Impacts of wastewater treatment plant effluent and pharmaceutical exposure on innate cytokine expression of darters (*Etheostoma* spp.) in the Grand River

by

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Author's Declaration

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

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Abstract

Aquatic organisms, such as fish, are in constant contact with various stressors and antigenic material present within their environments. The impact of stressors associated with wastewater-exposed environments on native fish species has become of particular interest in toxicology studies. The objectives of this thesis were to examine the effects of stressors associated with wastewater treatment plant (WWTP) effluents on innate cytokine expression within the gills of darter species (*Etheostoma* spp.), using both field and laboratory approaches. Male and female darters (rainbow, greenside, fantail, and johnny darters) were collected upstream and downstream of the Waterloo WWTP in the Grand River, ON. Gill samples were collected from fish in the field and from a second subset of fish brought back to the laboratory. Laboratory fish were acutely exposed (96-hours) to an environmentally relevant concentration of a commonly prescribed antidepressant, venlafaxine (1.0 µg/L). To assess the impacts of effluent and venlafaxine exposure on the innate immune response of darters, the expression of key innate cytokines was examined. No significant effects on innate cytokine expression were observed within the gills between upstream and downstream sampled fish. Moderate effects on cytokine expression were observed in fish exposed to 1.0 µg/L of venlafaxine compared to their control counterparts however, changes were not indicative of a biologically significant immune response occurring because of the exposure. Although the results of this thesis did not display major impacts of effluent and pharmaceutical exposure on the expression of innate cytokines within the gills, they provide a novel avenue of study, illustrating the importance of examining the potential impact that wastewater-associated stressors can have on fundamental immune responses of native fish species.

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List of Abbreviations

BOD – biological oxygen demand
CBZ – carbamazepine
CECs – contaminants of emerging concern
DAMPs – danger-associated molecular patterns
DCF – diclofenac
DO – dissolved oxygen
EIT – Economic Insurance Trailway, downstream of the Waterloo WWTP
FTD – fantail darter
GSD – greenside darter
IL – interleukin
JD – Johnny darter
KIW – Kiwanis, upstream of the Waterloo WWTP
LC-MS/MS – liquid chromatography and tandem mass spectrometry
NSAIDs – non-steroidal anti-inflammatory drugs
O-VEN – O-desmethyl venlafaxine
PAMPs – pathogen-associated molecular patterns
PPCPs – pharmaceutical and personal care product(s)
PRRs – pattern recognition receptors
RBD – rainbow darter
SNRI – serotonin norepinephrine reuptake inhibitor
TSS – total suspended solids
VEN – venlafaxine
WMR – West Montrose, further upstream of the Waterloo WWTP
WWTPs – wastewater treatment plant(s)

Chapter 1

General Introduction

1.1 The Grand River watershed

The Grand River watershed is the largest watershed in Southern Ontario, covering 6,965 km^2 (Grand River Conservation Authority). This encompasses all land drained by the Grand River and four major tributaries: the Nith, Conestogo, Speed, and Eramosa River, which support over 1 million people living across 39 municipalities and 2 First Nations territories. Due to a large and increasing population, the Grand River watershed is highly influenced by agricultural activity, urban development, and wastewater treatment plant (WWTP) effluents (Cooke, 2006; Loomer & Cooke, 2011). The watershed receives discharged effluent from 30 municipal WWTPs, all with varying levels of wastewater treatment, that can ultimately affect the overall quality of the watershed (Cooke, 2006; Srikanthan, 2019). The upper region of the Grand River watershed often has water of good quality due to the low intensity of agricultural activity and minimal urban influences (Cooke, 2006; Loomer & Cooke, 2011). Conversely, water quality begins to progressively deteriorate as it descends into the central and lower regions of the watershed due to the large influence of high intensity farming and urban development that accumulate downstream (Cooke, 2006; Loomer & Cooke, 2011).

1.1.1 Municipal wastewater treatment plants

Municipal WWTPs are a crucial part of waste management strategies within our community. Their purpose is to treat incoming wastewater, removing and reducing total suspended solids (TSS), phosphorus, ammonia, and chemical contaminants, before the resulting effluent is discharged into the Grand River (Cooke, 2006; Samer, 2015; Srikanthan, 2019). In Canada, the treatment regiment used in wastewater facilities is site-specific and the level of treatment is therefore, highly variable with some plants producing lower quality effluents that have the potential to threaten aquatic receiving environments (Srikanthan, 2019). Most WWTPs use

conventional wastewater treatments that can be generally classified as primary and secondary treatments (Samer, 2015). Primary treatment involves physical and chemical processes that remove suspended solids and other organic matter from the incoming wastewater (Samer, 2015). Wastewater then undergoes secondary treatment, where various biological processes are utilized to decrease nitrogen levels along with the biological oxygen demand (BOD) (Samer, 2015). Ultimately, these conventional treatments remove most suspended solids, nitrogen, and phosphorus from released effluents however, they are less effective at reducing the amount of ammonia and contaminants released (Samer, 2015). As a result, some WWTPs including those in Kitchener and Waterloo, have undergone upgrades to their treatment processes adding an advanced chemical or tertiary treatment which utilizes various filtration processes to further improve the quality of effluent and remove chemical contaminants (Cooke, 2006; Samer, 2015; Srikanthan, 2019). These upgrades have reduced harmful chemicals such as ammonia and further aid in the reduction of BOD and TSS (Keegan A. Hicks et al., 2017; Srikanthan, 2019).

1.2 Study species

1.2.1 Darters in the Grand River watershed

Darters (*Etheostoma* spp.) are small and often colourful perch species that inhabit benthic regions of aquatic environments such as small shallow creeks and wide rivers (Carlson & Wainwright, 2010). Within these environments, darters can be highly sympatric as multiple species interact with one another, although each species occupies its own unique microhabitat ranging from sandy or vegetation-filled pools to fast-flowing rocky or gravely riffles (Carlson & Wainwright, 2010). Their diet consists mainly of small, aquatic insect larvae such as midges, mayflies, and caddisflies while they themselves are major food sources for larger fish species and act as parasitic hosts (Carlson & Wainwright, 2010; Crane et al., 2011; Tetreault et al., 2011).

