact with DNA and proteins resulting oxidative damage (Marnett, 1999). Oxidative stress in human brains with Alzheimer's disease has been

shown through increased nuclear DNA damage caused by oxygen-derived radicals (Gabbita et al., 1998). In mice with a Parkinson's disease phenotype, brain oxidative stress was observed through decreased antioxidative capacity and increased protein and lipid peroxidation (Palacino et al., 2004). Moreover, the brain has limited antioxidative abilities as it's protected by the blood-brain barrier which reduces passage of toxins but limits the mobility of antioxidative enzymes (Pinol-Ripoll et al., 2006; Li et al., 2010). Thus, as oxidative stress is a major mechanism of neurodegeneration, then neurotoxicants such as several pharmaceuticals which are capable of eliciting ROS, are candidates for risk factors for neurological impairment in exposed organisms (Cannon and Greenamyre, 2011). Although extensive research has been performed on mammals to observe the link between oxidative stress and neurodegenerative diseases, there are very few studies on the impacts of pharmaceutical neurotoxicants found in effluent on aquatic species. However, the study of fish behaviour is being increasingly used to evaluate the effects of chronic exposures to wastewater effluent (Simmons et al., 2017a). For example, male fathead minnows and male three-spine sticklebacks were observed to have reduced abilities in building nesting sites following effluent exposure (Martinovic et al., 2007; Garcia-Reyero et al., 2011; Sebire et al., 2011; Weinberger II and Klaper, 2014; Simmons et al., 2017a). In addition, the neuroendocrine disrupting effect of VEN has caused changes to predator avoidance (Painter et al., 2009) and predation behaviour in fish (Bisesi et al., 2014; Bisesi et al., 2016; Simmons et al., 2017a; Martin et al., 2019). Therefore, as behavioural impairment has been observed following effluent exposure, neurological impairment may be responsible for these effects, however further investigation on the mechanisms in the brain is required. Studying model, non-target aquatic organisms such as Darters (Etheostoma sp.) which are endemic to the Grand River is crucial to better understand these impacts and the relationship between oxidative stress and contaminated effluent.

1.3.4 Darters as Model Species

Darters are small and colorful benthic perch-like fish from the Percidae family that often co-exist, and experience varied reproductive behaviour and life history traits making these species optimal for comparative studies (Paine, 1990). There is an estimated 190 described and 30 undescribed species of darters that can found in freshwaters, representing around 20% of darters recognized diversity in North America (Carlson and Wainwright, 2010). The species is short lived with a breeding period from April to June and therefore, put a lot of energy and effort into reproduction resources (Paine, 1990). They feed on small invertebrates such as midges, caddisflies, and mayflies, while they are prey to larger fish as well as hosts to parasites, thus making them key species in the food wed (Brown et al., 2011; Crane et al., 2011; Hodgson et al., 2020). The species can be found in habitats ranging from sandy pools to fast flowing rocky riffles and deep sand raceways (Carlson and Wainwright, 2010).

Rainbow darters (RBD; *Etheostoma caeruleum*) are known to remain within the same area throughout their lifetime which result in them being constantly exposed to contaminated effluent if living downstream from WWTPs (Brown et al., 2011; Hicks and Servos, 2017). In contrast, greenside darter *(*GSD; *Etheostoma blennioides*) are highly mobile, allowing them to remain in faster running water and change between cleaner or more contaminated sites (Bunt et al., 1998). Fantail darter (FTD; *Etheostoma flabellare*) tend to move in response to habitat change, and much like the RBD, they remain within similar habitats (Hodgson et al., 2020). Lastly, Johnny darters (JD; *Etheostoma nigrum*) are the least abundant and they inhabit rocky shores (Propst and Carlson, 1989). RBD have displayed a more competitive advantage at downstream sites through carbon and nitrogen stable isotope analyses and therefore, RBD are most likely to be more tolerant to contaminated effluent (Brown et al., 2011). Darters are model organisms as they are found in

abundance in the Grand River watershed, they have high site fidelity, meaning they remain in one location throughout their lifetime, and they have been observed to have high sensitivity to anthropogenic activities (Hicks and Servos, 2017; Mehdi et al., 2018; Hodgson et al., 2020).

Anecdotally, RBD have been the easiest species to capture in high numbers, however following WWTPs upgrades, other darter species such as the GSD and FTD have seemingly increased in numbers, suggesting increased sensitivity to effluent exposure. RBD have been used in previous studies as sentinel species to study WWTP effluent effects on reproduction (Bahamonde et al., 2015), metabolic rates (Mehdi et al., 2018), changes to gill physiology and morphology (Hodgson et al., 2020), as well as several studies on WWTP effluent effects (Tetreault et al., 2011; Fuzzen et al., 2016, McCallum et al., 2019). The advantage of using RBD are due to their abundance, their high site fidelity owing to their small movements, and their increased sensitivity to pollution (Tetreault et al., 2011; Hicks and Servos, 2017; Mehdi et al., 2018; Hodgson et al., 2020). Although, RBD have been observed to have effects from WWTP effluent, like high degree of intersex, increased metabolic rates and changes to gill morphology (Bahamonde et al., 2015; Mehdi et al., 2018; Hodgson et al., 2020), investigating one species does not provide a comprehensive investigation into wastewater effluent effects, considering there are several other species with varying tolerances to pollution in the Grand River.

1.4.0 Objectives & Hypotheses

In summary, in the natural environment, wastewater effluent consists of several mixtures of contaminants and thus, effects of chronic exposures may be convoluted with additive, synergistic and/or antagonistic effects (Liney et al., 2006; Backhaus, 2014; Simmons et al., 2017a; Hodgson et al., 2020). There is an array of studies on the effects of pharmaceuticals in effluent on reproductive fitness in fish (Fuzzen et al., 2015; Tetrault et al., 2011), however, there is very little

research on the effects of pharmaceuticals in effluent on other physiological responses such as stress, metabolic processes, and neurological mechanistic responses in the brain (Liney et al., 2006; Mehdi et al., 2018). There is additionally a substantial number of studies which observe behavioural impairment (Sebire et al., 2011; Weinberger II and Klaper, 2014; Simmons et al., 2017a), suggesting neurological imbalances most likely attributed to contaminant exposure. Thus, this research will aim to further our knowledge about the effects of effluent exposure on non-target organisms by investigating how the impacts of pharmaceutical concentrations affect the metabolic processes in the brain of fish as studies are limited. Therefore, this thesis aims to:

1. Determine the antioxidant stress response in four darter species living in effluent contaminated waters of the Grand River by measuring changes in antioxidant enzyme activity and changes to their specific transcript markers. It is hypothesized that fish living downstream of the Waterloo, WWTP effluent release point will have greater prevalence of ROS related impacts due to chronic exposure of effluent.
2. Investigate antioxidant stress response following an acute exposure to varying concentrations of venlafaxine directly on isolated brain cells by measuring antioxidant enzyme activity and cell viability. It is hypothesized that brain cells acutely exposed to venlafaxine will have greater ROS related impacts due to overloaded antioxidant response.

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Chapter 2

Impacts on antioxidative enzymes and transcripts in darter (*Etheostoma* spp.) brains in the Grand River exposed to wastewater effluent.

2.0.0 Impacts on antioxidative enzymes and transcripts in darter (*Etheostoma* spp.) brains in the Grand River exposed to wastewater effluent.

2.1.0 Introduction

The Grand River watershed is the largest watershed in Southern Ontario, Canada, covering approximately an area of 6900 km^2. Several major municipalities, including two First Nations are located within the watershed which is drained by the Grand River and four major tributaries: the Nith, Speed, Eramosa, and Conestogo Rivers, supporting a population of over one million. Along the Grand River watershed and its tributaries, 30 different wastewater treatment plants (WWTPs) with varying degrees of sewage treatment are found supporting mainly urbanized areas (Tetreault et al., 2013; Srikanthan, 2019). Treated effluents discharged into the Grand River contain a number of contaminants of emerging concern (CECs), such as pharmaceuticals, personal care products (PPCPs) and endocrine disrupting compounds (EDCs), many of which have been demonstrated to have sub-lethal effects on resident fish populations found below effluent outfalls, including impacts on growth, reproduction, feminisation, and metabolism (Bahamonde et al., 2015;; Fuzzen et al., 2016; Mehdi et al., 2018, Hodgson et al., 2020). Within the past decade, major, multi-million-dollar upgrades to the WWTPs in Kitchener-Waterloo have significantly improved effluent discharge in the Grand River, reducing both estrogenic, nitrogenous compounds and few pharmaceuticals such as ibuprofen and naproxen (Srikanthan, 2019). However, many residual pharmaceuticals (e.g., venlafaxine (VEN), a prescribed antidepressant and carbamazepine (CBZ), an anticonvulsant) are not efficiently removed and remain in the effluent discharge leading to measurable concentrations (ng/L – μg/L) in the surface waters (Srikanthan, 2019; Hodgson et al., 2020).

CECs found in effluent have been observed to cause complications across all levels of biological organizations due to their ability to disrupt endocrine, neurological, and metabolic

pathways, leading to behavioural impairments (Lange et al., 2009; Mennigen et al., 2011; Mehdi et al., 2018, Hodgson et al., 2020). In hybrid striped bass (*Morone saxatilis x Morone chrysops*), exposure to VEN (1-500 μg/L) resulted in increased predation time and decreased brain serotonin levels (Bisesi et al., 2014). Moreover, decreased growth rate, slowed reaction times, and anti-anxiety behaviour was exhibited in juvenile and adult brown (*Salmo trutta*) trout after exposure to 10 μg/L VEN (Ziegler et al., 2021). Embryonic and larval fathead minnows (*Pimephales promelas*) also demonstrated reduced predator avoidance measured through delayed latency period when exposed to VEN at environmentally relevant concentrations of 0.5 μg/L for embryos and 2 μg/L in larval minnows (Painter et al., 2009). Additionally, VEN exposures from 0.001 to 3 μg/L during critical developmental windows in fathead minnows (Thompson and Vijayan, 2020a) and larval zebrafish (*Danio rerio*) (Thompson et al., 2017; 2021; 2022) demonstrated significant impacts on behavioural responses such as reduced travelling distance and reduced activity in light with hyperactivity in darkness. These behavioural changes are likely attributed to disruption in brain serotonergic levels due to antidepressant exposure (Thompson and Vijayan, 2020b). At 10 μg/L, CBZ exposure in zebrafish has demonstrated reduced feeding times and reproductive impairment (da Silva Santos et al., 2018). Several CECs can bioaccumulate in organs, supported with few studies that have quantified CECs such as fluoxetine, sertraline and CBZ within brain tissue of native fish populations in USA streams and the Niagara River (Brooks et al., 2005; Schultz et al., 2010; Valdés et al., 2016; Arnnok et al., 2017). These studies suggest that waterborne exposure to pharmaceuticals have significant neurological impairment with major behaviour disruptions, which can lead to population level impacts on fish species. These effects can be supported with few studies that have demonstrated the measurable presence of antidepressants such as fluoxetine and sertraline within brain tissue of native fish populations in USA streams and the Niagara River,

which flows out of Lake Erie (Brooks et al., 2005; Schultz et al., 2010; Arnnok et al., 2017). Currently, mechanisms for behavioural impairment following wastewater exposure are poorly understood, however the generation of reactive oxygen species (ROS) and related damage may play a role in understanding these changed behaviours.

CECs can trigger the production of ROS, an array of molecules and free radicals found within a chemical species with one unpaired electron originating from molecular oxygen (Turrens, 2003; Beckhauser et al., 2016). ROS can be generated through electron transfer reactions resulting in sequential reduction of reactive by-products, e.g., superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (·OH) (Turrens, 2003; Apel and Hirt, 2004; Beckhauser et al., 2016). These oxygen derived molecules are produced under normal physiological functions, however, overproduction may trigger oxidative stress affecting membrane properties such as fluidity, as well as enzyme activities, protein synthesis and DNA damage resulting in cell death (Bandyopadhyay et al., 1999; Apel and Hirt, 2004; Fedorova et al., 2014). ROS exists in equilibrium with the antioxidant defense system which involve several antioxidative enzymes such as glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT) (Turrens, 2003; Apel and Hirt, 2004). These enzymes can detoxify ROS molecules via oxidation with the help of reducing agents, resulting in the production of water and oxygen, and thus, protection from oxidative stress (Apel and Hirt, 2004). When the balance between antioxidants and ROS production is disrupted, oxidative stress occurs through varying cellular events leading to problems such as cardiovascular dysfunction, neurodegenerative diseases, metabolic dysfunctions, and surrounding tissue damage (Bandyopadhyay et al., 1999; Fedorova et al., 2014). In humans and mammals, oxidative stress has been linked to several neurological diseases such as Alzheimer's and Parkinson's disease observed as decreased antioxidant capacity, DNA damage and LPO (Gabbita et al., 1998; Palacino

et al., 2004; Forero et al., 2006). The vertebrate brain has limited antioxidative abilities as it is protected by the blood-brain barrier which reduces passage of toxins but limits the mobility of antioxidative enzymes (Pinol-Ripoll et al., 2006; Li et al., 2010). Moreover, teleosts are especially vulnerable to oxidative stress, as their brains are composed of a large amount PUFA, thus increasing levels and chances of LPO (Day, 2009; Yan et al., 2013). Therefore, as oxidative stress is a major mechanism of neurodegeneration, then neurotoxicants such as several pharmaceuticals which are capable of eliciting ROS, are candidates for neurological impairment in exposed organisms (Cannon and Greenamyre, 2011). Although extensive research has focused on humans and mammals to observe the link between oxidative stress and neurological impairment, there is a gap in knowledge on the impacts of effluent exposure containing mixed pharmaceuticals and contaminants on non-target aquatic organisms such as Darters (*Etheostoma* spp.) which are endemic to the Grand River.

Darters are small benthic fishes that experience varied reproductive behaviour and life history traits making these species optimal for comparative studies (Paine, 1990). Rainbow darters (RBD; *Etheostoma caeruleum*) have been used in previous studies as sentinel species to study WWTP effluent effects on reproduction (Bahamonde et al., 2015), metabolic rates (Mehdi et al., 2018), changes to gill physiology and morphology (Hodgson et al., 2020), as well as several studies on WWTP effluent effects (Tetreault et al., 2011; Fuzzen et al., 2016, McCallum et al., 2019). The advantage of using RBD are due to their abundance, their high site fidelity owing to their small movements, and their increased sensitivity to pollution (Tetreault et al., 2011; Hicks and Servos, 2017; Mehdi et al., 2018; Hodgson et al., 2020). While RBD have displayed effluent impacts from WWTP effluent exposure, like high degree of intersex, increased metabolic rates and changes to gill morphology (Bahamonde et al., 2015; Mehdi et al., 2018; Hodgson et al., 2020), investigating

one species does not provide a comprehensive investigation into wastewater effluent effects, considering there are several other species with varying responses to pollution in the Grand River. Therefore, this study includes three additional darters, greenside darter (GSD; *Etheostoma blennioides*), fantail darter (FTD; *Etheostoma flabellare*) and Johnny darter (JD; *Etheostoma nigrum*), to expand the knowledge on how different darter species are impacted by WWTP effluent. RBD tend to remain within the same area throughout their lifetime which results in constinuous exposures to contaminated effluent (Brown et al., 2011; Hicks and Servos, 2017). In contrast, GSD are highly mobile, allowing them to remain in faster running water without major disruptions to feeding (Bunt et al., 1998). FTD live in silty/rocky areas, tend to move in response to habitat change, and much like the RBD, they remain within similar habitats (Hodgson et al., 2020; Hicks and Servos, 2017). Lastly, JD are the least abundant and they inhabit silty sites near the river shores (Propst and Carlson, 1989). RBD have displayed a more competitive advantage at downstream sites through carbon and nitrogen stable isotope analyses and therefore, RBD are most likely to be more tolerant to contaminated effluent (Brown et al., 2011).

The objective of this study was three-fold: 1) to examine the antioxidant response in the brains of four darter species (*Etheostoma* spp.) living downstream of the Waterloo WWTP, measured through changes in antioxidant enzyme activity and changes to their specific transcript markers; 2) to determine whether environmental variables, pharmaceutical contaminants and biological responses predict responses to effluent via principal component analysis for individual species; and 3) assess if these antioxidant markers remain consistent from year to year. We hypothesized that fish living downstream from the effluent release point would have greater prevalence of ROS related impacts compared to upstream reference species due to chronic exposure of effluent, and yearly differences would be minimal. This would be associated with

increased gene expression of ROS antioxidants, as well as increased activity of corresponding enzymes compared to fish living upstream.

2.2.0 Materials and Methods

2.2.1 Site Descriptions & Fish Collection

In October 2020, four darter species were collected at two sites relative to the Waterloo WWTP (Fig. 1A). The first collection site (sampled October 19th before noon) is approximately 1km downstream of the Waterloo WWTP effluent outfall located at the Economical Insurance Trailway (EIT; 43°47’26’’N, 80°47’41’’W) and is located within an urbanized area. The second collection site (sampled October 20th before noon) is located at Kiwanis Park (KIW; 43°50’26’’N, 80°47’57’’W) which is approximately 4km upstream of the Waterloo WWTP. The same sites were sampled in Fall 2021 (Oct 13th), however only two of four species were collected. Using a backpack electro-fisher and dip nets, RBD, GSD, FTD and JD (at least 15 males and 15 females of each species; Fig. 1B) were collected in Fall 2020. In Fall 2021, 8-11 males and 4-8 females of RBD and GSD were collected. Sex as an explanatory variable was investigated as expected morphological differences are present and thus, investigating antioxidant response between sexes may provide better understand of future population responses.

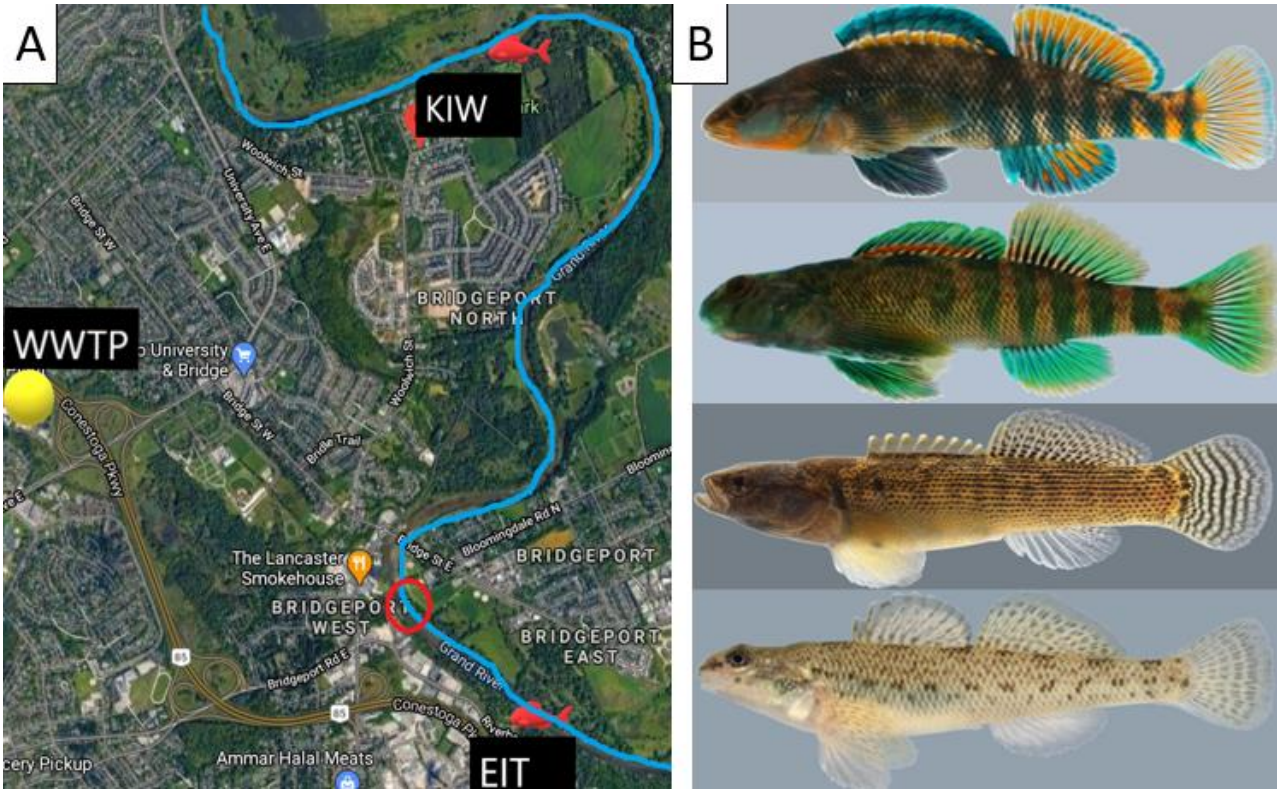

Figure 2.1. A) Map of sampling locations along the Grand River where darter species were collected for this study relative to the Waterloo WWTP (represented by the yellow circle), the WWTP outlet is indicated by the red circle. KIW = Kiwanis is the upstream sampling site and EIT = Economical Insurance Trailway is the downstream sampling site (Google Maps; Map data ©2021, CNES/Airbus, Landsat, Copernicus, Maxar Technologies). **B)** The four species of darter (*Etheostoma* spp.) collected. Top to bottom; rainbow darter (RBD), greenside darter (GSD), fantail darter (FTD) and Johnny darter (JD). These species are all found in the Grand River. Pictures taken from Google Images (2021).