While there are over 200 darter species found across freshwater ecosystems in North America (Carlson & Wainwright, 2010), four species, native to the Grand River watershed, are of particular interest. Rainbow darters (*Etheostoma caeruleum*; RBD) are the most abundant, demonstrating high site fidelity and moderate mobility (Hicks & Servos, 2017). Consequently, RBD tend to remain in the same area throughout their lifetime and as a result, are continuously exposed to effluents if found downstream of WWTPs (Brown et al., 2011). Although continuously exposed, RBD have displayed competitive advantages at these sites, utilizing the greater amounts of nutrients, and are likely to be more tolerant to contaminated effluents (Brown et al., 2011). Unlike RBD, greenside darters (*Etheostoma blennioides*; GSD) are highly mobile and are able to remain and move through fast flowing water (Bunt et al., 1998; COSEWIC, 2006). This mobility allows them to move more freely from contaminated sites into cleaner sites (Brown et al., 2011). Similarly to RBD, fantail darters (*Etheostoma flabellare*; FTD) tend to remain within one area but will move however, in response to habitat changes (Roberts & Angermeier, 2007; Hodgson et al., 2020). Lastly, Johnny darters (*Etheostoma nigrum*; JD) much like RBD and FTD, remain in one habitat during their lifetime, preferably in shallow, sandy areas (Krause et al., 2010). Based on the observed sampling history of these fish at sites within the Grand River, JD are the least abundant of the four species.

Overall, darters are an emerging model organism due to their high abundance within the Grand River watershed and their high tendency to remain in the same habitat. Although RBD are the most abundant and frequently used to investigate the impacts of WWTP effluents, examining one species does not provide a comprehensive understanding of their impacts. Within these effluent-receiving environments, there are several other species all of which have varying degrees of tolerance to the same anthropogenic stressors. Darter species in the Grand River watershed have

already been observed to be sensitive to stressors associated with contaminated WWTP effluents, displaying impacts of metabolism, intersex, and gill morphology (Tetreault et al., 2011; Mehdi et al., 2018; Hodgson et al., 2020) however, it is unknown how these stressors may impact other physiological aspects such as their immunity.

1.3 Immunity in fishes

The teleost immune system, like that of other vertebrates, can be divided into two major responses: the innate and the adaptive (Smith et al., 2019). Together, these responses recognize and defend against a variety of pathogens and foreign contaminants that a fish may encounter. Although most vertebrates have similar immune mechanisms, teleost fish live predominantly in water, an environment in which they are in continuously contact and exposed to antigenic material (Smith et al., 2019). This aquatic environment poses additional challenges to the immune system of fish and as a result, requires the implementation of an array of unique immunological defences.

1.3.1 Innate immunity

Physical and chemical barriers, the first innate defence mechanisms utilised by fish, separate foreign entities in the outside environment from an organism's internal environment (Ángeles Esteban, 2012; Smith et al., 2019). Maintaining the integrity of these barriers is vital since without interaction to the internal environment, damage and subsequent infections become particularly difficult. Although their most prominent roles include respiration and osmoregulation, the gills are an important barrier of the immune system as they are the first to encounter toxicants and antigenic material (Smith et al., 2019). The gills not only physically block the entry of antigenic material, but they also secrete a protective mucus layer as well (Ángeles Esteban, 2012; Smith et al., 2019). The mucus layer acts as a natural, chemical barrier that is able to trap and immobilize pathogens or contaminants through the use of various antimicrobial agents including

lectins, lysozymes, complement proteins, and antimicrobial peptides (Uribe et al., 2011; Ángeles Esteban, 2012).

If antigenic material does penetrate the external defences, immune cells, namely macrophages and neutrophils, within the epithelium initiate the innate immune response to recognize and eliminate the threat (Smith et al., 2019). To identify foreign pathogens or contaminants, immune cells possess germline-encoded pattern-recognition receptors (PRRs) in their cell membranes that are responsible for binding specific pathogen associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) released from infected or damaged cells (Medzhitov, 2007; Rebl et al., 2010; N. C. Smith et al., 2019). Once bound, these phagocytic immune cells become activated and initiate phagocytosis. This is accomplished through the production of reactive oxygen species in a respiratory burst or using lysozymes and other hydrolytic enzymes present in their lysosomes (Grayfer et al., 2018; Mokhtar & Abdelhafez, 2021). Furthermore, activated immune cells produce small, secreted proteins known as cytokines to promote and regulate additional responses such as inflammation, cell movement, and further activation of immune cells (Commins et al., 2010; Zou & Secombes, 2016). Key cytokines of the innate immune system include interleukin 1 beta (*il-1β*), interleukin 6 (*il-6*), interleukin 8 (*il-8*), and interleukin 10 (*il-10*) (Fig. 1). *il-1β* and *il-6* are proinflammatory cytokines produced by activated macrophages and damaged cells to promote inflammation (Zou & Secombes, 2016; Semple & Dixon, 2020). These cytokines induce fever in endotherms and cause the damage site to swell, increasing the surrounding vascular permeability to allow additional immune cells from the blood to move into the damaged tissue and produce more proinflammatory cytokines (Semple & Dixon, 2020). In addition, the damaged tissue also releases chemokines, such as *il-8*, that are responsible for recruiting neutrophils to the site of injury through chemotaxis (Alejo & Tafalla,

2011; Semple & Dixon, 2020). Once present, these cells will continue to promote inflammation and recruit additional cells to eliminate the damage (Semple & Dixon, 2020). Although inflammatory responses are extremely important, they can also be very damaging to the surrounding tissues (Semple & Dixon, 2020). To control these responses, anti-inflammatory cytokines, such as *il-10* and also *il-6*, are produced by macrophages and other immune cells to limit inflammation and redirect the reaction towards tissue repair once the threat is removed (Zou & Secombes, 2016; Semple & Dixon, 2020). Cytokine producing immune cells are not only important in regulating various innate immune responses but are also important in adaptive immune responses as well as they provide an essential link between both immunities to ensure the appropriate immune responses are carried out (Semple & Dixon, 2020).

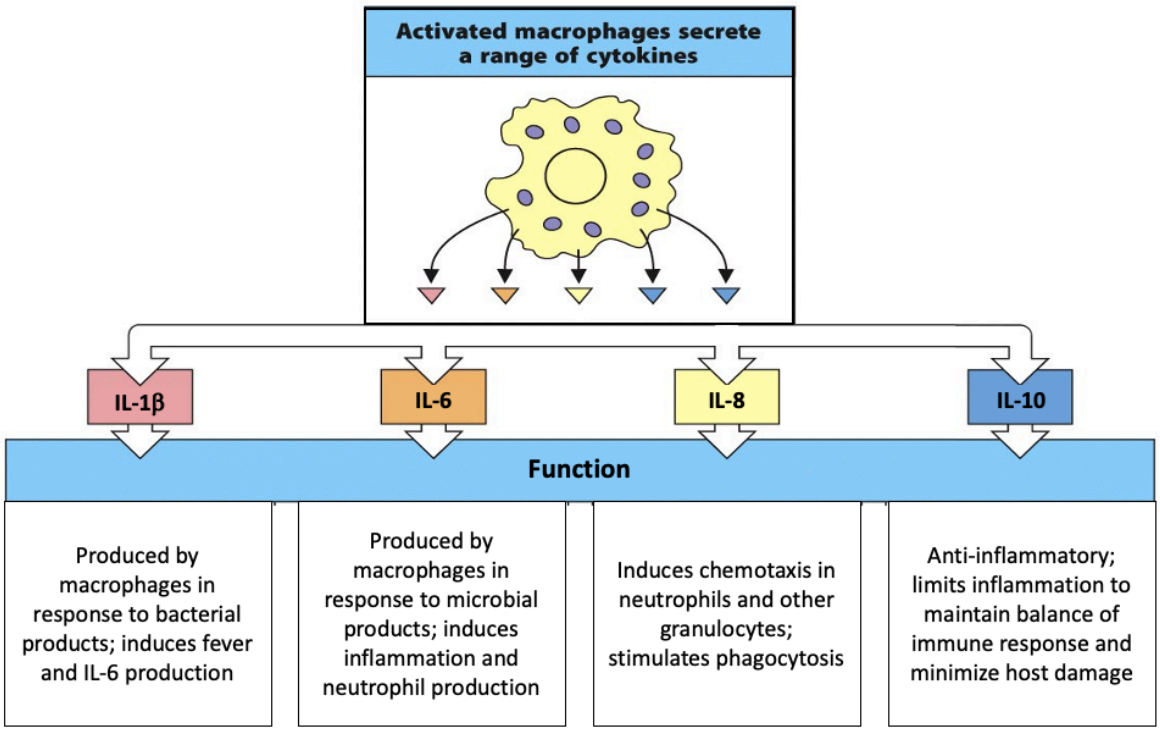

Figure 1: Important cytokines and chemokines of the innate immunity adapted from (Murphy et al., 2012).

1.3.2 Adaptive immunity

The adaptive immune system becomes activated if a pathogen persists despite the defence mechanisms of the innate immunity (Smith et al., 2019). Compared to the innate immune system, the adaptive immune system uses more specialized defence mechanisms, employing specialized cells, proteins, and signals to respond to specific antigens and antibodies (Uribe et al., 2011; Smith et al., 2019). Although the responses of the adaptive immune system are more advanced than the innate, they take considerably more time to develop and mount a response against a pathogen (Ellis, 2001; Magnadóttir, 2006). For this reason, adaptive immunity was not a focus in this thesis as the objectives focused on a short time frame (96 hours) where innate immunity is more relevant and essential towards pathogen defence in teleost fishes.

1.4 Objectives and hypotheses

In summary, the Grand River watershed has become highly influenced by anthropogenic sources, mainly those associated with discharged WWTP effluents (Cooke, 2006). There is an increasingly wide array of studies investigating the impacts of these effluents on various physiological aspects of local fish species such as darters (Tetreault et al., 2011; Mehdi et al., 2018; Hodgson et al., 2020) however, there is less research on the impact of other physiological processes namely those associated with immunity. As predominately aquatic animals, fish are in constant intimate contact with an environment that may contain potentially harmful toxicants or other antigenic material (Smith et al., 2019). If toxicants damage vital, primary immunological barriers like the gills, fishes are more likely to have impaired immune responses leading to greater susceptibility of infections and possibly death. For these reasons, this thesis will aim to further our understanding of about the effects of WWTP effluent exposure on local fish species by

investigating their impact on fish immunity since little is currently known. Thus, this thesis aims to:

1. Determine the impact of effluent-associated stressors on the innate immune response in four darter species living in downstream effluent waters of the Grand River by measuring changes in innate cytokine transcript expressions. It is hypothesized that effluent-exposed darters living downstream of the Waterloo WWTP outfall will have impaired transcript expressions of key innate cytokines in the gills as they are the first innate immune barrier exposed to environmental stressors.

2. Investigate the impacts of a specific effluent-associated stressor, pharmaceutical exposure, on the innate immune response in four darter species by measuring changes in innate cytokine transcript expressions following an acute exposure to an environmentally relevant concentration of venlafaxine. It is hypothesized that darters acutely exposed to venlafaxine will have impaired transcript expressions of key innate cytokines in the gills as they are the first innate immune barrier exposed to pharmaceutical toxicants.

Chapter 2

Impacts on innate cytokine expression in wastewater effluent exposed darters (*Etheostoma* spp.) in the Grand River

2.1 Introduction

2.1.1 Stressors associated with wastewater treatment plant effluents

WWTPs aim to treat wastewater received from the surrounding communities and remove contaminants to improve the water quality before its release into subsequent rivers. Unfortunately, discharged effluents from WWTPs are not the same quality as the receiving bodies of water, prompting various adverse modifications (Carey & Migliaccio, 2009). The high nutrient content of WWTP effluents is the main factor attributed to the rise of eutrophication in numerous aquatic environments (Carey & Migliaccio, 2009; Preisner et al., 2020). Continuous loading of nitrogen and phosphorus into the environment promotes problems associated with eutrophication including increased agal growth, decreased amounts of dissolved oxygen (DO), and increased fish mortalities due to exacerbated ammonia toxicity (V. H. Smith et al., 1999; Carey & Migliaccio, 2009). Additionally, adverse effects of nutrient loading can also influence various aspects of the immune system in fish. Hypoxic conditions can supress respiratory bursts used in phagocytosis that will ultimately impact the removal of foreign pathogens and substances present within the organism (Ortuño et al., 2002). Furthermore, the temperature of released WWTP effluents has also been observed to increase the water temperature of receiving environments (Kinouchi et al., 2007). Changes in water temperature are an important stressor of poikilothermic fish as they equate to changes in their body temperature (Bowden, 2008; Abram et al., 2017). Undesirable changes in body temperature can trigger stress responses that ultimately impact a variety of physiological aspects and processes, including the immune system (Abram et al., 2017). Within the immune system, higher temperatures can reduce phagocytosis and impact the expression of various cytokines (Le Morvan et al., 1997; Raida & Buchmann, 2007). Another stressor linked to WWTP effluents is the entrance of contaminants of emerging concern (CECs) such as pharmaceuticals and

personal care products (PPCPs), and pesticides (Park & Park, 2015; Mehdi et al., 2018). These chemicals persist in aquatic environments due to their resilience to current wastewater treatment strategies and have the ability to act as endocrine, neuroendocrine, and metabolic disruptors in non-target species (Sumpter, 2005; Mennigen et al., 2011; Mehdi et al., 2018).