Fish were placed into aerated buckets filled with river water. Fish smaller than 4.0 cm were not included as they were considered immature. Fish were measured for total length (+/- 0.1 cm) and wet weight (+/- 0.01 g) and sacrificed via spinal severance. Gonads and liver were dissected and weighed (mg). Weights and lengths of each species were used to calculate morphological

parameters such as hepatosomatic index ((HSI) = [liver mass/body mass] x 100), gonadosomatic index ((GSI) = [gonad mass/body mass] x 100), and Fulton's condition factor ((K) = body mass/length^3] x 100). Whole brains were collected in cryotubes and immediately snap frozen in liquid nitrogen for enzymatic and molecular analyses. Animal collections were approved by the Animal Care Committee at the University of Waterloo under AUPP#40318 and followed the guidelines set out by the Canadian Council of Animal Care.

2.2.2 Water Quality

At each collection site, water measurements (temperature, conductivity in Fall 2020 and specific conductance (SPC) in Fall 2021, and pH) were recorded using a YSI Professional Plus multimeter (YSI Incorporated, OH, USA). The same day of fish collection, river water samples were collected for analysis of pharmaceuticals and general contaminants using LC-MS techniques (Fuzzen et al., 2016). Briefly, samples were collected in triplicate (n = 3 for each location), representing near, centre, and far bank of the river, or within the plume of the effluent for the downstream site. Samples were preserved (1 g/L sodium azide; 50 mg/L ascorbic acid) in 500 mL amber glass bottles and stored at 4^{o}C until extraction. Analysis of the water samples can be found detailed by Tanna et al., (2013). Pharmaceuticals were analyzed through solid phase extraction followed by liquid chromatography and tandem mass spectrometry (LC-MS/MS) using an Agilent 1200 HPLC (Mississauga, ON) coupled to an Applied Biosystems 3200 QTRAP mass spectrometer (ABSciex; Concord, ON) using appropriate standards. The classification of contaminants measured were NSAIDs, fibrates (gemfibrozil), Antiepileptics, Antibacterials, a pain reliever (acetaminophen), a beta-blocker (atenolol), a stimulant (caffeine), SNRI, SSRI, Antibiotics, DPP inhibitor (linagliptin), Statins and an herbicide (atrazine).

2.2.3 Molecular Analysis

Whole brains (4-8 females and 6-11 males) per site per species were used for total RNA extraction following RNeasy Mini Kit (Qiagen; Toronto, Canada) protocol, after homogenization using an OMNI tissue homogenizer (NW Kennesaw, GA, USA). RNA concentrations and purity (280:260 μm and 260:230 μm) were measured using the SpectraMax 190 from Molecular Devices (San Jose, California, USA). Due to the small brain sizes (3-10 mg), RNA concentrations were lower and therefore, each reaction were adjusted to contain 250 ng of template RNA for cDNA synthesis. RNA was converted into cDNA following the protocol provided by the QuantiTect Reverse Transcription Kit (Qiagen) and then diluted 10x with RNA free water. Primers were developed by gathering known sequences of closely related species (Orangethroat darter (*Etheostoma spectabile*), Arkansas darter (*Etheostoma cragini*) and Yellow perch (*Perca flavescens*)) using NCBI's Nucleotide database for glutathione peroxidase (catalytic subunit – *gpx4,* NCBI accession no. XM_032526863.1, XM_034880992.1, XM_028588175.1), superoxide dismutase (catalytic subunit – *sod1,* NCBI accession no. XM_032534291.1, XM_034890501.1, XM_031280860.1), and catalase (*cat,* NCBI accession no. XM_032523453.1, XM_034879178.1, XM_028584855.1) and tryptophan hydroxylase (catalytic subunit – *tph1,* NCBI accession no. XM_032522818.1, XM_032522817.1, XM_032522816.1). *Tph1* was chosen because it is a precursor of serotonin synthesis and changes to *tph1* can result in major neurological imbalances (Rahman et al., 2011). In addition, two housekeeping genes (β-actin and Glyceraldehyde 3-phosphate dehydrogenase) which are known to have consistent expression were used to normalize expression of target genes using the Vandesompele method, utilizing a geometric mean of each reference gene (Appendix A). Primer sequences were created by aligning multiple sequences using CLUSTALW (Kyoto University Bioinformatics Center) and blasted in NCBI's Primer design tool to find forward and reverse primers (Table A.1 in appendix A). Following cDNA preparation, RT-

qPCR was used with SSo advanced SYBR green with Biorad CFX 96 machine linked to CFX Maestro software (Version 2.0; 2020) from Biorad (Hercules, California, USA). Reactions were carried out in duplicates on Biorad low-profile clear plates with Biorad Microseal B Adhesive sealer (Hercules, California, USA). Pipetting errors were validated using quality control (QC%) set to 0.35. Primers were validated using a dilution series of pooled template RNA for each species to test primer efficiency and dimerization. Primers were considered efficient when the standard curves displayed an efficiency between 90-110% and an R^2 value >0.96. In addition, primers were validated with thermal gradients (55°C-65°C) to determine optimal annealing temperature of primers. The PCR conditions were a 30 second activation at 95°C, a 10 second denaturation at 95°C, an annealing and extension period for 25 seconds at 60°C for 40 cycles. The melt curve was at 60°C to 95°C every 0.5°C to verify for amplification of just one product.

2.2.4 Enzymatic Analysis

Whole brains collected in Fall 2020 from the total 30 initially collected (9 females and 9 males) per site per species were homogenized using an electric homogenizer (OMNI tissue homogenizer) in 10x volume per weight of brain in cold 20 mM HEPES buffer, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose, pH 7.2. The homogenate was then centrifuged at 1500 x g for 5 minutes at 4°C and centrifuged again at 10000 x g for 15 minutes at 4°C to divide cytosolic and mitochondrial fractions. The supernatant (cytosolic fraction) was removed and stored on ice. The remaining pellet (mitochondrial fraction) was then diluted with 100 µL of cold 20 mM HEPES buffer, pH 7.2 and stored on ice. Enzyme activity was not measured in brains collected Fall 2021 due to pandemic restrictions and limited fish collected.

Cytosolic and mitochondrial fractions were assayed for GPX activity with the Glutathione Peroxidase Assay Kit (Cayman chemicals, MI, USA) and for SOD activity following the

Superoxide Dismutase Assay Kit (Cayman chemicals, MI, USA), following manufacturer's instructions. Samples were run in duplicates on 96 well-microplates and read using the Molecular Devices Spectramax 190 spectrophotometer linked to SoftMax Pro 6.4 software. GPX activity (nmol/min/mL) was calculated according to kit instructions; however, results were divided by total protein (mg) multiplied by buffer dilution (mL) resulting in units of nmol/min/mg protein and then divided by 1000 to convert to μmol/min/mg. SOD activity (units/mL) was calculated according to kit instructions; however, results were divided by total protein (mg) multiplied by buffer dilution (mL) resulting in units of units/mg/mL.

Cytosolic and mitochondrial fractions were assayed for CAT following the protocol of Craig et al. (2007). Reactions were initiated using 10 μL of 800 mM stock H_2O_2 with final concentration of 40 mM. Samples were run in duplicates on 96-well UV spec plates and read at 240 nm for 5 minutes using the Molecular Devices Spectramax 190 spectrophotometer with SoftMax Pro 6.4 software. CAT activity (μmol/min/mL) was calculated using the extinction coefficient of 43.6 and pathlength of the well. Activity was then divided by total protein (mg) multiplied by buffer dilution (mL) resulting in final units of μmol/min/mg protein. Protein concentrations for all samples were quantified using the BCA assay (Sigma-Aldrich) and bovine serum albumin (BSA) as a standard.

2.2.5 Statistics

Data was analyzed using GraphPad Prism Version 9.1.2. Two-way ANOVAs and Tukey's post-hoc were used to determine any significance between sexes and sites within a species. Morphological indices were analyzed using Two-Sample Independent T-test. Normality of data was assessed using a Shapiro-Wilk's normality test and the homogeneity of variance was assessed using F-test for homogeneity of variance. When normality or variance assumptions were not met,

data was transformed using logarithm base 10 or square root functions, or a non-parametric Mann-Whitney was conducted. Finally, a Principal component analysis (PCA) was performed to assess the relationship between morpho-physio-biochemical characteristics of darter species in response to wastewater effluent. All p-values were compared to alpha (α) value of 0.05 and considered significant if less than α. Fold changes were calculated as B/A (B = EIT and A = KIW). Figures present data as means +/- standard error of the mean (SEM).

2.3.0 Results

2.3.1 Water Quality

Water quality measurements in the Grand River are recorded in Table 1. Means are ± SEM and were calculated from three measurements at each site (Table 1). In Fall 2020, there was an increase in temperature by 1.2°C, conductivity by 162.27 μS/cm, and TDS by 144.8 ppm downstream at EIT compared to the upstream KIW reference site. There was a decrease in dissolved oxygen (DO) by 0.9 mg/L and pH by 0.35 downstream compared to upstream. Moreover, water samples were analyzed for 24 different contaminants at both sites relative to the Waterloo WWTP. Upstream of the WWTP, only 11 out of 24 contaminants screened for were detected compared to 19 out of 24 detected downstream of the WWTP. An increase can be observed for naproxen, diclofenac (DCF), desmethyl-VEN, VEN, CBZ, ibuprofen, triclosan, sulfamethoxazole, trimethoprim, and p-hydroxy atorvastatin at EIT compared to KIW (Table 2). However, there was a decrease is observed for atrazine downstream compared to upstream.

In Fall 2021, water temperature at both KIW and EIT were roughly 10°C higher than the previous year with only a 0.6°C difference and 0.25 mg/L in DO were observed between sites (Table 1). No major differences were observed in flow discharge between both years (Table 1). Water samples were analyzed for the same contaminants at both sites with 17 out of 24 being

detected upstream and 19 out of 24 detected downstream of the WWTP. The same contaminants that were increased the previous year at EIT were also increased at EIT in Fall 2021 (Table 2). In contrast to Fall 2020 where VEN and DCF were the highest pharmaceuticals at EIT, desmethyl-VEN and VEN were the observed highest contaminants at EIT compared to KIW in 2021 by 44-fold and 16-fold, respectively.

Table 1. Water quality parameters for the Grand River from upstream (KIW) and downstream (EIT) collection sites of the Waterloo WWTP during the month of Fall 2020 and Fall 2021. Values presented as means (n = 3/site) with EIT samples taken on the side of the effluent plume. Flow data was retrieved from National Hydrometric Data from sites 15 km above KIW and below EIT (Government of Canada, 2022).

Date	Site	Water Temp. (°C)	DO (mg/L)	Flow (m^3/s)	SPC (µS/cm at 25° C)	Conductivity (µS/cm)	pH
Oct 20th, 2020	KIW	7.1	11.7	7.92	—	335.7	8.5
Oct 19th, 2020	EIT	8.3	10.8	14.6	—	497.97	8.15
Oct 13th, 2021	KIW	17.3	8.91	7.87	635	—	8.2
Oct 13th, 2021	EIT	17.9	8.66	17.5	898	—	8.2

Table 2. Concentration trends of the pharmaceuticals screened for upstream (KIW) and downstream (EIT) sites in Fall 2020 and Fall 2021. Three samples were taken (near the bank, in the middle of the river and far away from the bank). Data is presented as means ± SEM (n=3/site).

		Oct-2020		Oct-2021	
Classification	Pharmaceutical	KIW (ng/L)	EIT (ng/L)	KIW (ng/L)	EIT (ng/L)
NSAIDs	Naproxen	0.00	15.48	0.95	9.19
	Diclofenac	27.53	133.72	6.55	67.76
	Ibuprofen	7.66	16.08	5.26	9.17
Fibrates	Gemfibrozil	0.00	0.32	6.55	67.76
Antiepileptics	11,12-epoxide carbamazepine	0.32	1.51	0.00	0.00
	Carbamazepine	7.99	45.23	4.34	34.54
Antibacterials	Triclosan	0.00	9.51	3.20	5.44
	Triclocarban	0.00	0.00	0.24	0.31
	Sulfamethoxazole	0.00	1.15	9.77	47.67
	Sulfamethazine	5.28	31.99	44.90	33.19
	Trimethoprim	0.52	5.98	0.00	15.24
Pain relievers	Acetaminophen	0.00	0.00	0.00	0.00
Beta-blockers	Atenolol	0.00	0.00		
Stimulant	Caffeine	16.17	25.11	11.73	15.68
SNRI	Desmethyl-venlafaxine	4.99	44.01	15.80	695.34
	Venlafaxine	11.33	153.58	6.35	104.36
SSRI	Fluoxetine	0.00	4.55	0.30	3.08
	Norfluoxetine	0.00	0.00	0.00	0.00
Antibiotic	Lincomycin	3.72	2.44	0.00	0.00
	Monensin	0.00	0.00	0.54	0.75
Statins	p-hydroxy atorvastatin	0.00	15.04	0.00	2.59
	o-hydroxy atorvastatin	0.00	11.41	0.42	2.81
	Atorvastatin	0.00	5.79	2.85	1.57
Herbicide	Atrazine	120.03	92.67	35.56	22.69

2.3.2 Molecular analysis

Whole brains were used to measure total RNA abundance of antioxidative gene transcripts (*gpx*, *sod*, *cat*) as well as *tph* a gene transcript involved with serotonin synthesis. RBD demonstrated no significant differences other than an interaction effect for *tph* ($F_{1,19}$ = 6.4, p = 0.02) analyzed by Two-way ANOVA (Fig. 2D).

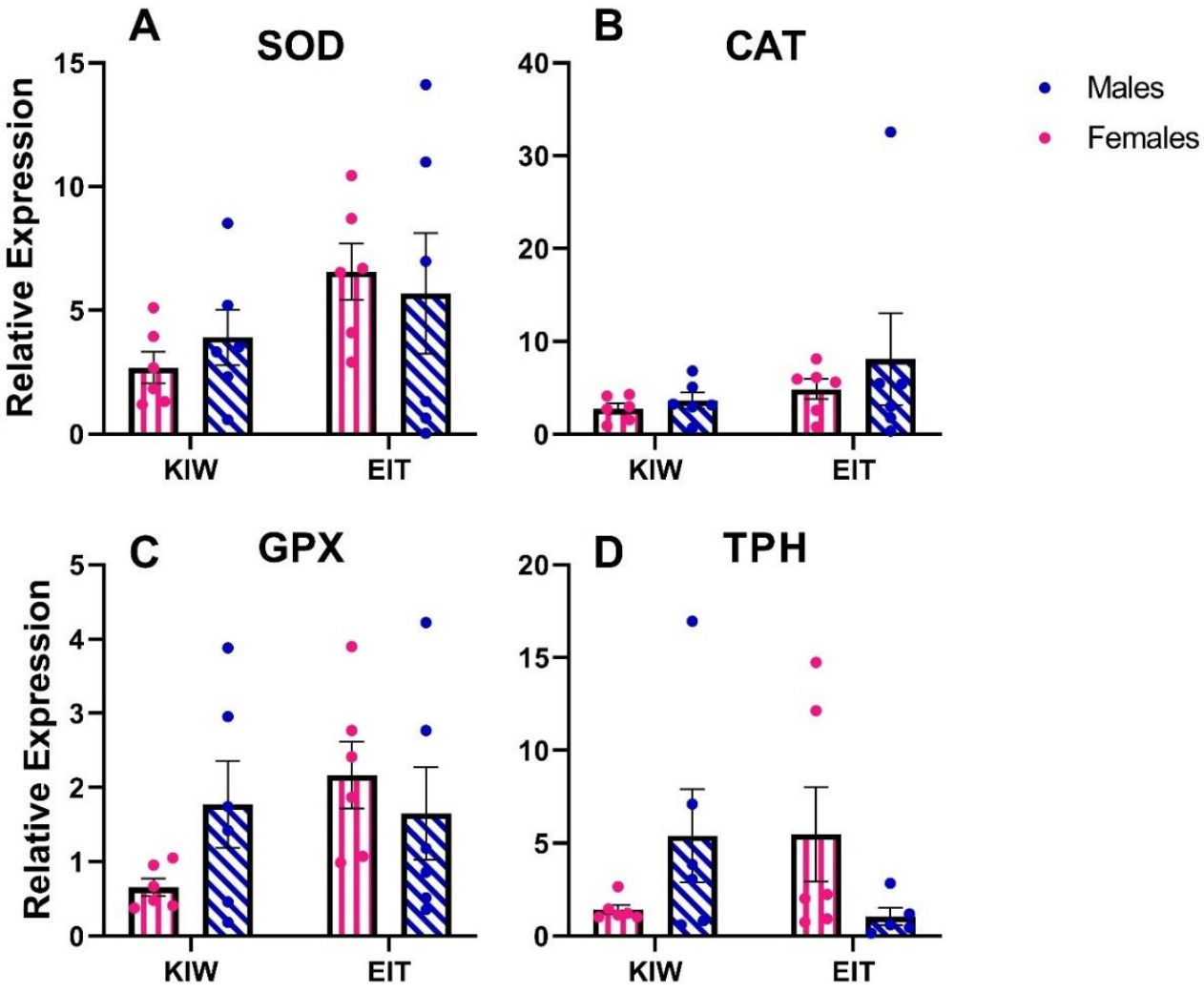

Figure 2.2. Normalized gene expression of A) superoxide dismutase (*sod*), B) catalase (*cat*), glutathione peroxidase (*gpx*) and tryptophan hydroxylase (*tph*) measured in the whole brain of rainbow darters (RBD) upstream (KIW) and downstream (EIT) from the WWTP effluent. Data presented as means + SEM, compared using Two-way ANOVA. Only an interaction effect for *tph* was found ($p = 0.02$, $n = 10$-12).