2.1.2 Impacts of effluent-associated stressors of fishes

Multiple stressors associated with wastewater effluents have shown several physiological impacts on fishes. Male and female RBD taken from downstream of WWTP effluent displayed higher rates of oxygen consumption (Mehdi et al., 2018). Additionally, females collected downstream had significantly higher baseline cortisol levels than any other group (Mehdi et al., 2018). Male RBD and GSD collected downstream of effluent discharges also demonstrated elevated rates of intersex and reduced capacities of testosterone and 11-ketotestosterone production (Tetreault et al., 2011). Furthermore, effluent exposed RBD and FTD had increased maximum metabolic rates which lead to increases in aerobic scope compared to reference sites (Hodgson et al., 2020). Gill samples collected from effluent-exposed RBD and GSD also exhibited more pathologies and variations in their morphology including hyperplasia and secondary lamellae fusion (Hodgson et al., 2020). Moreover, transcript expressions and enzymatic activity of the antioxidative enzymes superoxide dismutase and catalase, were seen to be increased in several darter species collected downstream of effluent outfall (Gauvreau et al., 2022). Overall, these studies suggest that exposure to WWTP effluents, and therefore their corresponding associated stressors, have significant impacts on various physiological aspects of fishes that can ultimately affect their overall populations. While the impact of effluent associated stressors has become of great interest, their impact on the immune system is still poorly understood.

2.1.3 Objectives and hypotheses

The objectives of this study were to investigate the impacts of environmental stressors associated with WWTP effluents on the innate immune systems of darter species in the Grand River since effects on other physiological aspects such as metabolism, and stress have already been demonstrated (Mehdi et al., 2018). Immune responses require a lot of energy and can become supressed under chronic stress events, so it is hypothesized that if these processes are affected by effluent-associated stressors then the immune system would be as well. It is hypothesized that effluent-exposed darters living downstream of the Waterloo WWTP will have impaired transcript expressions of key innate cytokines in the gills as this is the first innate immune barrier exposed to environmental stressors.

2.2 Material and methods

2.2.1 Fish collection

In October 2020, male and female RBD, GSD, FTD, and JD were collected at two sites in the Grand River, Ontario, Canada. The sites were located upstream (Kiwanis; KIW) and downstream (Economic Insurance Trailway; EIT) of the Waterloo Municipal WWTP effluent outfall, serving as reference 'clean' and 'contaminated' sites respectively (Fig. 2). Sites were selected based upon their proximity to the Waterloo WWTP in addition to accessibility for sampling. Moving in a zig-zag pattern across the width of the river, fish were stunned using a backpack electrofisher (Smith Root) and collected with dip nets before being placed into aerated buckets of river water. Fish were then brought to an on-site sampling trailer where total length ($\pm$ 0.1cm), weight ($\pm$ 0.001g), and sex were recorded prior to tissue collection. Fish smaller than 4.0 cm were not sampled as they were considered as immature. Males and females were differentiated through external features, and were further verified internally, by either the presence of testes or

ovaries respectively. Fish were stunned by a quick blow to the head before being euthanized, following which, gill tissues were collected in cryotubes and immediately snap-frozen in liquid nitrogen for further molecular analysis. For health status of sampled fish, please refer to Appendix A, Table A1. All animal use and handling protocols followed the Canadian Council of Animal Care guidelines and were approved by the University of Waterloo's Animal Care Committee (AUPP #40318).

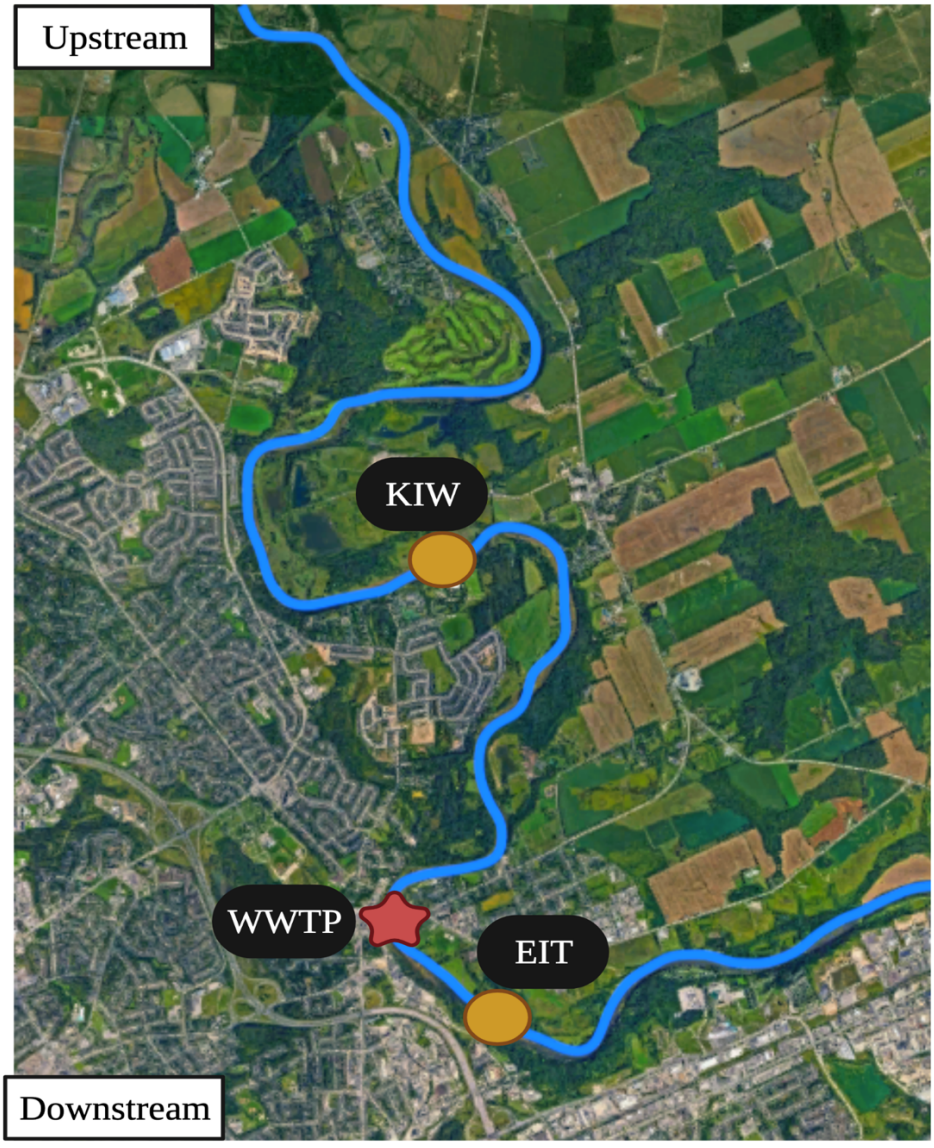

Figure 2*:* Map of Grand River sampling locations used in this study. Yellow markers indicate general locations of sites used to capture and collect darters; red indicates the general location of effluent outfall from the Waterloo municipal WWTP. KIW, Kiwanis is the upstream 'clean' collection site and EIT, Economic Insurance Trailway is the downstream 'contaminated' collection site (Google Maps; Map data ©2022, CNES/Airbus, Landsat, Copernicus, Maxar Technologies).

2.2.2 Water quality

Water quality measurements including temperature, pH, DO, and conductivity were recorded at each site in the Grand River using a YSI Professional Plus multimeter (Yellow Springs,

USA). For pharmaceutical and contaminant analysis, grab samples of surface river water were collected in triplicate at each site from the near, center, and far bank of the river, or within the effluent plume of downstream sites following the previously described techniques of (Fuzzen et al., 2016). Water samples were preserved with 1 g/L sodium azide and 50 mg/L ascorbic acid in 500 mL amber glass bottles before being stored at 4 °C until extraction. The methods used for extraction and analyses of water samples are described in detail by (Tanna et al., 2013). Pharmaceuticals and contaminants were were analyzed using solid phase extraction followed by liquid chromatography and tandem mass spectrometry (LC-MS/MS) using an Agilent 1200 HPLC (Mississauga, Canada) coupled to an Applied Biosystems 3200 QTRAPmass spectrometer (ABSciex, Concord, Canada).

2.2.3 Molecular analysis

Whole gill tissues (n = 12, 6 males: 6 females) per site per species were used for RNA extraction using Qiagen miRNAeasy kits (Hilden, Germany). The protocol was followed according to manufacturer guidelines after samples were homogenized using an OMNI TH handheld tissue homogenizer (Kennesaw, USA). RNA quantification and purity (280:260 μm and 260:230 μm) was determined using a SpectraMax 190 from Molecular Devices (San Jose, USA) and the SpectraDrop Abs DNA Quant protocol. Following extraction, RNA was converted into cDNA using Qiagen QuantiTect Reverse Transcription kits (Hilden, Germany). Some gills had lower RNA concentrations and thus the total quantity of template RNA per reaction tube was adjusted to contain 500 ng. The cDNA synthesis protocol was followed according to manufacturer guidelines and then samples were diluted 5x with RNA free water. Primers were developed from known sequences of two closely related species (Arkansas darter – *Etheostoma cragini*; orangethroat darter – *Etheostoma spectabile*) using the Nucleotide database from NCBI. Primer

sequences were created by blasting known gene sequences in NCBI's primer design tool, Primer-BLAST, with primer length parameters of 75-125 bp. The primers created and used for molecular analysis in this study can be seen in Table 1.

Following cDNA synthesis, RT-qPCR was conducted using Bio-rad CFX Maestro software linked to a Bio-rad CFX96 Touch Thermal Cycler (Hercules, USA). SSo Advanced Universal SYBR green was used to stain DNA for analysis (Hercules, USA). Following manufacturer guidelines for a 10 µL reaction volume: 1 µL of nuclease-free water, 1 µL of forward primer, 1 µL of reverse primer, 5 µL of SYBR green, and 2 µL of sample cDNA were used in each reaction. Reactions were performed in duplicate using Bio-rad low-profile, hard-shell plates covered with optically clear, Bio-rad Microseal B Adhesive seals (Hercules, USA). Primer validations and efficiencies were determined using a 5-point dilution series of pooled template RNA for each species, increasing by a dilution factor of 4 each time (4x, 16x, 64x, 256x, 1024x). Primers were determined to be efficient when the standard curves displayed efficiencies between 80-120% and R^2 values > 0.90. Furthermore, optimal annealing temperatures of primers were determined using thermal gradients (55 – 65°C). Selected house-keeping genes (*ef1a*, *β-actin*, and *5S rRNA*) were also validated using the Bio-rad CFX Maestro software's reference gene selection tool determining the gene's stability (Hercules, USA). House-keeping genes that displayed minimal variation across samples tested were determined to be stable and selected as ideal genes. The thermal cycling conditions used for PCR were: 30 s polymerase activation at 95 °C, 10 s denaturation at 95 °C, followed by a 15 s annealing and extension phase at a primer's optimal temperature. The annealing and extension phase were cycled for a total of 40 cycles. Following amplification, a melt curve was determined between 65 and 95 °C with recordings taken every 0.5 °C, to verify the amplification of only one product.

Table 1: Primers of interest for this study

Transcript Target	Accession Number	Amplicon Size (bp)	Primer Efficiency	Sequence (5'– 3')
b-actin	XM_032537833	114	RBD – 113.5	F: GTACCCCGGCATCGCA
			GSD – 110.5	
			FTD – 110.6	R: CCTCCGATCCAGACAGAGTATT
			JD – 120.2	
5S rRNA	XR_004332256	80	RBD – 101.0	F: CCTGAACACGCCCGATCTC
			GSD – 102.8	
			FTD – 103.6	R: TAGGCGGTCTCCCATCCAAG
			JD – 111.1	
ef1a	XM_034897828	106	RBD – 97.7	F: TGGTGACAACGTGGGCTTTA
			GSD – 100.1	
			FTD – 108.4	R: GTTGGCAGCTTCTTGTGGTG
			JD – 108.5	
caspase 9	XM_034877932	92	RBD – 92.1	F: GACCAGGCCAGACAGTTAGT
			GSD – 102.2	
			FTD – 98.3	R: TGCTGACCTGTCTCCTGAAG
			JD – 102.8	
il-1β	XM_034896187	80	RBD – 88.4	F: AATGCCGCGAGGAGGATTTA
			GSD – 105.4	
			FTD – 107.5	R: CCCCCTGGTCCTCTTGTAGA
			JD – 119.9	
il-6	XM_034891383	80	RBD – 119.9	F: TGCGCCAGATCCACTACTTC
			GSD – 116.1	
			FTD – 89.0	R: AGTCCTGAAGGCCAAACGTC
			JD – 109.4	
il-8	XM_034872933	112	RBD – 91.7	F: TCTGCACGCGAGGTGTATC
			GSD – 99.7	
			FTD – 98.7	R: GATTTCAGTCCGGCGACAGT
			JD – 102.6	
il-10	XM_034869787	100	RBD – 100.0	F: CAGCATGACTCCTCGGTCTC
			GSD – 102.8	
			FTD – 103.8	R: ACAGCACTGGTTATTGCACG
			JD – 107.4	

2.2.4 Statistical analysis

All statistical analyses were completed using Prism 8 software from Graphpad (San Diego, USA). Within each species, Two-way ANOVAs and post-hoc Tukey tests were used to determine any significance in innate cytokine transcripts between sexes and sites. Statistical differences were determined using an alpha (α) value of 0.05; p-values less than α were considered significant. Fold changes in transcript abundance were calculated as B/A (B = EIT, A = KIW). Data is presented as the mean $\pm$ the standard error of the mean (SEM).