GSD displayed a significant site effect for *sod* (Fig. 3A, $F_{1,19} = 5.9$, $p = 0.025$) and a Tukey test displayed a near significant increase for downstream females by 1.8-fold ($p = 0.052$). GSD additionally displayed a significant site effect for *cat* (Fig 3B, $F_{1,19} = 10.7$, $p = 0.004$), as well as a

sex effect (Fig 3B, $F_{1,19} = 10.2$, $p = 0.005$). Tukey test displayed significant increase of downstream males compared to upstream males by 2.88-fold ($p = 0.03$), compared to upstream females by 7.4-fold ($p = 0.001$) and compared to downstream females by 3-fold ($p = 0.033$).

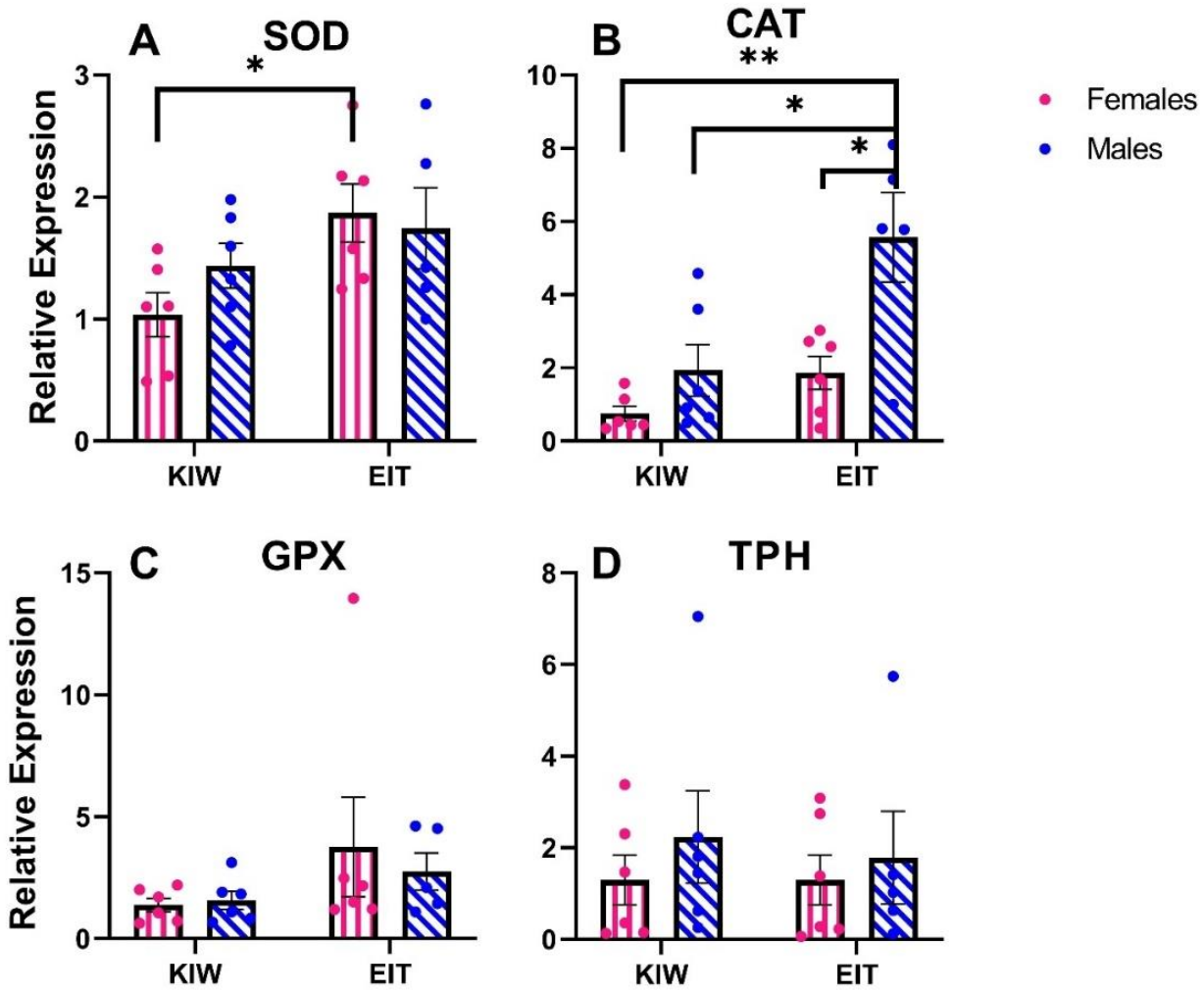

Figure 2.3. Normalized gene expression of A) superoxide dismutase (*sod*), B) catalase (*cat*), glutathione peroxidase (*gpx*) and tryptophan hydroxylase (*tph*) measured in the whole brain of greenside darters (GSD) upstream (KIW) and downstream (EIT) from the WWTP effluent. Data presented as means + SEM, compared using Two-way ANOVA. A site effect ($p < 0.05$) was found

for *sod*. Effect of site ($p < 0.01$) and effect of sex ($p < 0.01$) were found for *cat*. A post-hoc Tukey test was conducted. (* = $p < 0.05$, ** = $p < 0.01$, n = 10-12).

Two-way ANOVAs demonstrated no significant differences for any transcript in FTD (Fig. 4). JD only demonstrated a significant site effect for *sod* (Fig 5A, $F_{1,18} = 7.31$, $p = 0.015$).

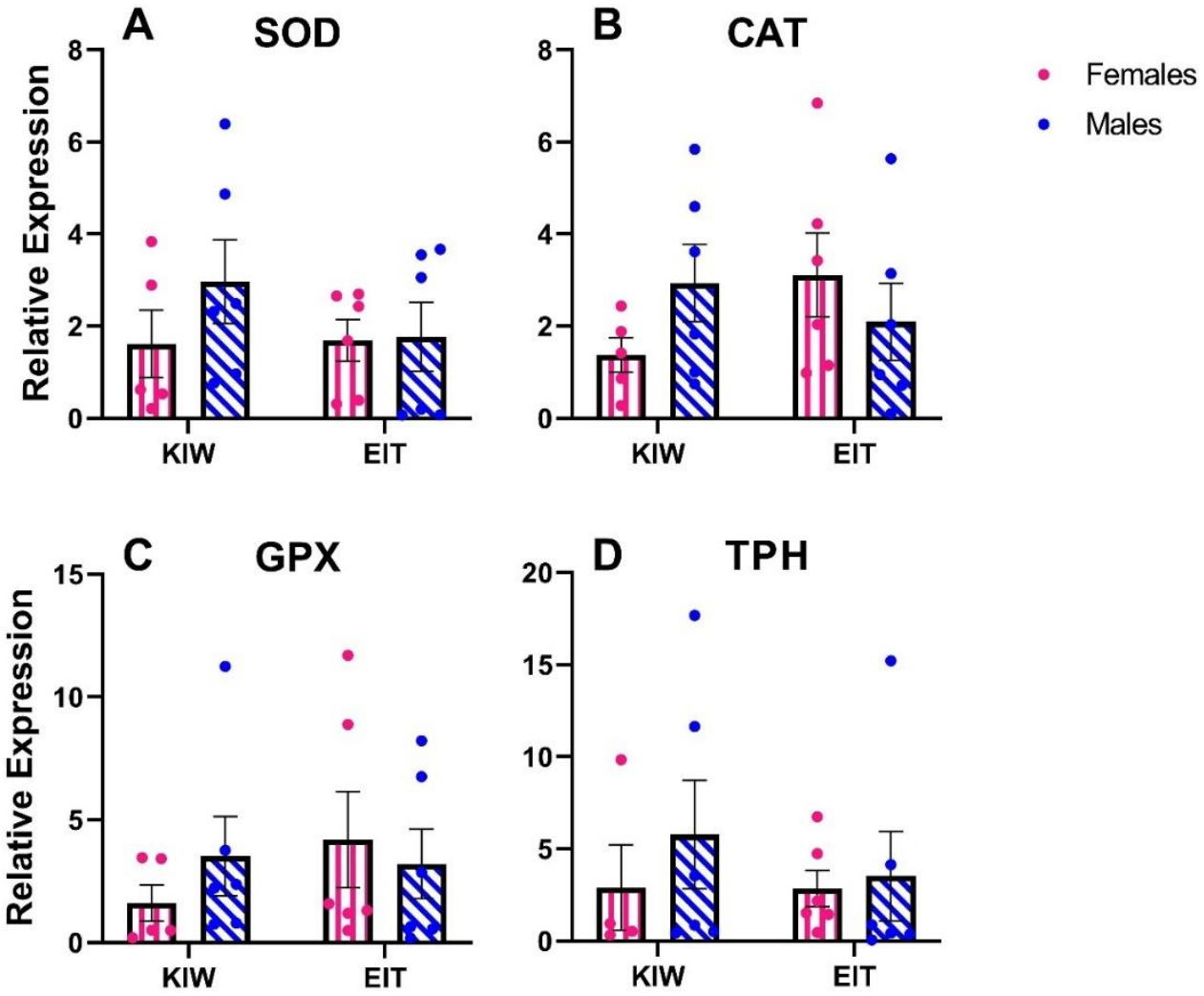

Figure 2.4. Normalized gene expression of A) superoxide dismutase (*sod*), B) catalase (*cat*), glutathione peroxidase (*gpx*) and tryptophan hydroxylase (*tph*) measured in the whole brain of fantail darters (FTD) upstream (KIW) and downstream (EIT) from the WWTP effluent. Data presented as means + SEM, compared using Two-way ANOVA ($p > 0.05$, n = 10-12).

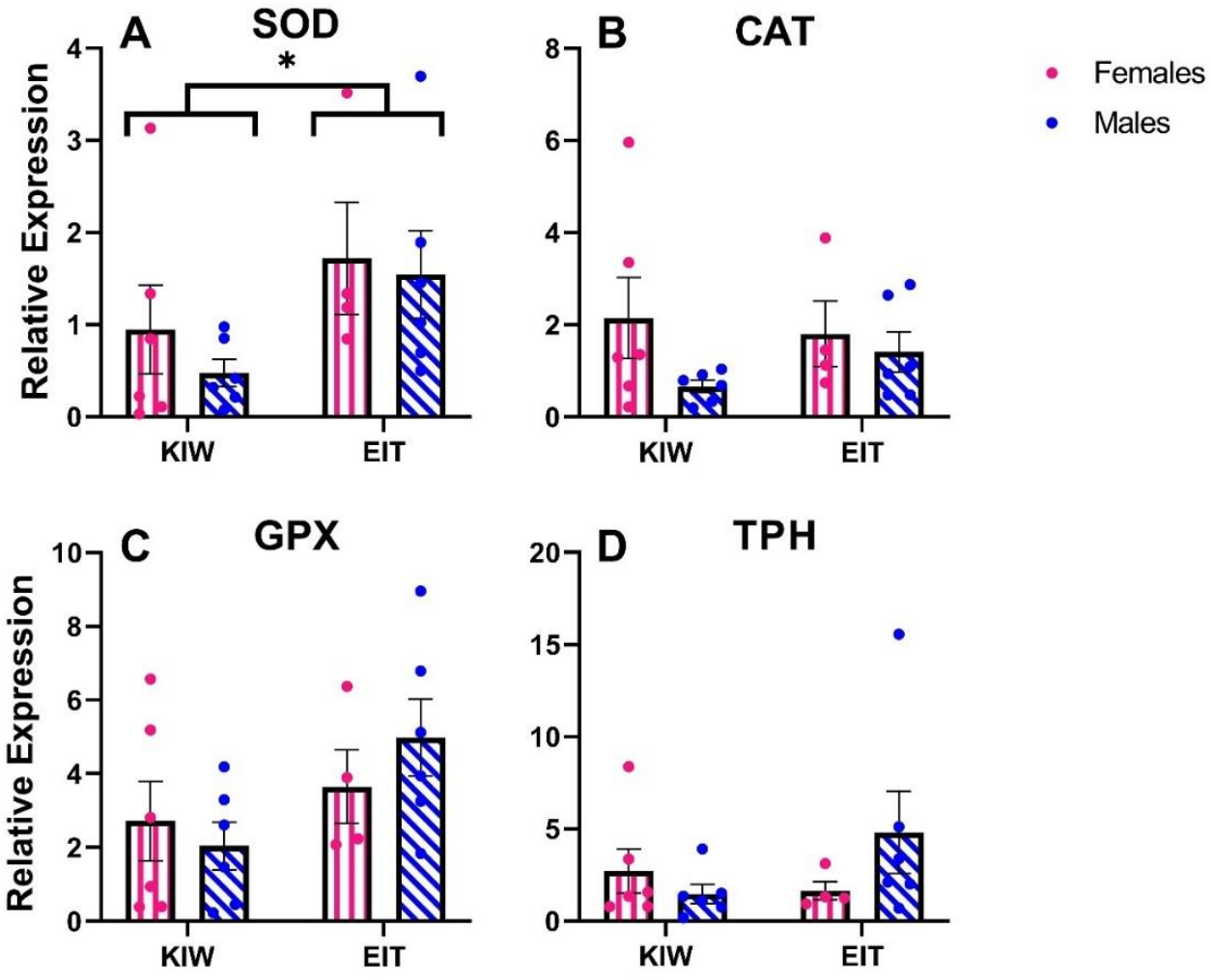

Figure 2.5. Normalized gene expression of A) superoxide dismutase (*sod*), B) catalase (*cat*), glutathione peroxidase (*gpx*) and tryptophan hydroxylase (*tph*) measured in the whole brain of Johnny darters (JD) upstream (KIW) and downstream (EIT) from the WWTP effluent. Data presented as means + SEM, compared using Two-way ANOVA. (* = $p < 0.05$, n = 10-12).

Whole brains of RBD and GSD collected in Fall 2021 were used to measure total RNA abundance of the same antioxidative gene transcripts observed previously to validate consistency across years for these transcripts. In contrast to the previous year, RBD demonstrated a significant

interactive effect between sex and site for *sod* (Fig 6A, $F_{1,28} = 5.2$, $p = 0.031$). Additionally, RBD displayed a site effect for *cat* (Fig 6B, $F_{1,28} = 6.46$, $p = 0.017$), however no significance was found following a Tukey test. To add to the opposing observations, a site effect was found for *gpx* (Fig. 6C, $F_{1,28} = 4.5$, $p = 0.043$) and *tph* (Fig. 6D, $F_{1,28} = 7.05$, $p = 0.013$) in RBD, with upstream males and females showing higher expression than downstream males and females. However, Tukey test only found a significant difference between RBD males for *tph* with upstream males having 0.3-fold higher expression than downstream ($p = 0.023$).

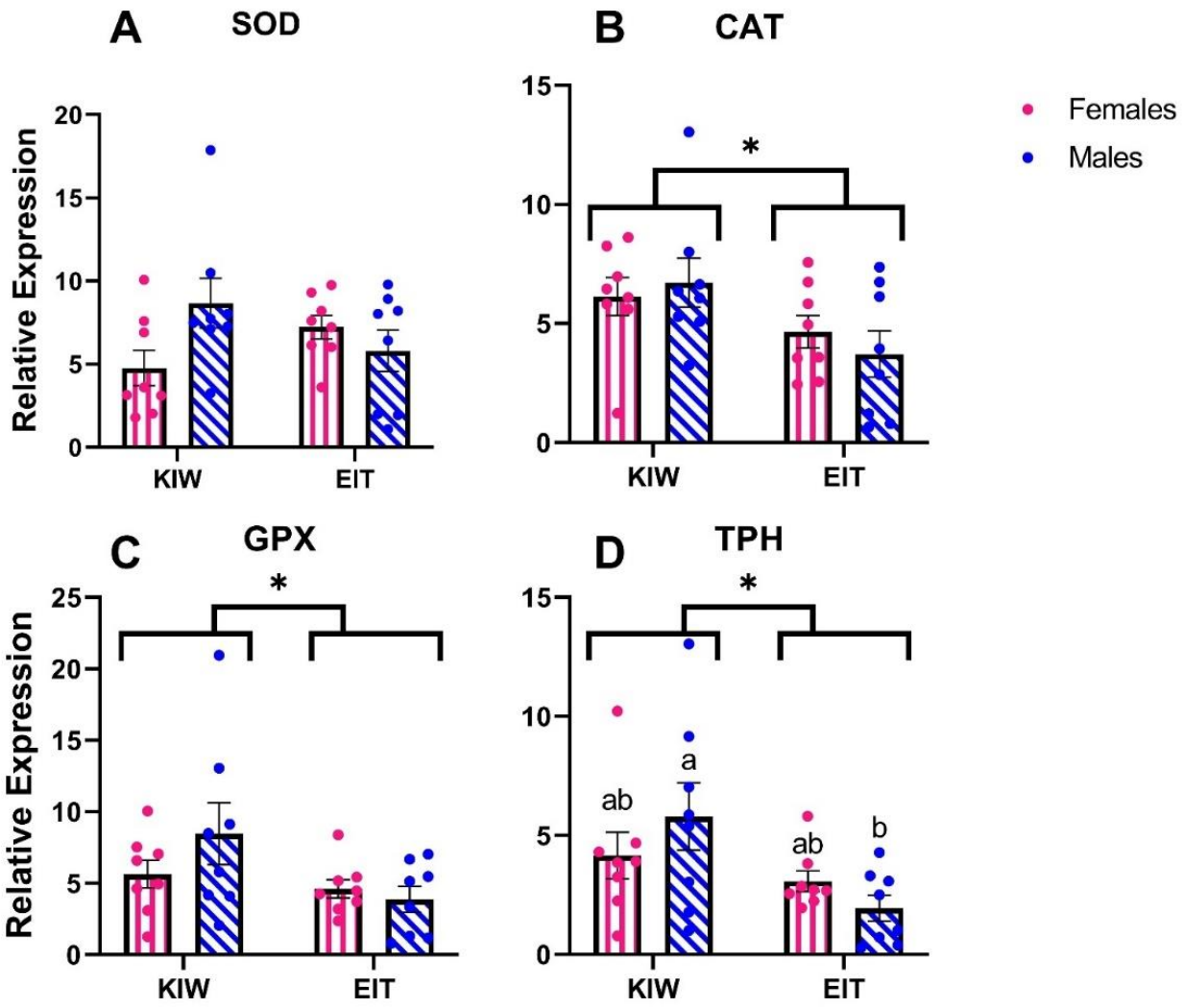

Figure 2.6. Normalized gene expression of A) superoxide dismutase (*sod*), B) catalase (*cat*), glutathione peroxidase (*gpx*) and tryptophan hydroxylase (*tph*) measured in the whole brain of rainbow darters (RBD) upstream (KIW) and downstream (EIT) from the WWTP effluent in Fall 2021. Data presented as means + SEM, compared using Two-way ANOVA. (* = $p < 0.05$, n = 13-16).

Following this trend, GSD displayed no significant effect for *sod* or *cat* following two-way ANOVAs (Fig. 7). Additionally, a significant site effect was observed for *gpx* ($F_{1,22} = 15.5$, $p <$

0.001; Fig. 7C) with upstream males and upstream females displaying 0.4-fold higher expression compared to downstream males ($p < 0.01$).

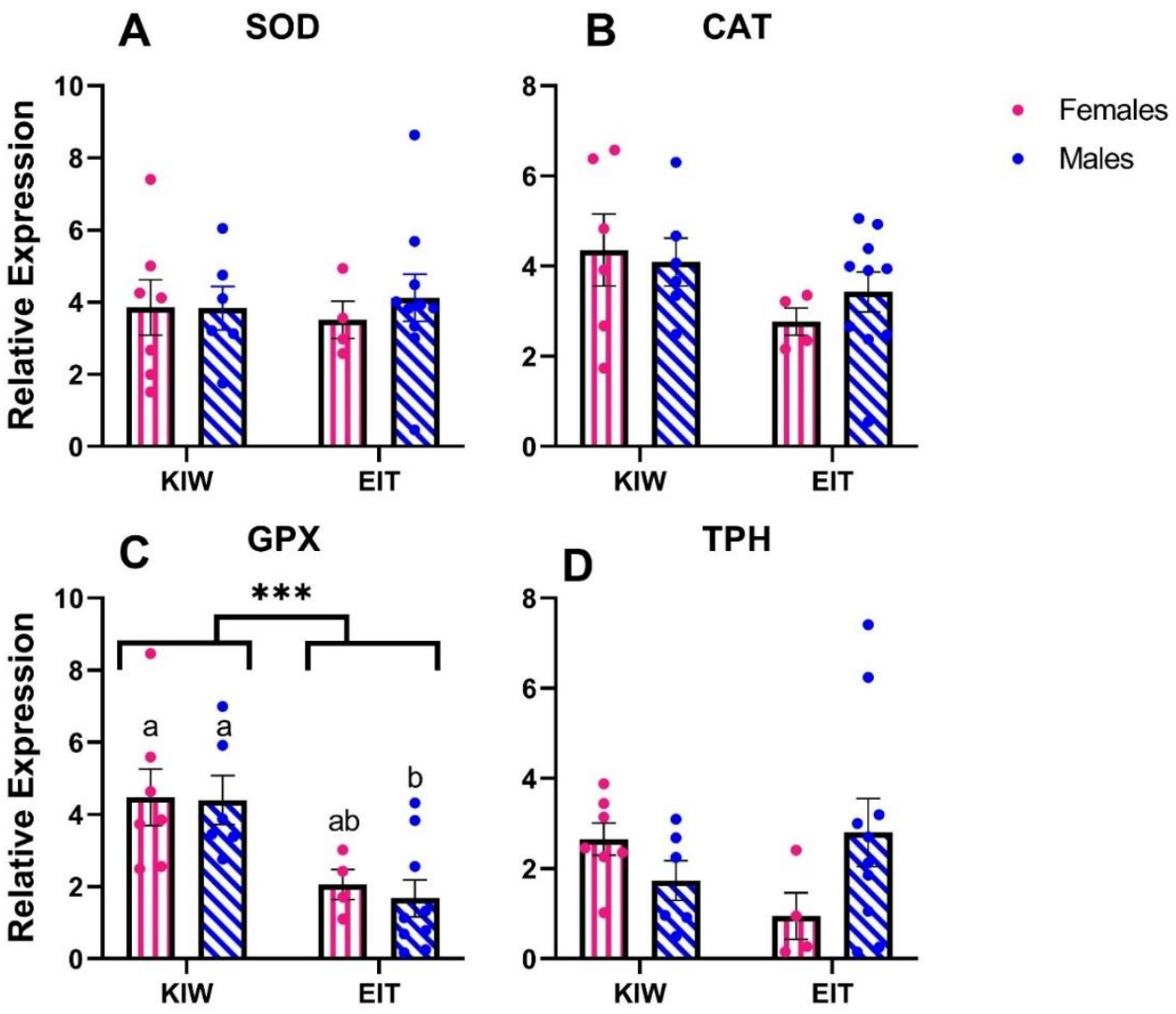

Figure 2.7. Normalized gene expression of A) superoxide dismutase (*sod*), B) catalase (*cat*), glutathione peroxidase (*gpx*) and tryptophan hydroxylase (*tph*) measured in the whole brain of greenside darters (GSD) upstream (KIW) and downstream (EIT) from the WWTP effluent in Fall 2021. Data presented as means + SEM, compared using Two-way ANOVA. (* = $p < 0.05$, n = 13-16).

2.3.3 Enzymatic analysis

Whole brains of species collected in Fall 2020 were used to measure enzymatic activity of antioxidative enzymes involved with ROS reduction (GPX, SOD and relative CAT) in mitochondria (MITO) and cytosol (CYTO) fractions (Table 3). RBD displayed a significant interaction effect for SOD-MITO ($F_{1,30} = 11.6$, $p < 0.001$) and a post-hoc Tukey test showed a significant difference between downstream females and males with females displaying higher activity by 2.4-fold ($p = 0.014$). Similarly, RBD displayed a significant interaction effect for both CAT-MITO ($F_{1,30} = 4.4$, $p = 0.045$) and CAT-CYTO ($F_{1,30} = 13.6$, $p < 0.001$). Tukey test only showed a significant difference in CAT-CYTO between downstream females and males with females displaying higher activity by 2-fold ($p = 0.006$) and between upstream and downstream males with upstream males displaying higher activity by 0.6-fold ($p = 0.034$). GSD displayed a significant interaction for SOD-MITO ($F_{1,31} = 6.1$, $p = 0.02$) and a Tukey test displayed a significant difference between downstream females and males with females displaying higher activity by 2.7-fold ($p = 0.013$). GSD also displayed a significant site effect for CAT-CYTO ($F_{1,31} = 15.3$, $p < 0.001$). Tukey test demonstrated differences between upstream and downstream females with upstream females displaying higher activity by 2.0-fold ($p = 0.04$) and between upstream males and downstream females with upstream males displaying higher activity by 2.1-fold ($p = 0.03$). FTD demonstrated a significant interaction effect ($F_{1,30} = 7.55$, $p = 0.01$ for SOD-MITO. Tukey test displayed only a significant difference between upstream females and males with females displaying higher activity by 3.8-fold ($p < 0.01$). A significant sex effect was found for SOD-CYTO ($F_{1,29} = 7.0$, $p = 0.01$), however no significance found by the post-hoc. FTD additionally demonstrated a significant sex effect ($F_{1,30} = 4.3$, $p = 0.039$) and site effect ($F_{1,30} = 4.7$, $p = 0.047$) for CAT-CYTO. Tukey test showed differences between upstream females and

downstream males with upstream females displaying higher activity by 1.5-fold ($p = 0.027$) and between downstream females and males with females displaying higher activity by 1.0-fold ($p = 0.048$). Lastly, JD demonstrated a significant interaction for CAT-MITO ($F_{1,31} = 4.3$, $p = 0.047$) with no significance found by the post-hoc. Additionally, a significant sex effect was found for CAT-CYTO ($F_{1,31} = 6.9$, $p = 0.013$) with only a difference between upstream females and upstream males with females displaying higher activity by 2.2-fold ($p = 0.034$).

Table 3. Enzyme activity of superoxide dismutase (SOD), relative catalase (CAT) and glutathione peroxidase (GPX) measured in mitochondrial (MITO) and cytosolic (CYTO) fractions, in the whole brain of rainbow darters (RBD), greenside darters (GSD), fantail darters (FTD) and Johnny darters (JD) upstream (KIW) and downstream (EIT) from the WWTP effluent in Fall 2020. Data presented as means (SOD μmol/$mg_{protein}$/mL) and (CAT and GPX μmol/min/$mg_{protein}$) + SEM, analyzed via Two-way ANOVA and post-hoc Tukey test. Different letters display significant differences.

	MITO					***CYTO***				
	Males		*Females*		***Stats***	*Males*		*Females*		***Stats***
RBD	KIW	EIT	KIW	EIT		KIW	EIT	KIW	EIT	
GPX	1.8 ± 0.2	2.06 ± 0.39	2.1 ± 0.3	2.6 ± 0.28		6.6 ± 0.34	6.16 ± 0.38	6 ± 0.5	8.4 ± 1.8	
SOD	6.3 ± 0.8^{ab}	3.75 ± 0.84^{a}	4.9 ± 1.6^{ab}	9.05 ± 1.8^{b}	**Interaction** $F_{1,30}$ = 11.6, p < 0.01	157.5 ± 21.5	140.1 ± 13.7	134.8 ± 15.5	195 ± 27.1	
CAT	966.3 ± 178.6	973.2 ± 249.3	540.5 ± 155.8	1256.8 ± 367.8	**Interaction** $F_{1,30}$ = 4.4, p = 0.045	42.6 ± 6.4^{a}	25.7 ± 2.2b	30.5 ± 1.7^{ab}	49 ± 8.3^{a}	**Interaction** $F_{1,30}$ = 13.6, p < 0.001
GSD										
GPX	56.3 ± 4	48.6 ± 5.5	52.6 ± 3.8	59.5 ± 6.5		19.4 ± 1.2	19.9 ± 0.7	20.8 ± 1.7	23.8 ± 1.6	
SOD	1.2 ± 0.2^{ab}	0.67 ± 0.08^{a}	1.03 ± 0.2^{ab}	1.8 ± 0.5^{b}	**Interaction** $F_{1,32}$ = 7.4, p = 0.01	122.6 ± 25	110.2 ± 20.9	120.9 ± 18.2	183.8 ± 32.5	
CAT	192.5 ± 28	151.7 ± 14.9	171 ± 18.2	181.5 ± 23.8		81.5 ± 5.3^{a}	108.2 ± 9.4^{ab}	167.6 ± 13.2^{a}	81.5 ± 38.1^{b}	**Site** $F_{1,32}$ = 6.9, p = 0.01
FTD										
GPX	4.5 ± 1.4	3.8 ± 1.1	7.5 ± 2.2	3.4 ± 0.6		6.9 ± 0.9	6.8 ± 1.1	6.9 ± 0.6	7.1 ± 0.5	
SOD	9.4 ± 1.9^{a}	16.8 ± 5.3^{ab}	35.8 ± 8.7^{b}	13 ± 2.4^{ab}	**Interaction** $F_{1,30}$ = 7.5, p = 0.01	250.8 ± 64.1	277.8 ± 91.8	374.7 ± 74	364.1 ± 71.5	**Site** $F_{1,30}$ = 4.6, p = 0.04
CAT	1104.8 ± 196.1	2847.6 ± 711.2	2992.8 ± 918.5	2516.2 ± 776.7		51.9 ± 4^{ab}	37.5 ± 5.1^{a}	55.8 ± 5.2^{b}	53.3 ± 5.3^{b}	**Sex** $F_{1,30}$ = 4.3, p = 0.046 **Site** $F_{1,30}$ = 4.6, p = 0.039
JD										
GPX	2 ± 0.8	0.9 ± 0.2	1.2 ± 0.2	1.1 ± 0.2		12.8 ± 3.3	12.1 ± 3	16.6 ± 2.1	13.7 ± 2.6	
SOD	5.6 ± 1.5	5.3 ± 0.5	6.3 ± 1.1	11.7 ± 3.5		416.3 ± 153.7	432.2 ± 70.6	611.4 ± 190	896 ± 293.6	
CAT	790.6 ± 120.8	638.9 ± 78.4	489.9 ± 38.6	768.4 ± 134	**Interaction** $F_{1,31}$ = 4.3, p = 0.047	58.6 ± 10.4^{a}	81.6 ± 13.1^{ab}	127.5 ± 20.5^{b}	107.2 ± 22.5^{ab}	**Sex** $F_{1,31}$ = 6.9, p = 0.01

2.3.4 Morphological Indices

Morphological measurements of species collected in Fall 2020 varied between sexes at both sites for each species (Table 4). RBD males downstream were significantly heavier by 1.2-fold (T= 2.059, df = 37, p = 0.047) compared to upstream individuals. Downstream RBD males displayed higher liver investment indicated by 1.2-fold (T = 2.148, df = 37, p = 0.038) increased hepatosomatic index (HSI). Downstream GSD males were significantly longer (1.1-fold, T = 2.376, df = 42.48, p = 0.022), heavier (1.4-fold, T =2.306, df = 41.98, p = 0.026) and had higher gonadal investment indicated by 1.3-fold (T = 2.110, df = 27.73, p = 0.044) increased gonadosomatic index (GSI) compared to upstream males. Downstream GSD females only displayed lowered gonadal investment by 0.51-fold (T = 2.182, df = 24.97, p = 0.039) compared to upstream females. Downstream FTD males had significantly higher HSI (1.7-fold, T= 2.404, df = 31.35, p = 0.0223) and a higher condition factor (*K*, 1.1-fold, T = 2.879, df = 35, p = 0.0068) than upstream individuals. Downstream FTD females were significantly heavier by 1.3-fold (T= 2.647, df = 26, p = 0.0136) and had a higher K by 1.07-fold (T = 2.528, df = 26, p = 0.0179) than upstream females. Downstream JD males were significantly shorter (1.0-fold, U = 102, n = 24,14, p = 0.0449), lighter (0.7-fold, T = 3.664, df = 30.73, p = 0.0009) and had lower *K* (0.9-fold, p < 0.01) than upstream males. Similarly, downstream JD females were significantly shorter by 0.8-fold (T = 8.583, df = 22.9, p < 0.001) and lighter by 0.53-fold (T = 8.719, df = 20.90, p < 0.0001) than upstream females.

Table 4. Morphological indices of rainbow darter (RBD), greenside darter (GSD), fantail darter (FTD) and Johnny darter (JD) collected upstream and downstream of the Waterloo Wastewater Treatment Plant in the Grand River in Fall 2020. Values are presented as mean ± S.E.M. Site differences in total length (cm), total mass (g), GSI (gonadosomatic index = [gonad mass/body mass] × 100), HSI (hepatosomatic index = [liver mass/body mass] × 100), and *K* (Fulton's condition factor = [body mass/length^3] × 100) are presented in the table and separated based on sexes and location of collection. An asterisk represents a significant difference between upstream and downstream expression within a species, compared using Welch T-test. One JD male collected from the upstream site was removed as an outlier. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

RBD	*Males*		*Females*	
Measurement	Upstream (KIW) *n* = 19	Downstream (EIT) *n* = 20	Upstream (KIW) *n* = 15	Downstream (EIT) *n* = 15
Total length (cm)	5.58 ± 0.112	5.84 ± 0.103	5.62 ± 0.107	5.75 ± 0.11
Total mass (g)	2.15 ± 0.16	2.57 ± 0.158*	2.12 ± 0.144	2.35 ± 0.147
GSI	1.11 ± 0.144	1.21 ± 0.289	3.83 ± 0.117	3.53 ± 0.128
HSI	1.5 ± 0.101	1.82 ± 0.109*	2.4 ± 0.117	2.11 ± 0.192
K	1.2 ± 0.03	1.26 ± 0.02	1.16 ± 0.024	1.21 ± 0.02
GSD	*Males*		*Females*	
Measurement	Upstream (KIW) *n* = 20	Downstream (EIT) *n* = 25	Upstream (KIW) *n* = 15	Downstream (EIT) *n* = 12
Total length (cm)	6.4 ± 0.2	7.05 ± 0.21*	6.63 ± 0.23	6.13 ± 0.334
Total mass (g)	2.89 ± 0.432	3.98 ± 0.441*	3.05 ± 0.34	2.62 ± 0.491
GSI	0.976 ± 0.114	1.3 ± 0.089*	4.86 ± 1.57	2.47 ± 0.309*
HSI	1.77 ± 0.246	1.78 ± 0.317	2.13 ± 0.109	1.95 ± 0.191
K	1.0 ± 0.018	1.04 ± 0.016	0.985 ± 0.016	1.013 ± 0.027
FTD	*Males*		*Females*	
Measurement	Upstream (KIW) *n* = 20	Downstream (EIT) *n* = 17	Upstream (KIW) *n* = 14	Downstream (EIT) *n* = 14
Total length (cm)	5.93 ± 0.155	5.97 ± 0.21	5.1 ± 0.085	5.45 ± 0.151
Total mass (g)	1.8 ± 0.123	2.07 ± 0.189	1.21 ± 0.077	1.592 ± 0.122*
GSI	0.731 ± 0.15	0.549 ± 0.06	2.77 ± 0.155	2.58 ± 0.096
HSI	1.06 ± 0.077	1.84 ± 0.5*	3.16 ± 1.49	2.35 ± 0.822
K	0.843 ± 0.022	0.917 ± 0.01**	0.897 ± 0.02	0.959 ± 0.014*
JD	*Males*		*Females*	
Measurement	Upstream (KIW) *n* = 24	Downstream (EIT) *n* = 14	Upstream (KIW) *n* = 10	Downstream (EIT) *n* = 15
Total length (cm)	5.725 + 0.15	5.23 ± 0.084*	5.87 ± 0.09	4.81 ± 0.091***
Total mass (g)	1.73 ± 0.135	1.19 ± 0.06***	1.73 ± 0.094	0.917 ± 0.055***
GSI	1.2 ± 0.065	1.12 ± 0.094	4.05 ± 0.207	3.72 ± 0.146
HSI	1.24 ± 0.061	1.34 ± 0.12	1.84 ± 0.157	1.75 ± 0.126
K	0.87 ± 0.013	0.81 ± 0.014**	0.85 ± 0.019	0.814 ± 0.014

In contrast to morphological measurements of fish collected in the previous year, fish collected in Fall 2021 demonstrated less differences between sites and sexes (Table 5) RBD males downstream displayed higher *K* compared to males upstream by 0.06-fold ($U = 112.5$, $p = 0.029$). RBD males downstream were longer and heavier compared to upstream males but not significantly (Table 5). Downstream RBD females were significantly shorter than upstream females by 0.05-fold ($T = 2.19$, $df = 39$, $p = 0.035$). In addition, downstream males and females GSD were lighter, shorter and had lower *K* compared to upstream GSD, however differences were not significant.

Table 5. Morphological indices of rainbow darter (RBD) and greenside darter (GSD) collected upstream (KIW) and downstream (EIT) of the Waterloo Wastewater Treatment Plant in the Grand River in Fall 2020. Values are presented as mean ± S.E.M. Site differences in total length (cm), total mass (g), HSI (hepatosomatic index = [liver mass/body mass] × 100), and *K* (Fulton's condition factor = [body mass/length^3] × 100) are presented in the table and separated based on sexes and location of collection. An asterisk represents a significant difference between upstream and downstream expression within a species, compared using Welch T-test (* = $p < 0.05$).

RBD – Oct 2021 -	*Males*		*Females*	
Measurement	Upstream (KIW) *n* = 19	Downstream (EIT) *n* = 20	Upstream (KIW) *n* = 21	Downstream (EIT) *n* = 20
Total length (cm)	5.97 ± 0.115	6.07 ± 0.115	5.73 ± 0.09	5.44 ± 0.097*
Total mass (g)	2.64 ± 0.19	2.88 ± 0.168	2.23 ± 0.12	1.95 ± 0.134
HSI	0.93 ± 0.077	0.82 ± 0.087	1.1 ± 0.08	1.17 ± 0.1
K	1.2 ± 0.03	1.28 ± 0.032*	1.16 ± 0.03	1.18 ± 0.015
GSD – Oct 2021 -	*Males*		*Females*	
Measurement	Upstream (KIW) *n* = 24	Downstream (EIT) *n* = 21	Upstream (KIW) *n* = 16	Downstream (EIT) *n* = 12
Total length (cm)	7.29 ± 0.26	7.17 ± 0.26	7.4 ± 0.23	7.13 ± 0.32
Total mass (g)	4.66 ± 0.58	4.17 ± 0.53	4.45 ± 0.36	3.81 ± 0.48
HSI	0.9 ± 0.07	0.91 ± 0.07	1.14 ± 0.096	1.32 ± 0.08
K	1.07 ± 0.02	1.05 ± 0.04	1.05 ± 0.01	0.99 ± 0.04

2.3.5 PCA Analyses

To assess effects of wastewater effluent attributes on darters, morphological, physiological, water parameters and five differently behaving contaminants associated to their specific modes of action (DCF, CBZ, sulfamethazine (SMZ), SNRI and atrazine) were used to conduct a principal component analysis (PCA). Score and loading plots were synthesized for RBD and GSD of both years. Each calculated principal component (PC) value has an associated eigenvalue. PC1 always has the largest eigenvalue and therefore, explains the largest variation in the original data with each consecutive PC holding a decreasing eigenvalue (Hin Low et al., 2011). In this study, RBD collected in 2020, the first two PCs accounted for 67.6% of the variability associated with effluent exposure (Fig. 8A). The biplot clearly demonstrates two clusters, being divided by upstream and downstream and driven by pharmaceutical contents and water parameters. The second driving factors are weight, length and sex, with antioxidant gene transcripts falling in the between both PCs. However, when looking at the biplot for enzyme activity (Fig. 8B) where the first two PCs accounted for 61.2% of the total variation. Pharmaceuticals (SNRI, DCF, CBZ and SMZ), weight and length are strongly correlated with PC1 with eigenvector going towards EIT. SOD and GPX activity are strongly correlated with PC2 with its eigenvector going towards EIT, as well as HSI and sex.