2.3 Results

2.3.1 Water quality

Water quality measurements collected from the Grand River are recorded in Table 2. Means were calculated using three measurements from each collection site. There was an increase in water temperature of 1.17 °C, flow of 6.68 m^3/s, and conductivity of 162.27 µS/cm downstream at EIT compared to upstream at KIW. There was also a decrease in DO by 0.9 mg/L and pH by 0.35 downstream compared to upstream.

Table 2: Water quality measurements of Grand River collection sites upstream (KIW) and downstream (EIT) of the Waterloo WWTP in October 2020. Data is derived from (Gauvreau et al., 2022) where the same collection sites and water samples were used and analyzed. Samples were taken from the near, center, and far bank of the river or within the effluent plume of downstream sites and values are presented as means (n = 3 per site). Flow rate data was retrieved from Historical Hydrometric Data from stations 15 km above KIW and below EIT (Government of Canada, 2022).

Collection Site	Water temperature (°C)	DO (mg/L)	Flow (m^3/s)	Conductivity (µS/cm)	pH
KIW	7.10	11.70	7.92	335.70	8.50
EIT	8.27	10.80	14.60	497.97	8.15

Furthermore, grab samples of surface river water from both collection sites were analyzed for 24 pharmaceuticals and contaminants concentrations (Table 3). 11 out of the 24 screened for pharmaceuticals and contaminants were detected upstream of the Waterloo WWTP compared to downstream where 19 out of 24 were detected. Concentrations of naproxen, diclofenac, ibuprofen, atorvastatin, P-hydroxy atorvastatin, O-hydroxy atorvastatin, gemfibrozil, venlafaxine, desmethyl-venlafaxine, fluoxetine, triclosan, sulfamethoxazole, sulfamethazine, trimethoprim, carbamazepine, 11,12-epoxide carbamazepine, caffeine, and lincomycin increased downstream compared to upstream. Atrazine however, decreased downstream compared to upstream. The most prevalent concentrations detected were atrazine, 120.03 ng/L, upstream at KIW along with diclofenac, 133.72 ng/L, and venlafaxine, 153.58 ng/L, downstream at EIT.

Table 3: Comparison of pharmaceutical and contaminant concentrations detected at upstream (KIW) and downstream (EIT) collection sites in October 2020. Data is derived from (Gauvreau et al., 2022) as the same collection sites and water samples were used and analyzed. Three samples were collected at each site from the near, center, and far bank of the river or within the effluent plume of downstream sites. Values are presented as means (n = 3 per site).

Classification	Pharmaceutical or contaminant	KIW (ng/L)	EIT (ng/L)
NSAIDs	Naproxen	0.00	15.48
	Diclofenac	27.53	133.72
	Ibuprofen	7.66	16.08
Cholesterol lowering	Atorvastatin	0.00	5.79
	P-hydroxy atorvastatin	0.00	15.04
	O-hydroxy atorvastatin	0.00	11.41
	Gemfibrozil	0.00	0.32
Antidepressants	Venlafaxine	11.33	153.58
	Desmethyl-venlafaxine	4.99	44.01
	Fluoxetine	0.00	4.55
	Norfluoxetine	0.00	0.00
Antibacterial	Triclosan	0.00	9.51
	Triclocarban	0.00	0.00
	Sulfamethoxazole	0.00	1.15
	Sulfamethazine	5.28	31.99
	Trimethoprim	0.52	5.98
Antiepileptics	Carbamazepine	7.99	45.23
	11,12-epoxide carbamazepine	0.32	1.51
Herbicide	Atrazine	120.03	92.67
Pain relievers	Acetaminophen	0.00	0.00
Stimulant	Caffeine	16.17	25.11
Antibiotics	Lincomycin	3.72	2.44
	Monensin	0.00	0.00
Beta-blockers	Atenolol	0.00	0.00

2.3.2 Molecular analysis

Whole gill tissues of RBD, GSD, FTD, and JD collected in October 2020 were used to measure the RNA abundance of innate cytokine transcripts (*il-1β, il-6, il-8, il-10*) and *caspase 9*,

a transcript involved in regulating innate immune responses and cytokine signaling. RBD (Fig. 3A) and GSD (Fig. 3B) demonstrated no significant differences in *il-1β* expression. FTD displayed a significant site effect for *il-1β* ($F_{1, 20} = 7.764$, $p = 0.0114$) when analyzed by Two-way ANOVA however, no significant differences were determined when analyzed by a Tukey test (Fig. 3C). JD displayed a significant site effect for *il-1β* ($F_{1, 20} = 11.02$, $p = 0.0034$) when analyzed by Two-way ANOVA and a Tukey test displayed a significant ($p = 0.0305$) decrease in downstream females compared to upstream females by 0.51-fold (Fig. 3D).