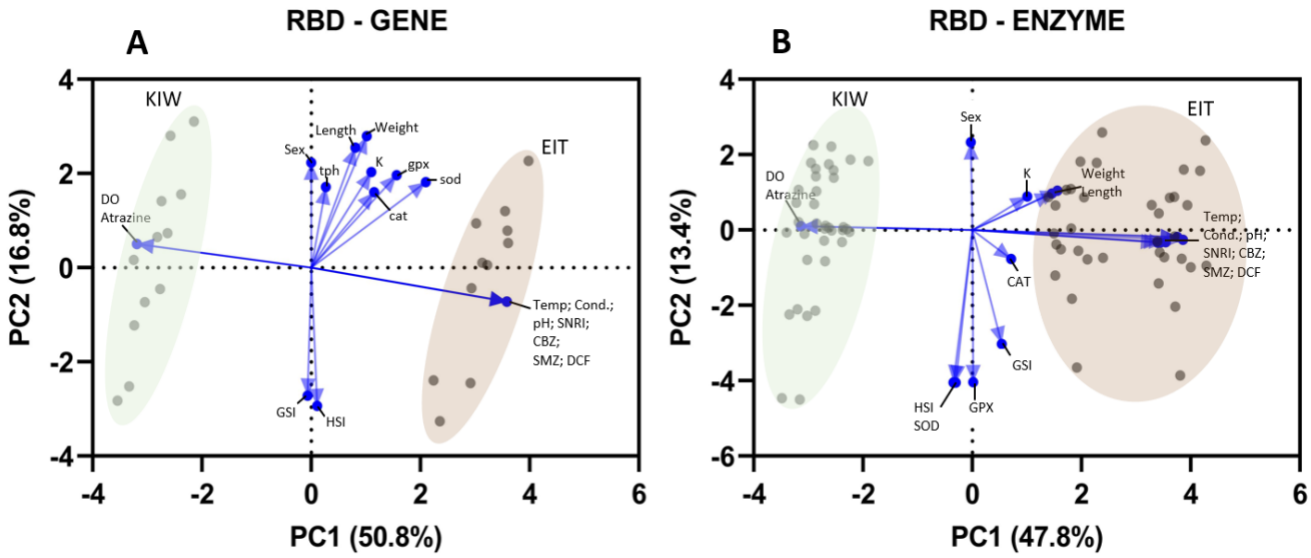

Figure 2.8. Principal component analysis (PCA) of morphological (sex, length, weight, HSI, GSI and K), physiological (*sod*, *cat*, *gpx* and *tph*) and biochemical (water temperature, conductivity, pH, SNRI, DCF, CBZ, SMZ and atrazine) characteristics of RBD collected from KIW and EIT in Fall 2020, A) gene transcripts and B) enzyme activity.

GSD collected in Fall 2020, displayed similar observations to RBD collected in the same season. PC1 explained 52.5% of the variability of the data and the biplot similarly demonstrated two clusters, divided by site (Fig. 9A). Once again PC1 was driven by pharmaceutical content and water parameters but its also strongly correlated with *sod* and *cat* transcript expression with their eigenvectors correlating with the downstream cluster (Fig. 9A). Looking at the corresponding enzyme biplot of 2020 (Fig. 9B), the first two PCs accounted for 63.1% of the total variation. Contaminants and water parameters are strongly correlated with PC1, and weight, length, CAT and GPX enzyme activity are strongly correlated with PC2 showing that downstream GSD are in fact heavier, longer but may be experiencing increased oxidative stress compared to upstream GSD due to the increased antioxidant response.

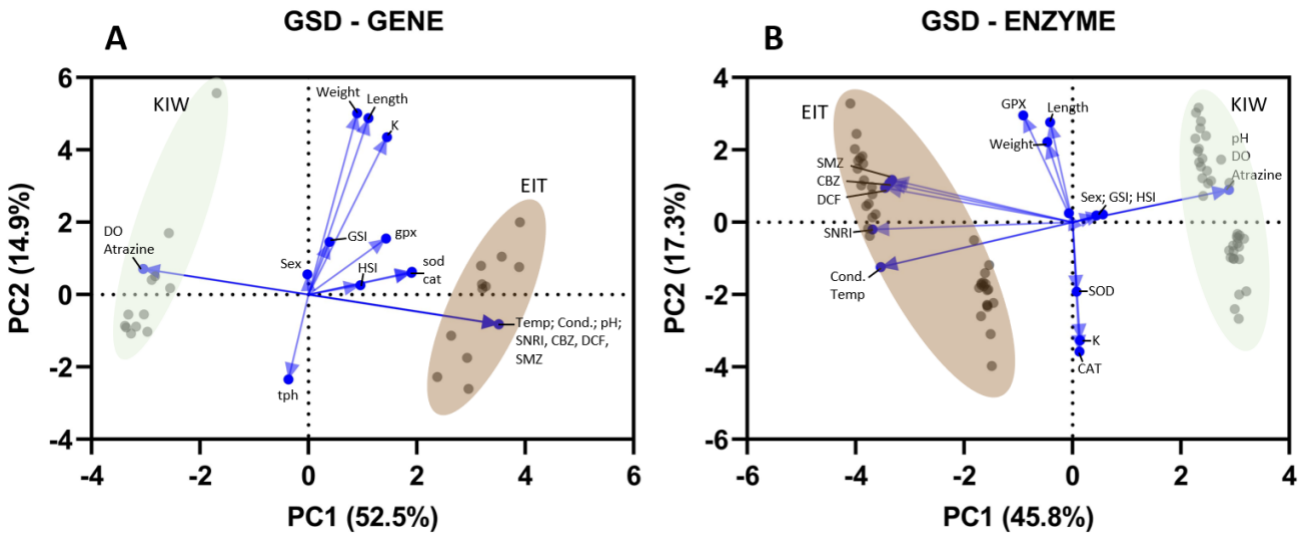

Figure 2.9. Principal component analysis (PCA) of morphological (sex, length, weight, HSI, GSI and K), physiological (*sod*, *cat*, *gpx* and *tph*) and biochemical (water temperature, conductivity, pH, SNRI, DCF, CBZ, SMZ and atrazine) characteristics of GSD collected from KIW and EIT in Fall 2020, A) gene transcripts and B) enzyme activity

In contrast, RBD collected in Fall 2021, experienced a different pattern in gene transcripts (Fig. 10A). Both PCs accounted for 69.9% of the variation in the data with water parameters and pharmaceuticals strongly correlated with PC1. Interestingly, in contrast to Fall 2020, SMZ's eigenvector was no longer towards the downstream cluster, instead the direction was towards the upstream cluster. Antioxidant transcripts *sod* and *gpx* were strongly correlated with PC2, along with weight and length all going towards upstream. PCs of GSD in Fall 2021 accounted for 72% of the variation in the data with water parameters and pharmaceuticals following the same pattern as RBD Fall 2021 (Fig. 10B). Gene transcript, *gpx*, was strongly correlated with both PCs with an eigenvector direction going towards KIW. Transcript's s*od* and *cat*, along with weight and K were strongly correlated with PC2.

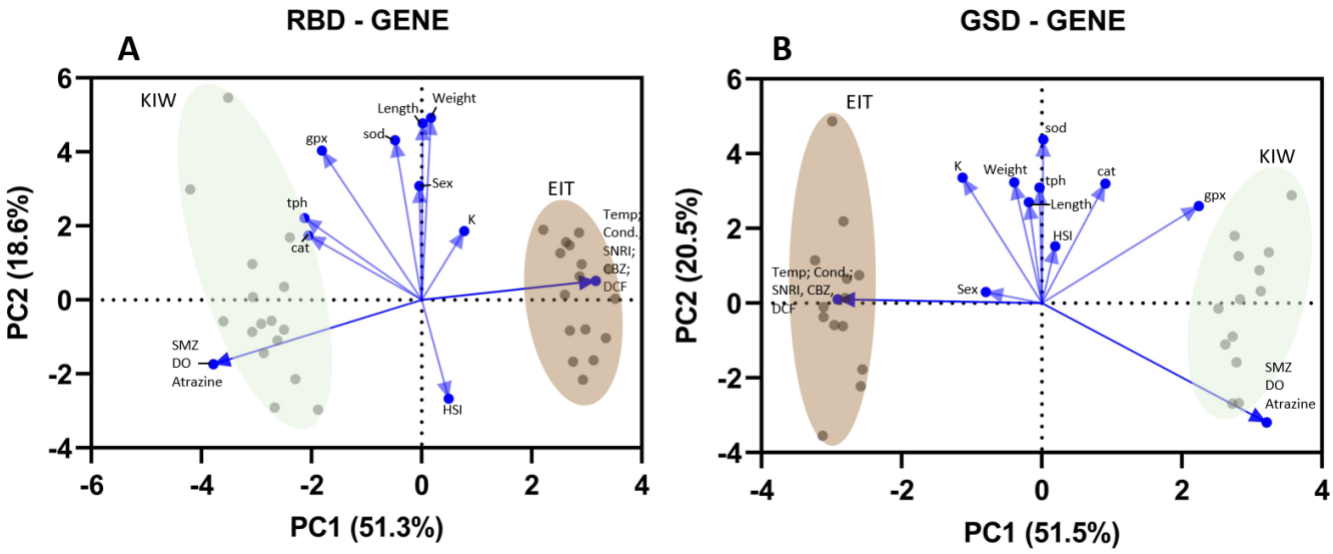

Figure. 2.10. Principal component analysis (PCA) of morphological (sex, length, weight, HSI, GSI and K), physiological (*sod*, *cat*, *gpx* and *tph*) and biochemical (water temperature, conductivity, pH, SNRI, DCF, CBZ, SMZ and atrazine) characteristics of RBD and GSD collected from KIW and EIT in Fall 2021 A) RBD gene transcripts and B) GSD gene transcripts.

2.4.0 Discussion

2.4.1 Water Quality

In Fall 2020, water samples collected downstream of the Waterloo WWTP revealed several pharmaceuticals and contaminants, including VEN and CBZ were present in high concentrations compared to upstream samples. The two highest contaminant concentrations found downstream were VEN and DCF, a nonsteroidal anti-inflammatory drug. Atrazine, a frequently used herbicide was the third highest downstream but also the highest contaminant upstream. CBZ was observed to be fourth highest. These findings agree with similar observations of the same contaminants found in the Fall of 2015 in the Niagara River which flows from Lake Erie to Lake Ontario (Arnnok et al., 2017). Several pharmaceutical contaminants (ie. ibuprofen, fluoxetine, venlafaxine, carbamazepine, naproxen, and diclofenac) have been previously detected with concentrations as

low as 0.11 ng/L to as high as 12.95 ng/L in various fish tissues such as brain, liver, and gonads (Brooks et al., 2005; Arnnok et al., 2017; McCallum et al., 2017). For instance, Arnnok et al. (2017) observed several SSRIs in brain, gonad, liver, and muscle tissues of various fish species ranging in concentrations of up to 17 ng/g of sertraline, between 0.2 to 40 ng/g of norfluoxetine and up to 57 ng/g of VEN. Thus, contaminants found downstream of WWTP are candidates that may trigger oxidative stress (Painter et al., 2006; Galar-Martínez et al., 2010; Beckhauser et al., 2016; Li et al., 2016; Thompson and Vijayan, 2020a). However, the abilities to trigger oxidative stress relies heavily on their bioavailability, bioaccumulation, retention time, uptake processes and ecological traits (Arnnok et al., 2017).

In Fall 2021, the highest pharmaceuticals downstream were VEN at and its metabolite desmethyl-VEN, with DCF and gemfibrozil, a fibrate, coming in third and fourth. Interestingly, the contaminant SMZ, an antibacterial, was notably higher upstream than the previous year. In addition, the water temperature of both KIW and EIT in Oct 2021, was almost 10 degrees higher than the previous year in Oct 2020. This increased temperature was sustained for weeks in result of a warmer Fall. Differences in water quality between sampling years will be discussed in the context of our data below.

2.4.2 Morphological indices

Each species displayed significant variations in morphological measurements between sites in fish collected Fall 2020. RBD males and females were heavier downstream, which is expected as they have been observed to take advantage of increased nutrients downstream (Brown et al., 2011; Hodgson et al., 2020). RBD males downstream displayed higher energy storage indicated by increased HSI, also most likely due to the advantage of increased nutrients (Tetreault et al., 2011; Mehdi et al., 2018). Similarly, GSD males downstream were longer, heavier and had

higher gonadal investment, indicated by increased GSI. This is additionally expected with the increased nutrient availability downstream. In contrast, GSD females downstream displayed lowered GSI, which is consistent with previously reported reductions in GSI in response to WWTP effluent (Vajda et al., 2008, Tetreault et al., 2011). Sex-related differences may be due to seasonal changes in reproductive endpoints, where Fall breeding has just ended and females exhibit lowest gonadal condition post-spawning (Brasfield et al., 2013; Tetreault et al., 2014). FTD males downstream had higher energy storage and higher *K*, indicating better fish conditions. As FTD live in similar habitats as RBD, their increased HSI and *K* may indicate their ability to effectively assimilate the nutrients downstream (Mehdi et al., 2018). The FTD females downstream were heavier and had higher *K*, thus demonstrated better fish conditions most likely due to their ability to utilize the increased nutrients downstream. In contrast, to the thriving RBD, GSD and FTD from increased nutrients, JD males and females downstream both were lighter and shorter, and males also displayed lower *K*, indicating poorer body condition. JD have recently returned to the Grand River since WWTP upgrades, suggesting they may be more susceptible to changes in water quality. These varying results may be indicative species-specific susceptibility to underlying effluent mixed stressors and be a result of their habitat preferences and ability to assimilate nutrients (Tetreault et al., 2014).

Interestingly, RBD and GSD collected in Fall 2021 opposed the findings of morphological measurements seen in RBD and GSD collected in Fall 2020. RBD females downstream were shorter and lighter. This opposes previous findings where downstream individuals normally can assimilate increased nutrients resulting in larger individuals (Tetreault et al., 2021; Mehdi et al., 2018). However, similarly to the previous year RBD males downstream also displayed higher *K* which supports the advantage of increased nutrients (Tetreault et al., 2011; Mehdi et al., 2018).

Also contrasting the previous year, GSD collected in 2021 were both shorter and lighter, however had higher *K*. Interestingly, both RBD and GSD downstream females had higher hepatic investment compared to upstream individuals which contrast findings from 2020. This increase in hepatic investment, albeit not statistically significant, can indicate either nutrient assimilation from the effluent or if individuals are incapable of converting internal energy reserves into growth (Tetreault et al., 2011). WWTP effluent is composed of mixed contaminants that vary spatially and temporally, thus its effects also differ within species and sexes (Alvarez et al., 2009; Mehdi et al., 2018). Overall, further research is required to provide a better understanding on the relationship between oxidative stress and morphological measurements.

2.4.3 ROS

In Fall 2020, downstream species generally displayed increased relative abundance of both *sod* and *cat* downstream of the Waterloo WWTP compared to upstream species. GSD displayed the most significant effects, with increased expression of *sod* and *cat*. Similarly, JD demonstrated increased *sod* expression in both sexes downstream of the WWTP. FTD displayed no significant differences between sites or sexes for gene expression, however followed similar sex-specific trends. These findings may be indicative of increased activity of ROS production in the brain cells of these species downstream of the WWTP and are therefore compensating with increased transcript expression. However, the observed results in FTD with some experiencing higher gene expression and enzyme activity upstream could be a result of the notably high atrazine concentration. Atrazine has been observed to induce elevated antioxidant responses in zebrafish exposed to as low as 0.3 µg/L following subchronic exposures (Blahová et al., 2013). Under normal physiological function, during the production of ROS radicals, the antioxidative defense system provides enzymes to reduce the radicals to water and oxygen, decreasing the chances of

creating an ideal environment for oxidative stress to occur (Hoseinifar et al., 2020). Overexpression of antioxidative enzymes in cells have been observed to provide protective support against the damaging effects of several oxidative stress pathways (Mirault et al., 1994; Warner, 1994; Day, 2009). Therefore, increasing transcript expression of the involved enzymes would provide a beneficial, plastic response to wastewater effluent exposure, although further investigation is warranted.

In contrast, RBD and GSD collected downstream of the effluent at EIT in Fall 2021 did not follow similar trends observed in Fall 2020. Although, an interactive effect for *sod* was displayed in RBD, increased gene expression of the antioxidants *sod* and *cat* were not as evident as the previous year. In fact, gene expression of the antioxidants was higher upstream at KIW. Considering notably high river temperatures were sustained for several weeks prior to and including the day of sampling at both sites (GRCA, 2022), it is possible that gene expression changes in Fall 2021 were in result of increased temperatures. Warming temperatures have been shown to enhance and/or attenuate changes elicited by venlafaxine exposure in juvenile fish (Maulvault et al., 2019). A similar study recently published demonstrated that increased water temperatures led to antagonistic effects of antidepressant exposure in *Daphnia magna*, where initial phenotypic changes were masked (Aulsebrook et al., 2022). Moreover, sustained elevated temperatures could have also increased levels of antioxidant activity in upstream species, as increased water temperatures have been previously shown to trigger ROS generation (Clotfelter et al., 2013; Banh et al., 2016). For instance, fish acutely exposed to temperature increases of 10-15°C, elevated lipid, and protein oxidation are observed, most likely attributed to limited rate capacity of the antioxidant enzymes (Banh et al., 2016 In addition, SMZ which was notably higher upstream in Fall 2021, has displayed inducing effects on antioxidant response at varying

developmental stages of zebrafish in concentrations as low as 0.2 ug/L (Yan et al., 2018). Therefore, considering a greater number of contaminants were detected upstream compared to the previous year, a higher concentration of SMZ and the combination of increased river temperatures, it is possible these parameters are acting as covariates and is resulting in upstream individuals experiencing greater than usual oxidative stress and thus is reflected by the increased antioxidant gene transcript.

Changes in gene transcript markers can be further supported by assessing enzyme activity within the cells to validate changes of expression (Sun et al., 2014). The results of this study demonstrated some significant variations of antioxidant enzyme activity in both the mitochondrial fraction and the cytosol in all species collected in Fall 2020. Corresponding, to the increase of transcripts, RBD and GSD females downstream demonstrated a trend of increased enzyme activity of SOD. Similarly, RBD, GSD and JD demonstrated a trend of increased CAT enzyme activity, however males downstream demonstrated a trending decrease in activity for SOD and CAT. In contrast, FTD downstream females showed a general decrease in SOD and CAT activity. The observed increased activity of SOD in females downstream of RBD and GSD support the observed increased gene expression of *sod*. SOD is the first line of defense of the antioxidative defense and the most abundant of the antioxidant enzymes, these results suggest increased activity may be a result to provide protection from ROS production as it functions to convert superoxide anions to hydrogen peroxide (H_2O_2) thus reducing toxicity and chances of oxidative damage (Zorov et al., 2020). In addition, following SOD in the antioxidative defense, CAT is the second line of defense with a primary function of reducing H_2O_2 production to water and oxygen (Li et al., 2016; Hoseinifar et al., 2020). Therefore, the increased SOD activity, resulting in increased production of H_2O_2, can be further supported by the observed increased activity of CAT in females

downstream and the observed increased gene expression of *cat*. As increased enzyme activity of CAT can be observed in three species, it may indicate an increased presence of H_2O_2 within the brain of downstream species, although this could not be measured in our samples. These findings agree with the observed increased activity of SOD and CAT in the brains of common carp chronically exposed to reservoir contaminants (Galar-Martínez et al., 2010). Furthermore, oxidative damage, measured through LPO, has been demonstrated in freshwater mussels (*Lasmigona costata*) following a 4-week exposure to wastewater effluent from the GR in 2010 (Gillis et al., 2014). Therefore, increased expression and enzyme activity of antioxidants may be an adaptive response to xenobiotics to induce stress to compensate for further oxidative damage (Galar-Martínez et al., 2010). However, when antioxidant enzymes are incapable of neutralizing or eliminating excess ROS, such as an imbalance, there is an increased risk of oxidative stress which can result in decreased enzyme activity through degradation which may be driven by increased concentrations of ROS molecules (Zhang et al., 2008; Sun et al., 2014; Hoseinifar et al., 2020). Under normal physiological function, females and males have different oxidant capacities and exhibit differences in enzyme activity, however in stressed conditions, these differences may be enhanced (Kamper et al., 2009). Similar results to this current study were observed when liver SOD and CAT activity were measured in brown trout collected from two streams, where males displayed lower activity compared to females (Parolini et al., 2019). These results can be supported by the observations that sex-hormones, namely testosterone promotes H_2O_2 production, where estrogen suppresses H_2O_2, which may provide a beneficial advantage for females against environmental stressors (Sullivan et al., 2007; Kamper et al., 2009: Parolini et al., 2019). Indeed, this may explain the trending decrease of antioxidative enzyme activity observed in males downstream as their antioxidant defense system may be overwhelmed by H_2O_2 resulting decreased

catalytic function of antioxidant enzymes due to protein degradation (Zhang et al., 2008). Moreover, a study on goodeid fish (*Ameca splendens*) exposed to polychlorinated biphenyls (PCBs) demonstrated in control males, antioxidant enzyme activity is higher than control females which supports the results observed in our study, as RBD and GSD control males also displayed higher activity compared to control females (Vega-López et al., 2009). Sex-specific responses in antioxidant activity may differ due to differences in cellular processes that regulate antioxidant responses which are affected by factors such as metabolism, behaviour, growth, and reproduction (Vega-López et al., 2007; 2009; Adeogun et al., 2020). Interestingly, GPX did not show any significant changes or trends across all species and sexes. This corresponds with a study conducted on common carp exposed to DCF where brain GPX activity displayed no changes (Islas-Flores et al., 2013). However, oxidizing agents such as pharmaceuticals can inactive or inhibit antioxidative enzymes if left to bioaccumulate (Modesto and Martinez, 2010). In addition, glutathione is inversely correlated with CAT, which may suggest that increased CAT activity can inactivate the requirement of GPX (Soorya et al., 2013). Its additionally important to note that species-specificity, tissue-specificity and time-specific sensitivty exist for oxidative stress and related damage, which may suggest some species, such as darters, might have an adaptive plastic response to live in these stressed environments without resulting damage or that other organs such as the liver, a major site of antioxidant activity may be more equipped to deal with these stressors (Ozcan Oruc et al., 2004; Kavitha and Venkateswara Rao, 2009; Islas-Flores et al., 2013; Brandão et al., 2013). Nevertheless, these results are in accordance with previous studies that demonstrate physiological changes in response to environmental stressors such as wastewater effluent containing pharmaceutical contaminants (Galar-Martínez et al., 2010; Maulvault et al., 2018; Maulvault et al., 2019; Adeogun et al., 2020; Li et al., 2020). In addition, the results of this study demonstrated

significant changes to the second isoform of SOD in the mitochondria and significant changes to CAT only in the cytosol. SOD1 in the cytosol is not the primary antioxidant of the cytosol, while SOD2 in the mitochondria is the primary antioxidant as it converts O_2^- into a more stable form of H_2O_2 (Benáková et al., 2021) Therefore, the response of SOD2 with no observed changes in SOD1 may be due to the prioritized requirement of the isoforms within the organelles. As H_2O_2 exits the mitochondria wall and enters the cytosol, catalase enzymes which are primarily found in peroxisomes, free floating in the cytosol, have an expected response to reduce H_2O_2 (Du et al., 2016; Benáková et al., 2021). These results are not specific to anyone CEC, however oxidative stress biomarkers and morphological parameters, may be indicative of species health following effluent exposure (Tetreault et al., 2021).

2.4.4 Antioxidant enzyme response to CECs

The evidence of increased antioxidant activity in this study are consistent with previous research which examined the direct exposure of pharmaceuticals on tissue and elicited increased antioxidant activity. For instance, waterborne VEN exposure at a concentration of 20 μg/L and combination to other stressors (warming and acidification) in the brains of meagre (*Argyrosomus regius*), a perch-like species, displayed increased levels of CAT, SOD and glutathione (GSH) activity, as well as increased levels of LPO after 28 days of exposure (Maulvault et al., 2019). Additionally, 0.5 ug/L of fluoxetine exposures on zebra mussels (*Dreissena polymorpha*) and zebrafish increased SOD, CAT and GSH activity after 14 days of exposure (Magni et al., 2017; Pan et al., 2018). In the brains of rainbow trout (*Oncorhynchus mykiss*) exposed to 200 μg/L and 2000 μg/L of CBZ, increased levels of CAT activity and LPO were observed after 21 days following exposure, demonstrating high oxidative stress damage (Li et al., 2020). At 200 μg/L and 7 days following exposure, the brains displayed increased activity of CAT, SOD and GPX,

however, SOD and GPX activity decreased after 21 days (Li et al., 2020), which may be due to oxidative stress causing cellular damage and therefore, enzyme degradation (Zhang et al., 2008; Li et al., 2020). Although research on interactive effects of pharmaceuticals and multi-stressors are limited, previous studies have observed increased activity of CAT, SOD and GSH in response to co-exposures of pollutants and warming temperatures or acidification which is a plausible cause for the oxidative stress occurring in this study (Freitas et al., 2016; Maulvault et al., 2018, Sampaio et al., 2018; Mauvault et al., 2019). While several studies use in-lab exposures (Magni et al., 2017; Pan et al., 2018; Li et al., 2020) to investigate the relationship between CECs and oxidative stress, comparing these studies to field studies can be difficult due to differences in length of exposures, co-pollutants, and time of sampling (Metcalfe et al., 2010; Yang et al. 2020). Due to the analytical limitations of this study, several other contaminants may not have been screened for and/or contaminant concentrations may differ between years , thus further studies should focus on direct exposure of pharmaceuticals and PPCPs on brain tissue to better understand the antioxidant responses to these stressors found in the wastewater effluent.

2.4.5 Tryptophan hydroxylase (*tph*)

Tph is the rate-limiting enzyme acting as the precursor of serotonin synthesis thus influencing the concentration of serotonin, as well as catalyzes reactions critical to support cellular function of serotonin neurons in the brain (Rahman et al., 2011). Previous studies have demonstrated that oxidizing conditions to *tph* can quickly destroy its catalytic function, resulting in changes to serotonin levels and function (Kuhn et al., 1979;1980). Hussain and Mitra (2004) investigated ROS-mediated effects on *tph* in the brains of rats and found that increased presence of CAT activity not only inhibited H_2O_2 from damaging the enzyme but also increased residual activity of *tph* by 50%. In this study, fall 2020 RBD downstream females and males demonstrated

opposing changes in gene expression of *tph*, where downstream females had increased expression and downstream males decreased. Also observed, where opposing changes in activity of CAT enzyme, where females downstream increased CAT activity and males downstream decreased. CAT enzyme activity in downstream females may be compensating for potential ROS-mediated damage to *tph* enzymes, however more studies are needed to understand the sex differences seen in *tph*. With the presence of certain antidepressants in the effluent such as venlafaxine and fluoxetine, both selective serotonin-reuptake inhibitors, they have been observed to increase the activity of *tph*, and therefore, changes in expression of *tph* in RBD may be linked to waterborne exposure of antidepressants found in the effluent discharge (Qiao et al., 2019). Interestingly, in fall 2021, *tph* expression was significantly higher in RBD males upstream compared to downstream. This observation may be an indication of changes in serotonin levels in the brain due to xenobiotic disruption. Moreover, high concentrations of antidepressants can reverse adverse effects of depression and anxiety in the brain, but they can also trigger ROS production (Elmorsy et al., 2017), thus pharmaceutical exposures can lead to counter-productive effects in the brain of non-target animals which could explain the opposing sex and seasonal differences observed between RBD males and females.

2.4.6 Limitations & Conclusions

Oxidative stress and the antioxidant defense in teleost and other aquatic species, may be triggered by several varying factors including age, diet, xenobiotics, and environmental factors such as temperature, oxygen and nutrient availability, salinity, and pH (Martínez-Álvarez et al., 2005; Ferreira et al., 2014; Birnie-Gauvin et al., 2017; Hoseinifar et al., 2020). In addition, antioxidant enzyme activity is highly influenced by complex pathways and may play a mechanistic role in how organisms adapt to environmental variability (Birnie-Gauvin et al., 2017). To our

knowledge, this is the first research to investigate antioxidative activity in the brain of *Etheostoma* species. The brain utilizes large amounts of oxygen and has limited anaerobic capacity; therefore, it relies heavily on aerobic oxidative phosphorylation, in turn leaving it to be especially sensitive to ROS-mediated damage (Day, 2009). Although responses are triggered to protect cells from oxidative stress, overexpression of ROS molecules and antioxidant defense can lead to neurological impairment due to the high sensitivity of the vertebrate brain relating to its increased oxygen consumption and composition of PUFA (Migliore and Coppede, 2009; Day, 2009; Yan et al., 2013). This can be seen in humans that suffer from neuronal diseases which have a link to overproduced ROS radicals contributing to symptom manifestation of neurodegenerative diseases such as Parkinson's, Huntington's, and Alzheimer's disease (Przedborski and Ischiropoulos, 2005; Browne and Beal, 2006; Forero et al., 2006).

In conclusion, effluent discharge contains complex mixtures of contaminants which make it challenging to assess effects due to synergistic, antagonistic, or additive effects of combined contaminants (Metcalfe et al., 2010). While these findings do not directly show cause and effect, they highlight the evidence of oxidative stress levels occurring in the brains of these species living near WWTP effluent contaminated sites and sex-related differences of antioxidative abilities., This study also demonstrates the large yearly variation in water parameters such as contaminants and river temperatures, as well as fluctuations in physiological responses within species demonstrated through the correlating patterns observed in PCAs. As field work presents its challenges in acquiring fish and equal sexes, particularly during the pandemic where personnel restrictions were implemented in Canada, repeated future studies should refer to the Appendix A, Table A.2, for suggested sample sizes for oxidative transcripts and enzymes for within/between group comparison following power analysis Moreover, the COVID-19 pandemic has negatively

impacted people's mental health and with antidepressants as the frontline of therapy, concentrations of these pharmaceuticals are predicted to increase in the years to come in aquatic environments (Chen et al., 2020; Khan et al., 2020; Castillo-Zacarías et al., 2021). Thus, future studies should aim to focus on the cause and effect within effluent that may be driving oxidative stress and its relationship with pharmaceuticals, as well as focus on sex-related differences in antioxidant abilities which may provide insightful knowledge into mechanisms related to behavioural impairment and a better understanding on more subtle effects caused by WWTP effluent and the CECs that inhabit the Grand River watershed.

Chapter 3

General Discussion

3.0.0 General Discussion

3.1.0 Conclusions

The primary objective of this study was to examine the antioxidant defense response to pharmaceutical contaminated WWTP effluent in *Etheostoma* spp. (darters) from the Grand River by investigating antioxidant enzymes through transcriptional and enzymatic analyses. Antioxidant enzymes are excellent biomarkers of oxidative stress as they respond rapidly and the impact of oxidative damage relies on the effectiveness of these enzymes (Bandyopadhyay et al., 1999). This is supported through several studies that demonstrate rapid antioxidant changes following acute exposures to several CECs in varying fish tissue (Stancova et al., 2017; Li et al., 2010; Beckhauser et al., 2016; Duarte et al., 2020). However, sustained increased antioxidant activity and oxidative stress can have detrimental impacts on physiological pathways, such as neurological pathways. Changes to neurological pathways may be leading to observed behavioural alterations in several aquatic species (Cannon and Greenamyre, 2011; Beckhauser et al., 2016; Simmons et al., 2017a). Behavioural analyses have been commonly used in ecotoxicology to determine sublethal effects to contaminants in aquatic species, with an increasing body of evidence of behaviour alterations following pharmaceutical exposures (Soler et al., 2021). However, the mechanisms causing these changes in behaviour are still poorly understood. Therefore, investigating the relationship between antioxidant activity, oxidative stress and waterborne pharmaceutical exposure to non-target species following contaminated effluent exposure could provide insight into these changed behaviours.

The study carried out in chapter two of this thesis demonstrated results related to antioxidant gene expression and enzyme activity following effluent exposure and water conditions in two separate years, as well as results between species and sex. Comparing the differences between species may be difficult due to species-specific responses and tolerances, as well as

comparison would require much larger sample sizes. Chapter two also highlighted, how the antioxidant defense can be heavily influenced by the varying magnitude of factors. These factors include xenobiotics, morphological parameters, and environmental factors such as temperature, oxygen, conductivity, and pH which can be observed to have varying changes in the literature (Martínez-Álvarez et al., 2005; Ferreira et al., 2014; Birnie-Gauvin et al., 2017; Hoseinifar et al., 2020).

Chapter two provides growing evidence of the role pharmaceuticals may have in driving changes in antioxidant activity in non-target species facing environmental stressors in rivers. This is supported with the strong correlation observed between antidepressants and antioxidant response demonstrated by PCA analyses. A study conducted by Tanoue et al. (2015) which demonstrated that psychotropic drugs have higher transportability to fish brain than NSAIDs and antibacterial agents in wild fish exposed to wastewater effluent suggest that the observed increased antidepressants in the Grand River could potentially be a driving factor in changes in antioxidant response in the brains of darters. However due to synergistic, antagonistic, and additive effects of all contaminants and water parameters found in the Grand River, it is nearly impossible to relate oxidative stress to pharmaceutical exposure and behavioural changes without investigating neurological mechanisms involved in pharmaceutical uptake and effects. The uptake of pharmaceuticals is determined by several interactive factors such as water quality parameters, exposure time, chemical properties, and species-specific factors (McCallum et al., 2017). The uptake pathway of CECs is quite complex and still poorly understood (Fig 3.1).

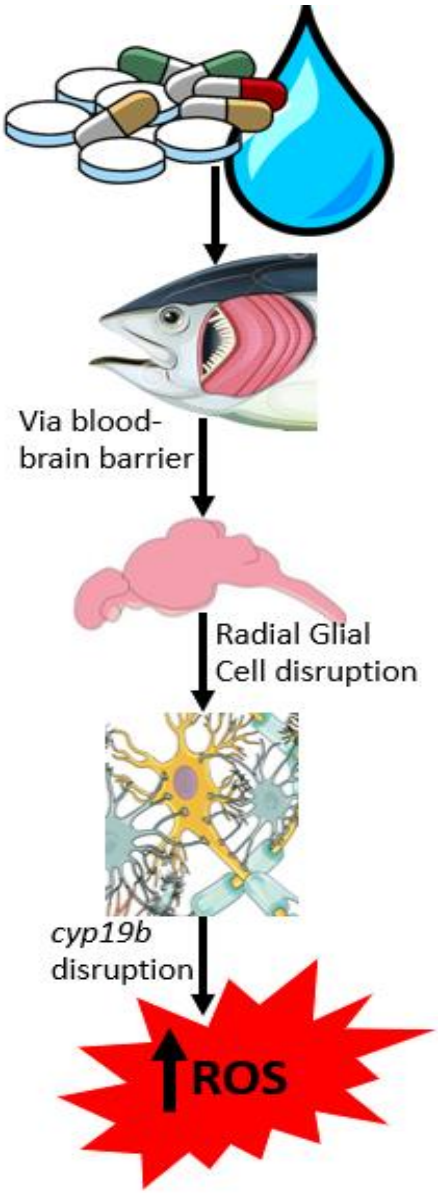

Figure 3.1. Suggested uptake pathway of CECs in the water through the gills, up to the brain and cellular disruption resulting in increased reactive oxygen species (ROS).

Pharmaceuticals and CECs are taken up via the gills and transported across the epithelium through transcellular or carrier-mediated processes (Stott et al., 2015). However, the concentration of pharmaceuticals that enter plasma and other tissues remain difficult to identify because some contaminants may bind to protein and be metabolized and/or excreted by the gills, leading to

inaccurate prediction models (Stott et al., 2015). Once a given concentration of pharmaceuticals are present in the plasma, some may be carried up to the brain and metabolized by cytochrome P450 aromatase enzymes (Miksys and Tyndale, 2002).

Cytochrome P450 enzymes (CYP) are mainly found in hepatic cells but have been identified in the brain of teleost with high activity (Miksys and Tyndale, 2002; Menuet et al., 2005). These enzymes are involved in oxidative activation and deactivation of endogenous and exogenous pharmaceuticals, meaning modifications to amino acid chains, as well as metabolism of neurotransmitters and steroids (Miksys and Tyndale, 2002). CYP enzymes are also involved in production of ROS molecules from 'leaky' branches from the normal physiological consumption of NADPH by microsomal CYP enzymes (Zangar et al., 2004). Teleost' have two duplications of cytochrome P450, *cyp19a* (AroA), mainly expressed in the gonads and *cyp19b* (AroB), mostly expressed in the brain (Menuet et al., 2005). AroB has been observed to be almost exclusively expressed in radial glial cells of the telencephalon, diencephalon, and mesencephalon of the teleost brain (Menuet et al., 2015). As CYP enzymes are involved in xenobiotic uptake and ROS molecule production, then AroB in the teleost brain is a potential target for disruption following pharmaceutical exposure and thus, may be an important factor in increased oxidative stress. As pharmaceutical concentrations are not well characterized in the brain tissue of non-target organisms, investigating different environmentally relevant concentrations on the various levels of biological organizations (e.g., cellular level) in subsequent experimentation, may provide a better understanding of the varying effects pharmaceuticals may have on the brain and whether pharmaceuticals may be responsible for behavioural changes.

3.2.0 Primary Cell Culture

An optimal method to investigate physiological mechanisms is the use of *in vitro* assays such as cell cultures, as it provides a key advantage in understanding cell functions and whole organism reactions (Scholpp, 2020). *In vitro* assays are additionally advantageous as the method uses lower tiers in biological levels allowing the assessment of given pharmaceuticals on cell biology and can supplement *in vivo* experiments (Chang et al., 2019). Fish brain cells can be used to investigate environmental endocrine disruptors and toxicology in response to environmental stressors (Zheng et al., 2015). As Chapter two provided evidence of changes in antioxidant activity in darters exposed to contaminated effluent, it is crucial to determine what is the causing these changes, . Although antidepressants were demonstrated to be some of the most common pharmaceuticals to be found in the Grand River in two separate years , it is not possible to suggest that antidepressants are the driving these changes in antioxidant activity in the brains of darters without investigating antidepressant exposure on isolated brain cells. For this reason, a preliminary study on zebrafish and darters isolated brain cells was conducted to investigate the antioxidant response to varying concentrations of venlafaxine (See Appendix B).