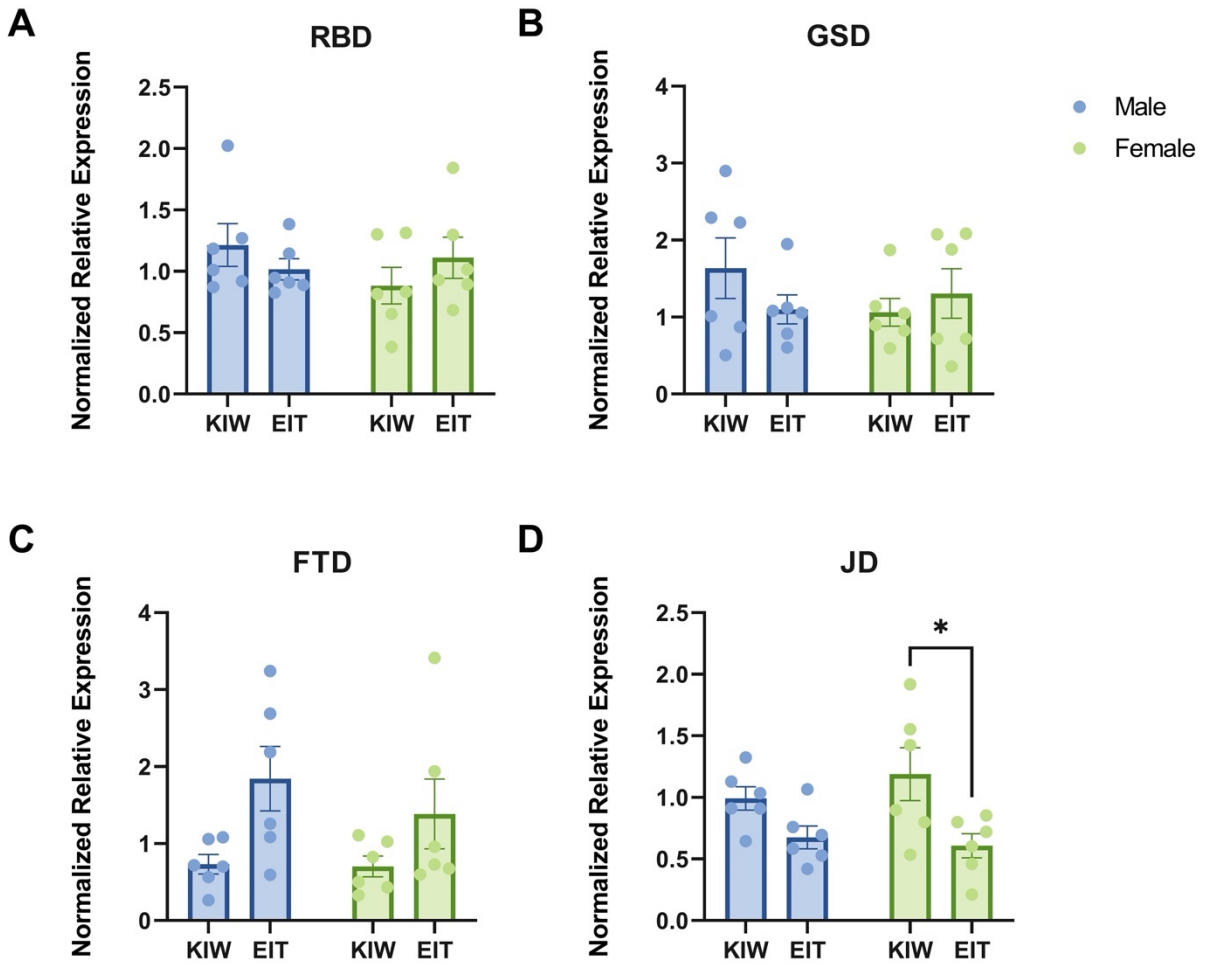

Figure 3: Normalized gene expression of interleukin 1β (*il-1β*) measured in whole gill tissues of A) rainbow darters (RBD), B) greenside darters (GSD), C) fantail darters (FTD), and D) Johnny darters (JD) caught upstream (KIW) and downstream (EIT) of the Waterloo WWTP effluent outfall in October 2020. Data is presented as means ± SEM, compared using a Two-way ANOVA and post-hoc Tukey test (* = $p < 0.05$, n = 12 per site per species [6 males: 6 females]).

RBD demonstrated no significant differences in *il-6* expression (Fig. 4A). GSD displayed a significant site effect for *il-6* ($F_{1, 20} = 4.451$, $p = 0.0477$) when analyzed by Two-way ANOVA however, no significant differences were found following a Tukey test (Fig. 4B). FTD demonstrated no significant differences in *il-6* expression (Fig. 4C). A significant site effect for *il-6* ($F_{1, 20} = 5.684$, $p = 0.0271$) was displayed by JD analyzed by Two-way ANOVA (Fig. 4D). Additionally, JD demonstrated no significant differences when analyzed by a Tukey test (Fig. 4D).