The teleost Zebrafish (*Danio rerio*) are an ideal candidate for primary cell culturing of brain cells because they are readily available and quickly grown to adult stages in lab. Since its debut as a model organism for studying cell biology in the 1980's, scientist now aware of its high genomic similarity to humans and other vertebrates, thus making the study of zebrafish applicable to humans (Scholpp, 2020). Zebrafish were initially chosen to develop a working protocol for isolating and culturing brain cells. The developed method was adapted from the primary culture protocol from the study by Sounders II et al., 2021. A detailed step-by-step protocol can be found in Appendix B. Briefly, the primary cell culture protocol involved pooling three to four dissected whole brains to acquire enough cells. Brains were rinsed in Hank's Balanced Salt Solution

containing antibiotic-antimycotic, then trypsinized using 0.25% trypsin for thirty minutes. Cell viability was counted using trypan blue exclusion and a haemocytometer to ensure over 90% viability prior to using cells for the experiment. Cells were equally divided in wells of a 48-well plate and left to incubate in Leibowitz medium containing 15% fetal bovine serum (FBS) at 37°C for 24 hours to allow cell proliferation. A higher percentage of FBS was used as it promotes glial cell proliferation and inhibits neuronal growth (Sounders II et al., 2021). Then, isolated brain cells were exposed to 0 μg/L, 0.01 μg/L, 0.1 μg/L and 1 μg/L of venlafaxine for 24 hours. Following the acute exposure, cells were counted to assess cell viability, then centrifuged into a pellet and homogenized in cold 20 mM HEPES buffer, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose, pH 7.2. Homogenates were then used to measure SOD and CAT enzyme activity, as well as BCA to normalize to protein abundance. Once the primary cell culture protocol was established and deemed successful, the protocol was then applied on greenside and rainbow darters to compare species specific responses. Antioxidant enzyme activity were of primary interest as acute pharmaceutical exposures elicit rapid phenotypic responses in cells. The rapid response of antioxidant enzyme activity is also indicative of potentially increased oxidative stress.

3.2.1 Primary Cell Culture Results & Implications

Descriptive results of cell culture experiments can be found in Appendix B. Following exposure to various concentrations of VEN, zebrafish brain primary cell cultures displayed a rapid 0.65-fold increase in SOD activity at 0.01 μg/L compared to the control, with no differences at higher concentrations (Fig. B.2A). However, CAT activity significantly peaked at the higher concentrations with a 44.6-fold increase at 1 μg/L compared to control (Fig. B.2B). When an imbalance between oxidative stress and antioxidant response occurs, antioxidants activity can be overwhelmed and incapable of neutralizing ROS molecules, which may lead to diminished enzyme

activity (Zhang et al., 2008; Sun et al., 2014; Hoseinifar et al., 2020). Therefore, a possible explanation for the rapid response in SOD with decreased activity may be due to diminished activity from increased oxidative stress. As SOD reduces anions to H_2O_2, CAT activity increases in response to the increased H_2O_2 (Li et al., 2016; Hoseinifar et al., 2020). This process may explain the increased CAT activity observed at the higher concentrations (Halliwell and Whiteman, 2004). This explanation corresponds with the decreased cell viability observed in zebrafish with increasing concentrations (Fig. B.1), most likely due to cells undergoing degradation through apoptosis (Zhang et al., 2008; Li et al., 2020), however, this was not measured.

In contrast to the zebrafish brain primary culture, GSD brain cells demonstrated a trend of gradual increase in both SOD and CAT antioxidant activity with the increase of VEN concentrations (Fig. B.3). Specifically, SOD activity increased by roughly 3-fold at 0.1 and 1 μg/L compared to control (Fig. B.3A). Similarly, CAT activity increased by 3-fold at the highest concentration compared to control (Fig. B.3B). In contrast, RBD demonstrated a more rapid increase at 0.01 ug/L compared to GSD and experienced a gradual decrease with increasing VEN concentrations in both antioxidants (Fig. B.4). RBD displayed a 1.8-fold increase in SOD (Fig. B.4A) and a 3.45-fold increase in CAT activity (Fig. B.4B) at 0.01 ug/L compared to control. The observed response of the darters appears to be different from the zebrafish which is potentially due to the darters being recently wild caught from the Grand River in November 2021, meaning they have been previously and recently exposed to VEN or other contaminants, although the darters used were collected from a relatively clean reference site (West Montrose; WMR) approximately 15km above KIW in Fall 2021 (pharmaceutical concentrations can be found in Appendix B; Fig. B.1). Previous exposures may have led to increased tolerance and therefore, a more stable antioxidant response compared to lab reared zebrafish that have never been pre-exposed. This is

supported by studies that have shown fish pre-exposed to sub-lethal concentrations of contaminants like ammonia and oxazepam, may develop increased tolerances to higher concentrations, therefore adopting an adaptive mechanism (Vossen et al., 2020; Soler et al., 2021). On the other hand, this experiment has a difference between temperate (darters) and tropical (zebrafish) species. Although all cell cultures were incubated at equal temperatures of 37°C for optimization purposes, and antioxidant assays conducted at 25°C, living species are found at different optimal temperatures with zebrafish living in ranges of 27°C and darters living in ranges between 10-20°C. Studies have shown that increased temperatures can have an impact on rate capacity of antioxidant enzymes (Carney Almroth et al., 2015; Banh et al., 2016), which may explain a more robust response zebrafish and a more modest response in darter antioxidant response. Further studies in antioxidant rate capacities at increased temperatures are required to assess the difference between tropical and temperate species.

Unfortunately, as this protocol was used with a primary cell culture, it is unclear as to which types of cells are present and which cells are being affected, however it is evident that VEN does increase ROS on a cellular level in the brain of teleost. Studies have shown that fish brain cells can induce different cell types in different cell-culture media (Zheng et al, 2015). Although, 15% FBS was used to facilitate glial cell growth and inhibit neuronal growth, additional methods are required to determine if glial cells were the affected cells. Therefore, it is important to take this protocol further with immunocytochemistry to determine which cells are responding the VEN exposure and to determine the role of *cyp19b*. Understanding which brain cells are responding to pharmaceutical exposures may provide greater insight into the mechanisms of action for VEN exposure and observed behavioural changes (Painter et al., 2009; Bisesi et al., 2014; da Silva Santos et al., 2018; Thompson and Vijayan, 2020a; Ziegler et al., 2021).

3.3.0 Recommendations & Future Work

Overall, there are several directions that could arise from the findings of this thesis. For instance, chapter two only provides evidence of changes to antioxidant response in darter brains following effluent exposure, as well as potential impacts arising from differences in water quality parameters, such as temperature. Both chapters also provide potential evidence for the increased antioxidant response of darters to be an adaptive response mechanism and may potentially be preventing and/or reducing oxidative damage. Further analysis on oxidative damage could be beneficial in investigating if the occurring antioxidant stress response is due to increased tolerance. It is would also be beneficial to determine r or if there may be future damage if pharmaceutical concentrations continue to increase in effluent, especially following the COVID-19 pandemic where mental health problems anecdotally increased antidepressant usage.

There are several methods of investigating oxidative damage as this type of damage can result in various effects such as lipid peroxidation, protein carbonyl groups and DNA damage (Craig et al., 2007). LPO attack the double bond of a methylene group in polyunsaturated fatty acids (Bandyopadhyay et al., 1999) and can result in several products formed such as malondialdehyde (MDA), which is the most common method to quantify oxidative damage (Halliwell and Whiteman, 2004). Protein carbonyls are created when aldehydes bind to proteins which generate products such as glutamate (Halliwell and Whiteman, 2004). Measuring protein carbonyls through spectrophotometry and ELISA techniques are additionally a common method of quantifying oxidative damage but is a little less specific as these methods also pick up aldehydes and glycated proteins (Halliwell and Whiteman, 2004; Craig et al., 2007). Therefore, using both above-mentioned techniques combined as a biomarker of oxidative damage would be more accurate then only using a single assay (Halliwell and Whiteman, 2004). As there is little evidence

in fish regarding ROS and brain neurodegeneration, it is essential to further study oxidative damage because the result of lipid peroxidation and protein carbonyls have been observed to have a role with behavioural alterations in fish (Halliwell and Whiteman, 2004).

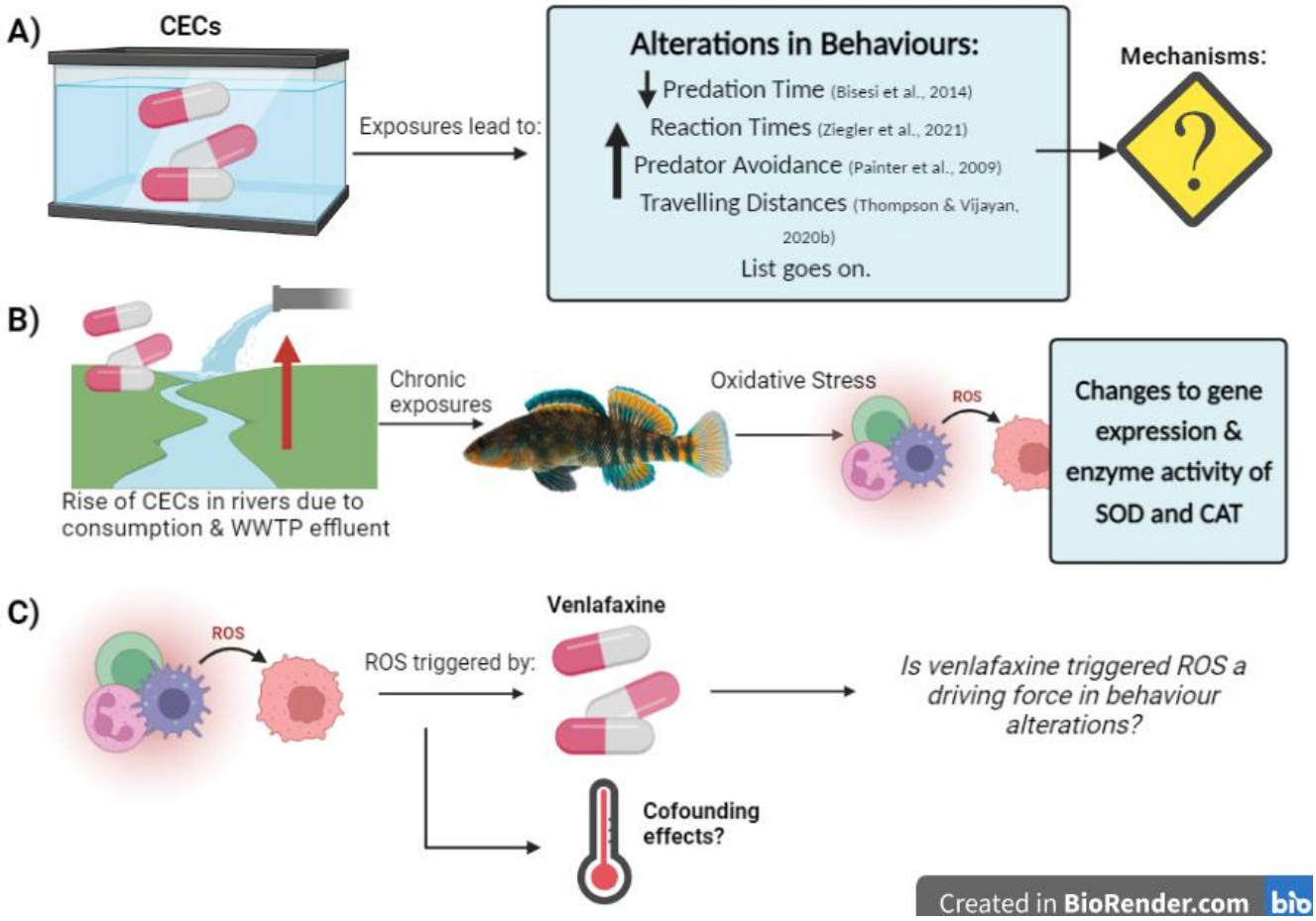

Figure 3.2. Summary diagram demonstrating A) what was known prior to this work, B) what this thesis investigated and C) conclusion of the work contributed and suggested future direction.

In summary, Fig. 3.2 displays what was known prior to this thesis and how this thesis contributed knowledge to previous literature on antioxidant response following effluent exposure. It is likely that the observed behavioural changes following pharmaceutical exposures demonstrated in brown trout, fathead minnows and zebrafish (Thompson et al., 2017; da Silva

Santos et al., 2018; Thompson and Vijayan, 2020a; Ziegler et al., 2021) may have a link to ROS generation and potential oxidative damage. For this reason, using *in vitro* assays in supplement to *in vivo* experiments can provide insights to links between oxidative stress, altered behaviours and ROS. Thus, repeating the above cell culture protocol but measuring apoptosis via lactate dehydrogenase (LDH) enzyme leakage (Cadonic, 2019), would provide insight into the observed decreased cell viability and would strengthen the argument of cell death via apoptosis due to increased oxidative stress. Finally, in addition to VEN, chapter two also demonstrated temperature may have played a significant role in upstream species as they generally experience significantly lower concentrations of pharmaceuticals but still experienced increased antioxidant gene expression. Therefore, not only is investigating antidepressants on isolated cells crucial, its just as important to study cofounding effects such as temperature and antidepressants. This would provide a more realistic approach to understand the true effects of wastewater effluent and potential future effects of climate change on brains of non-target species.

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Appendix A

Table A.1. Primer information. There are four target genes and two housekeeping genes. Information, species target, amplicon size, and sequences are given.

Target	Species target	Information	Amplicon size (b.p.)	Forward (5'-3') Reverse (3'-5')
actb	RBD, GSD, FTD, JD	β-actin Housekeeping gene	114	F: GTACCCCGGCATCGCA R: CCTCCGATCCAGACAGAGTATT
gapdh	RBD, GSD, FTD, JD	Glyceraldehyde 3-phosphate dehydrogenase Housekeeping gene	118	F: GAAGGGTGGTGCCAAGAGA R: GGAAGCGTTGCTTACAACC
gpx4	RBD, GSD, FTD, JD	Glutathione peroxidase Variant 4	104	F: CATGGGAAACAGTATCAAGTGGA R: TCTCCACCACACTGGGATCA
sod1	GSD, FTD, JD	Superoxide dismutase (1) Variant 1	150	F: TGCAGGCCCTCACTTCAATC R: GGGCCAGTGAGGGTGATTAT
sod1	RBD	Superoxide dismutase (2) Variant 1	115	F: TTTTGAGCAGGAGGACGGTT R: GCACCCGTTTGTATTGTCCC
cat	GSD	Catalase (1)	79	F: GAAGGCTGTCCATCCAGACT R: TGTGTTCTTCTGGGCCTTCG
cat	RBD, FTD	Catalase (2)	125	F: GCCATCGGAGACCTGTTCAA R: ACCTTGGTAACGTCGAAGGG
cat	JD	Catalase (3)	119	F: CCACATGAAAGACCCCGACA R: CGTTCATGTGACGGTAGCCA
tph1	GSD	Tryptophan hydroxylase (1) Isoform 1	149	F: GCCTGCCGGGAATACTTGAA R: GGGGACAGATAACCAGCCAC
tph1	RBD, FTD, JD	Tryptophan hydroxylase (2) Isoform 1	107	F: ACGGTGGAGTTTGGCCTATG R: CTTGCATTGCCAGAGAGTGC

Table A.2. Suggested sample size required for future studies for each darter species conducted by power analysis (GPower 3.9.1.4), using transcript markers and enzyme activity data from Fall 2020, with an alpha = 0.05, power = 0.8. df = 1, number of groups = 4 and calculated effect sizes from variances.

		RBD	GSD	FTD	JD
Transcript markers					
Error of variance (σ)		3.48	0.09	0.74	0.29
Variance explained by effect (σ^2)	Interaction	2.28	0.10	0.31	0.24
	Site	4.57	0.21	0.61	0.49
	Sex	4.57	0.21	0.61	0.49
Output	Total sample size	15	11	22	13
	Actual power	0.81	0.84	0.81	0.84
	Effect size	0.81	1.04	0.64	0.92
Enzyme activity					
Error of variance (σ)		4.67	0.21	77.9	10.07
Variance explained by effect (σ^2)	Interaction	3.92	0.18	104.12	6.78
	Site	7.84	0.35	208.23	13.57
	Sex	7.84	0.35	208.23	13.57
Output	Total sample size	13	13	10	15
	Actual power	0.84	0.84	0.86	0.82
	Effect size	0.92	0.92	1.16	0.82

Appendix B – Cell Culture Protocol & Results

Primary cell culture protocol

Dissection:

1. Prepare necessary tools for dissection (dissection plate, two forceps, scissors) by washing with 70% ethanol and prep a small beaker filled with 70% ethanol.
2. Prepare MS222 for anesthesia of fish by mixing roughly 180 mg/L in milliQ water and mix with a stirrer for 3-5 minutes.
3. Pipette 1 mL of HBSS (with Antibiotic-antimycotic) in each 2 mL tube placed on ice to later add the brains in.
4. Sanitize counter in the Water Aquatic Facility room to prepare for dissection.
5. Collect 2 fish at a time and place them in the MS222 for anesthesia.
6. Once fish are belly up and undisturbed, dry the fish with Kim Wipes and then quickly snip their spinal cord and then wash their bodies in 70% ethanol beaker.
7. Dissect fish by making two straight cuts from neck toward the eyes and lift the skull to expose the brain.
8. Place dissected brains in tubes on ice.

Cell culture:

1. Wash brains by rinsing with HBSS (with Antibiotic-antimycotic) solution in each 2 mL tube twice to discard of any impurities or cartilage, etc.
2. Remove about 0.8 mL of HBSS from each tube and cut up the brain tissue in the tubes to small explants.
3. Then add 1.5 mL of 0.25% Trypsin into each tube and let the brains sit in solution for 30 mins at room temperature.
4. After 30 minutes of trypsinization remove the brain explants from each tube and place them in a 50 mL falcon tube.
5. Transfer 10 mL of 15% Leibowitz serum (containing 15% Fetal Bovine Serum; Gibco) media into the falcon tube over the brain cells.
6. Pipette up and down using 1 mL pipette tip to dissociate the cells and break apart clumps to maintain similar concentrations in each transfer.
7. Remove 10 micro litres of media from the falcon tube for Trypan Blue Exclusion (Invitrogen™) cell counting and centrifuge it down.
8. After centrifugation, remove the serum media and replace it with serum free media because the serum proteins dye blue with trypan blue and will affect the results of counting
9. After mixing the pellet with serum free media, add a 1:1 ratio of trypan blue by adding 10 microlitres of 0.4% trypan blue and mix well by pipetting.
10. Prepare the hemocytometer by cleaning it and the cover slip with 70% ethanol and then pipetting 2 drops of water on each side of the glass to keep the glass in place
    a. The trypan blue exclusion method of counting followed can be found here: Strober, W. (1997). Trypan blue exclusion test of cell viability. *Current protocols in immunology*, *21*(1), A-3B.

11. After counting the cells in suspension, based on the number of cells preferred per well, dilute the original media, and calculate the number of cells.
12. After diluting the media (by adding more media) to the preferred amount, plate cells in media on a 48-well plate by adding 0.4 mL of media suspension in each well.
13. After plating, cover the plate and incubate the cells at 37 degrees Celsius in the lab incubator for approximately 24 hours.

Exposure:

1. Prepared the venlafaxine (VEN) concentrations of 0.01, 0.1 and 1 μ/L by diluting an initial stock solution of VEN of 20 μ/L.
2. After making 0.01, 0.1 and 1 μ/L VEN solutions, place each into a 15 mL falcon tube to use later for exposures.
3. After the 24-hour period of incubation, remove the plate from the incubator and place it into the cell culture room hood.
4. Then, carefully remove all the media and cells in each well and placed them into their corresponding already labelled tubes.
5. Centrifuged the tubes for 12 minutes at 1300 rpm
6. Then, collect the pellets, and discard the media by pipetting it out into a waste beaker
7. The samples were exposed to VEN by inserting 0.4 mL of the corresponding VEN solution into each tube and pipetting to ensure the pellet was properly mixed into suspension.
8. Following this, the samples were plated back into their positions on the 48-well plate.
9. The plate was then incubated at 37°C for another 24 hours.

Sample collection following 24 hr exposure:

1. Transfer samples from each well including the control wells into their corresponding tubes.
2. Centrifuged down for 12 minutes at 1300 rpm
3. Remove supernatant from each tube.
4. Homogenize pellets in cold 20 mM HEPES buffer, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose, pH 7.2 and freeze in -80°C for future use.

Descriptive results of cell culture experiments

Zebrafish cell viability (Fig B.1 in appendix B), demonstrated an expected decrease with increasing concentration of VEN. The controls had an average of 93.5% viability, 0.01 ug/L had viability of 83%, 0.1 ug/L had a viability of 79.1% and 1 ug/L had a viability of 62%. A significant difference was displayed by a one-way ANOVA ($F_{(3,23)} = 8.3$, $p < 0.001$) with Tukey test showing a difference between control and 1 ug/L by 0.66-fold ($p < 0.001$) and between 0.01 ug/L and 1 ug/L by 0.75-fold ($p = 0.023$). A significant difference was observed for SOD activity measured in zebrafish ($F_{(3, 20)} = 9.2$, $p < 0.001$; Fig B.2A in appendix B). Tukey test displayed a significant difference between control and 0.01 ug/L by 4-fold ($p < 0.001$) and between 0.01 ug/L and 1 ug/L by 0.5-fold ($p = 0.042$). Lastly, a significant difference was observed for CAT activity measured in zebrafish ($F_{(3, 20)} = 53.2$, $p < 0.0001$; Fig B.2B in appendix B). Tukey test displayed a significant difference between control and 0.1 ug/L by 12.6-fold ($p = 0.035$), between 0.01 ug/L and 1 ug/L by 8.5-fold ($p < 0.0001$) and between 0.1 ug/L and 1 ug/L by 3.6-fold ($p < 0.001$). GSD demonstrated a significant effect for SOD activity ($F_{(3,28)} = 36.4$, $p < 0.0001$; Fig. B.3A in appendix B). Tukey test displayed a significant difference between control and 0.01 ug/L by 2.6-fold ($p < 0.0001$), between control and 0.1 ug/L by 3.83-fold ($p < 0.0001$), between control and 1 ug/L by 3.83-fold ($p < 0.0001$) and between 0.01 ug/L and 0.1 ug/L by 1.5-fold ($p = 0.049$). A significant difference for CAT activity was also observed in GSD ($F_{(3,28)} = 11.4$, $p < 0.0001$; Fig B.3B in appendix B). Tukey test displayed a significant difference between control and 0.01 ug/L by 2.82-fold ($p = 0.0035$), between control and 0.1 ug/L by 3.2-fold ($p < 0.001$) and between control and 1 ug/L by 4-fold ($p < 0.0001$). RBD demonstrated a significant effect for SOD activity following a Kruskal-Wallis test ($H = 18.7$, $df = 24$, $p < 0.001$; Fig. B.4A in appendix B). A Post-hoc test displayed a significant difference between control and 0.01 ug/L by 2.82-fold ($p = 0.003$)

and between control and 0.1 ug/L by 2.76-fold ($p < 0.001$). In addition, a significant effect for CAT activity in RBD was also displayed following a Kruskal-Wallis test ($H = 16$, $df = 24$, $p = 0.0012$; Fig. B.4B in appendix B). A Post-hoc test displayed a significant difference between control and 0.01 ug/L by 4.45-fold ($p = 0.0151$), between control and 0.1 ug/L by 4.47-fold (p - 0.0014) and between control and 1 ug/L by 3.37-fold ($p = 0.0255$).

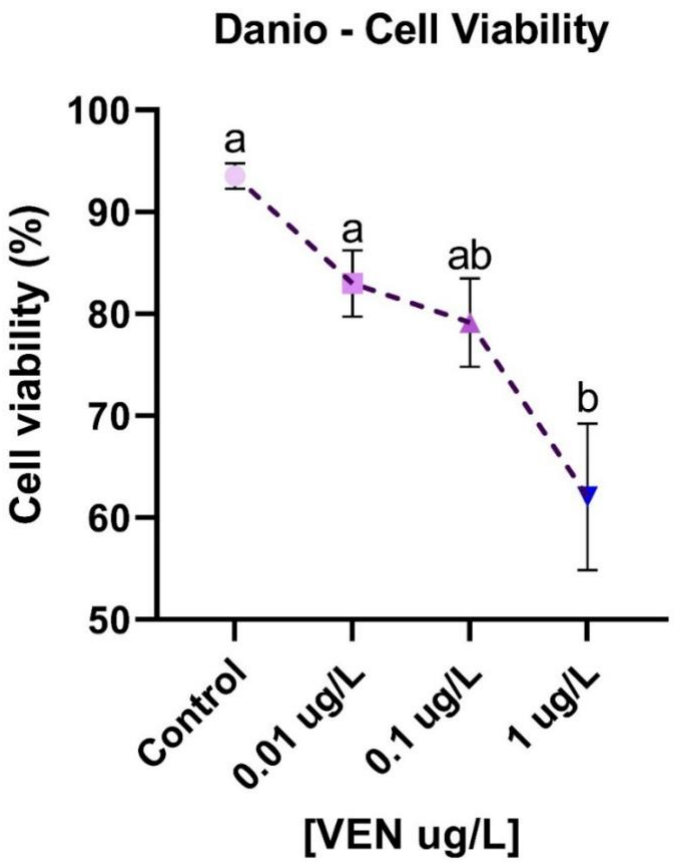

Figure B.1. Cell viability (%) of isolated brain cells of zebrafish (*Danio rerio*) following 24-hour exposure to different concentrations (0 µg/L, 0.01 µg/L, 0.1 µg/L and 1 µg/L) of venlafaxine. Data presented as means + SEM, compared using One-way ANOVA. Letters represent significant differences displayed by Tukey test (n = 6-7).

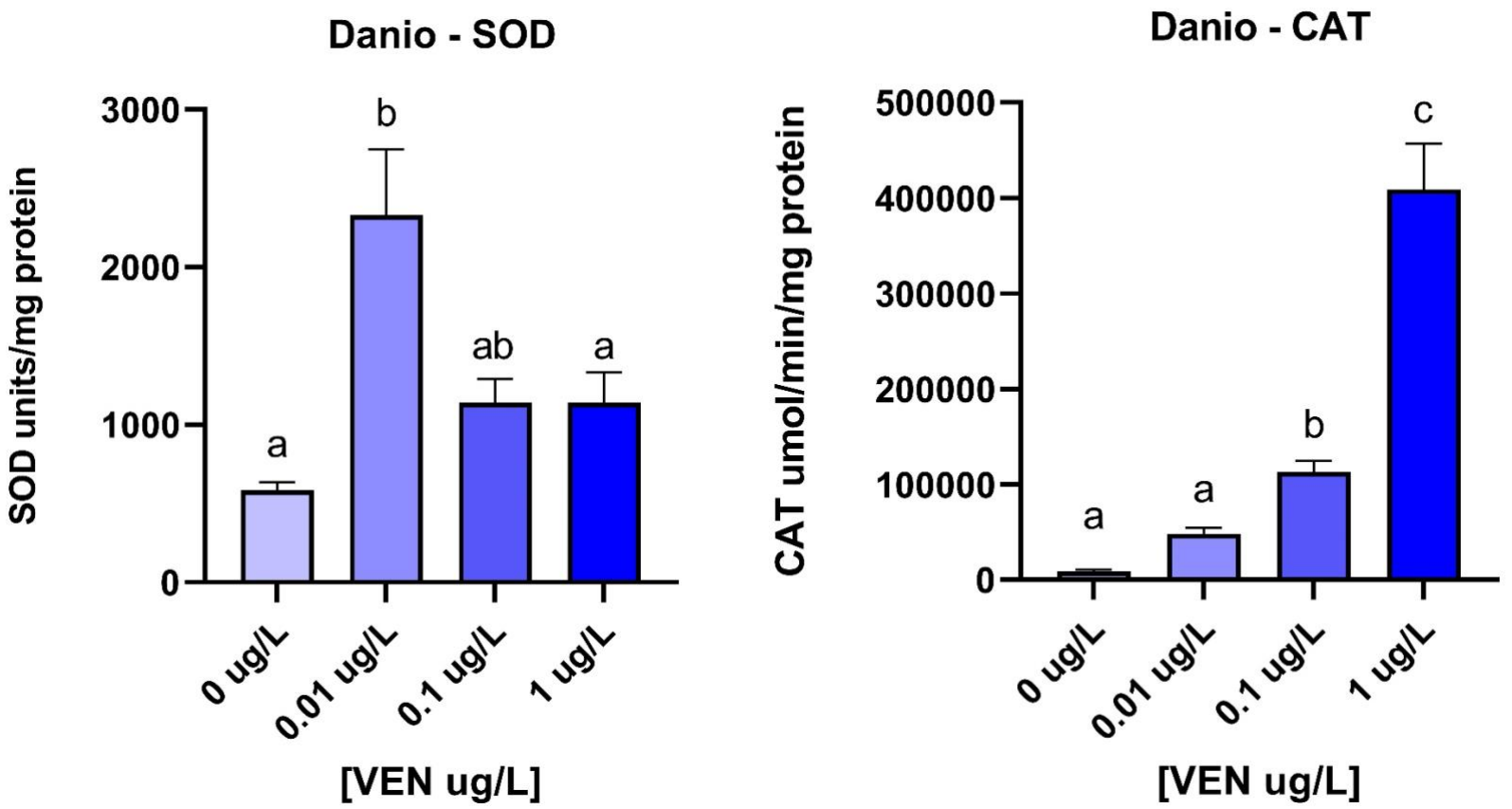

Figure B.2. A) Superoxide dismutase (SOD) enzyme activity and B) catalase (CAT) enzyme activity measure in isolated brain cells of zebrafish (*Danio rerio*) following 24-hour exposure to different concentrations (0 µg/L, 0.01 µg/L, 0.1 µg/L and 1 µg/L) of venlafaxine. Data presented as means + SEM, compared using One-way ANOVA. Letters represent significant differences displayed by Tukey test (n = 6).

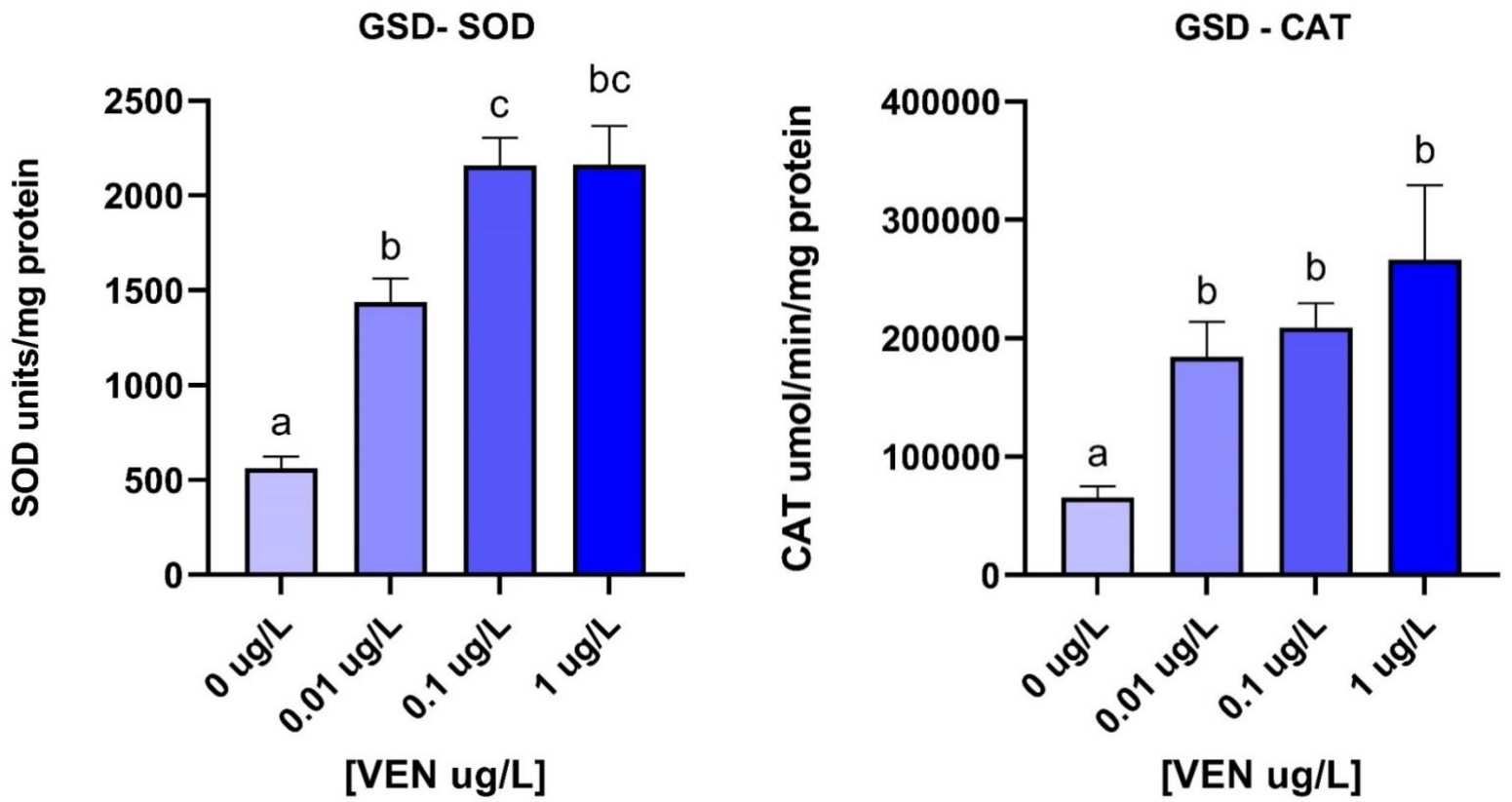

Figure B.3. A) Superoxide dismutase (SOD) enzyme activity and B) catalase (CAT) enzyme activity measured in isolated brain cells of greenside darters (*Etheostoma blennioides*) following 24-hour exposure to different concentrations (0 µg/L, 0.01 µg/L, 0.1 µg/L and 1 µg/L) of venlafaxine. Data presented as means + SEM, compared using One-way ANOVA. Letters represent significant differences displayed by Tukey test (n = 8).

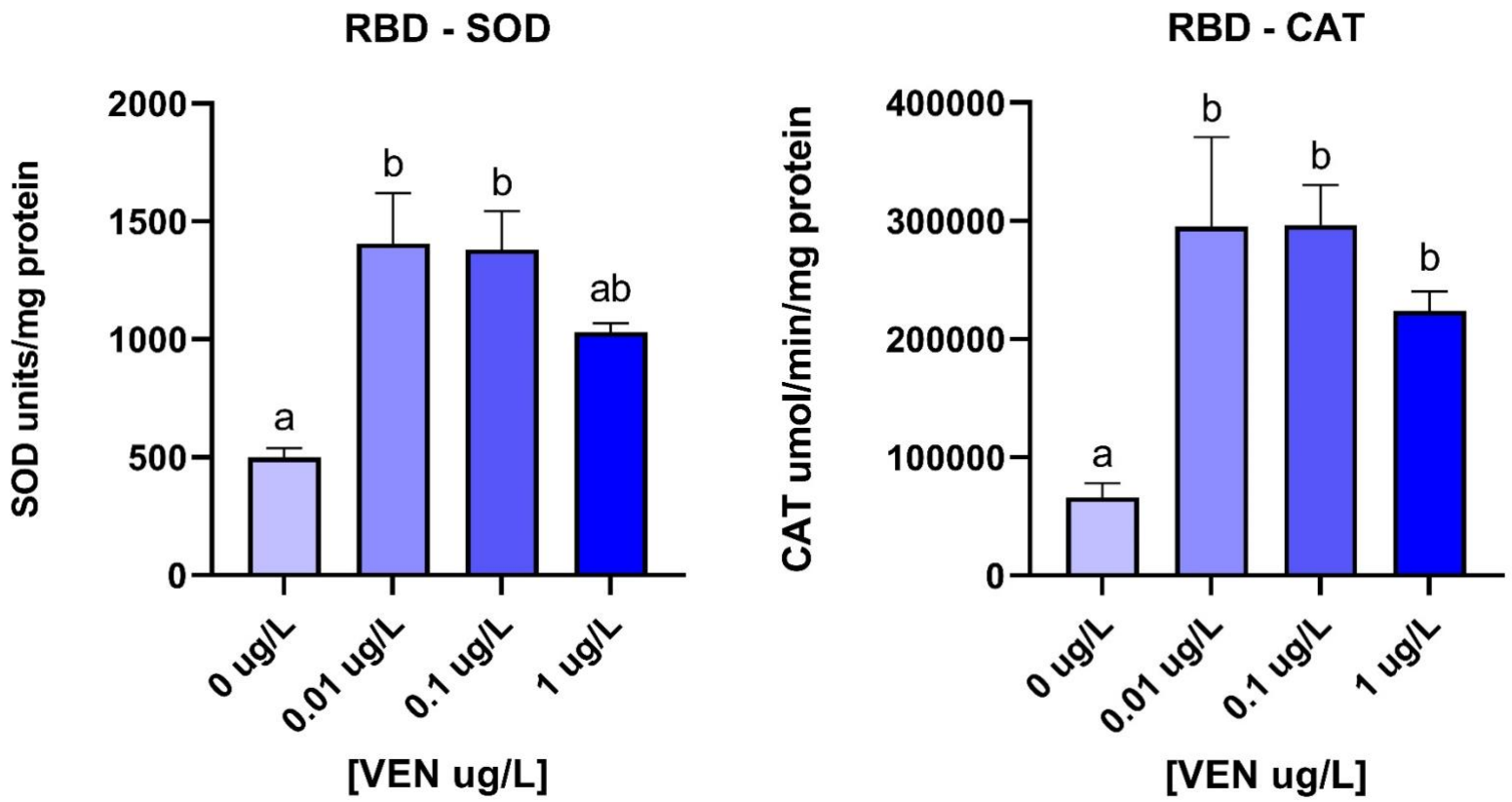

Figure B.4. A) Superoxide dismutase (SOD) enzyme activity and B) catalase (CAT) enzyme activity measured in isolated brain cells of rainbow darters (*Etheostoma caeruleum*) following 24-hour exposure to different concentrations (0 µg/L, 0.01 µg/L, 0.1 µg/L and 1 µg/L) of venlafaxine. Data presented as means + SEM, compared using Kruskal-Wallis Test. Letters represent significant differences displayed by Tukey test (n = 7).

Table B.1. Concentration trends of the pharmaceuticals screened on November 8th, 2021. Three samples were taken (near the bank, in the middle of the river and far away from the bank). Data is presented as means ± SEM (n=3/site).

Classification	Pharmaceutical	WMR (ng/L)
NSAIDs	naproxen	0
	diclofenac	10.75
	ibuprofen	0
Fibrates	gemfibrozil	10.75
Antiepileptics	T-type calcium channel blocker	0
	11,12-epoxide carbamazepine	0
	carbamazepine	4.09
Antibacterials	triclosan	1.62
	sulfamethoxazole	12.13
	sulfamethazine	20.41
	trimethoprim	0
	norflaxacin	0
Pain relievers	acetaminophen	0
Stimulant	caffeine	30.27
SNRI	desmethyl-venlafaxine	3.457
	venlafaxine	9.59
	fluoxetine	0.22
DPP inhibitor	linagliptin	0
Statins	p-hydroxy atorvastatin	1.06
	o-hydroxy atorvastatin	1.09
	atorvastatin	0.65
Herbicide	atrazine	14.87