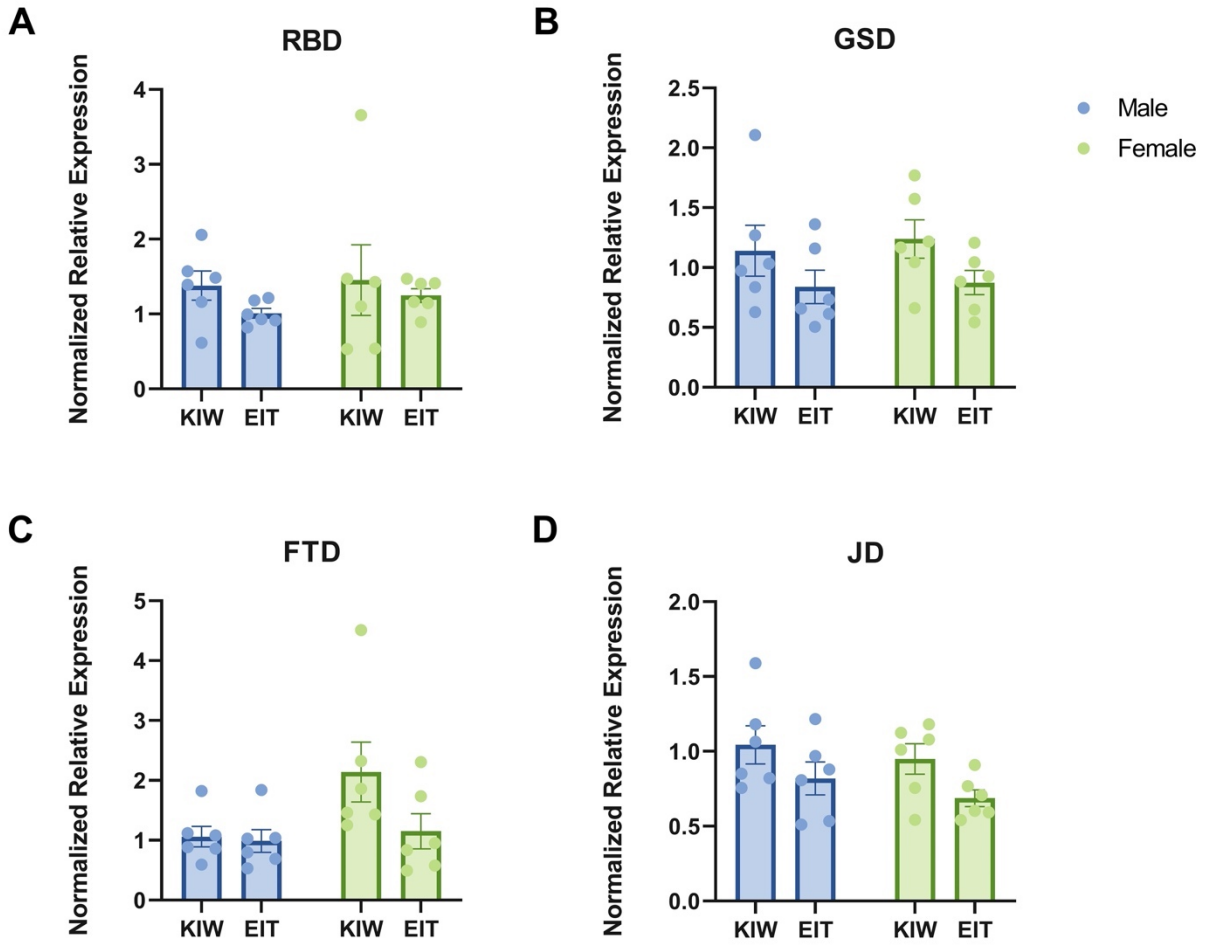

Figure 4: Normalized gene expression of interleukin 6 (*il-6*) measured in whole gill tissues of A) rainbow darters (RBD), B) greenside darters (GSD), C) fantail darters (FTD), and D) Johnny darters (JD) caught upstream (KIW) and downstream (EIT) of the Waterloo WWTP effluent outfall in October 2020. Data is presented as means ± SEM, compared using a Two-way ANOVA and post-hoc Tukey test ($p < 0.05$, $n = 12$ per site per species [6 males: 6 females]).

RBD, GSD, FTD, and JD all demonstrated no significant differences in *il-8* expression when analyzed by Two-way ANOVAs or Tukey tests (Fig. 5).

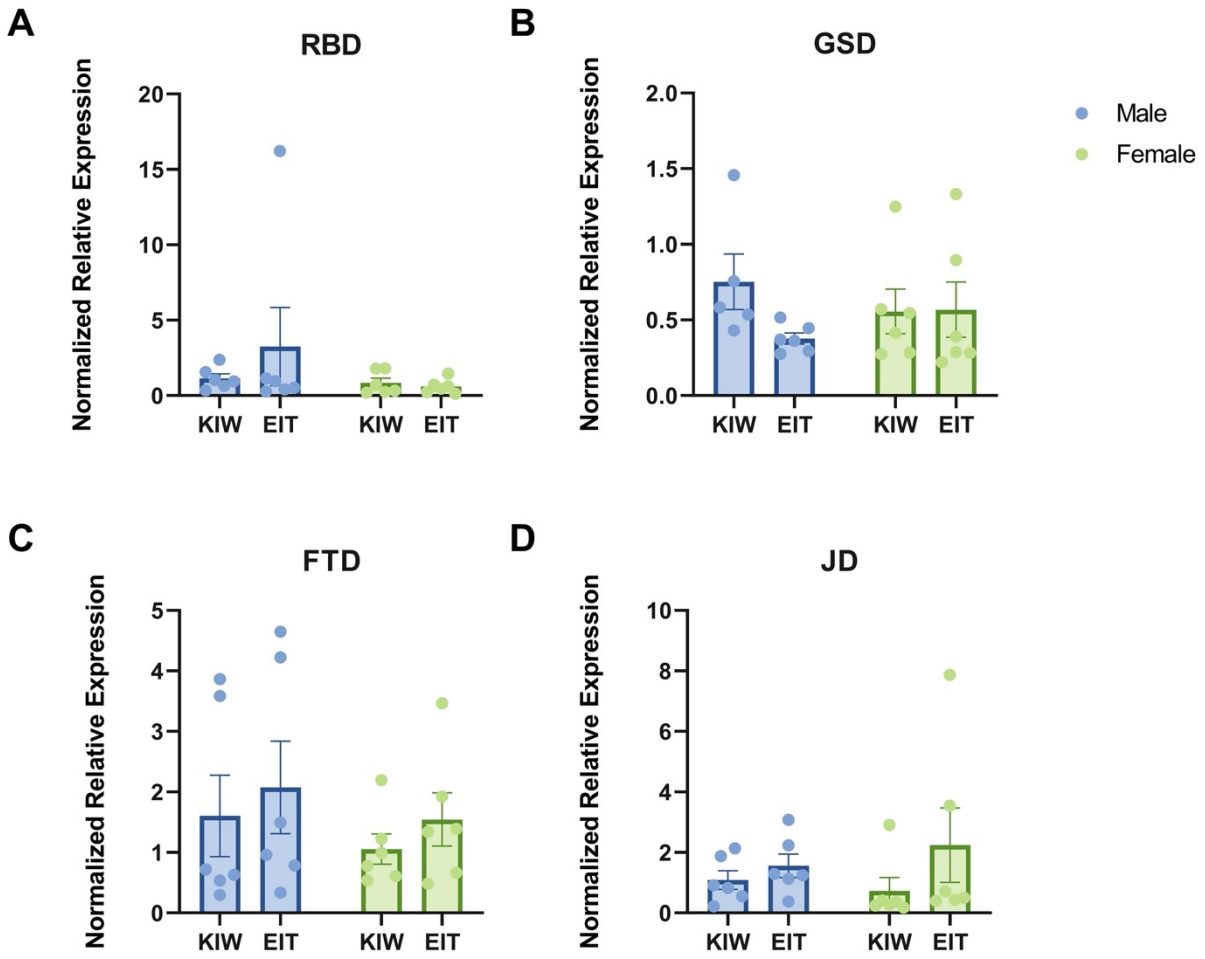

Figure 5: Normalized gene expression of interleukin 8 (*il-8*) measured in whole gill tissues of A) rainbow darters (RBD), B) greenside darters (GSD), C) fantail darters (FTD), and D) Johnny darters (JD) caught upstream (KIW) and downstream (EIT) of the Waterloo WWTP effluent outfall in October 2020. Data is presented as means ± SEM, compared using a Two-way ANOVA and post-hoc Tukey test ($p < 0.05$, n = 12 per site per species [6 males: 6 females]).

RBD (Fig. 6A) and GSD (Fig. 6B) demonstrated no significant differences in *il-10* expression. FTD displayed a significant site effect for *il-10* ($F_{1, 20} = 4.874$, $p = 0.0391$) when analyzed by Two-way ANOVA however, no significant differences were displayed when analyzed by a Tukey test (Fig. 6C). JD also demonstrated no significant differences in *il-10* expression when analyzed by Two-way ANOVA or a Tukey test (Fig. 6D).

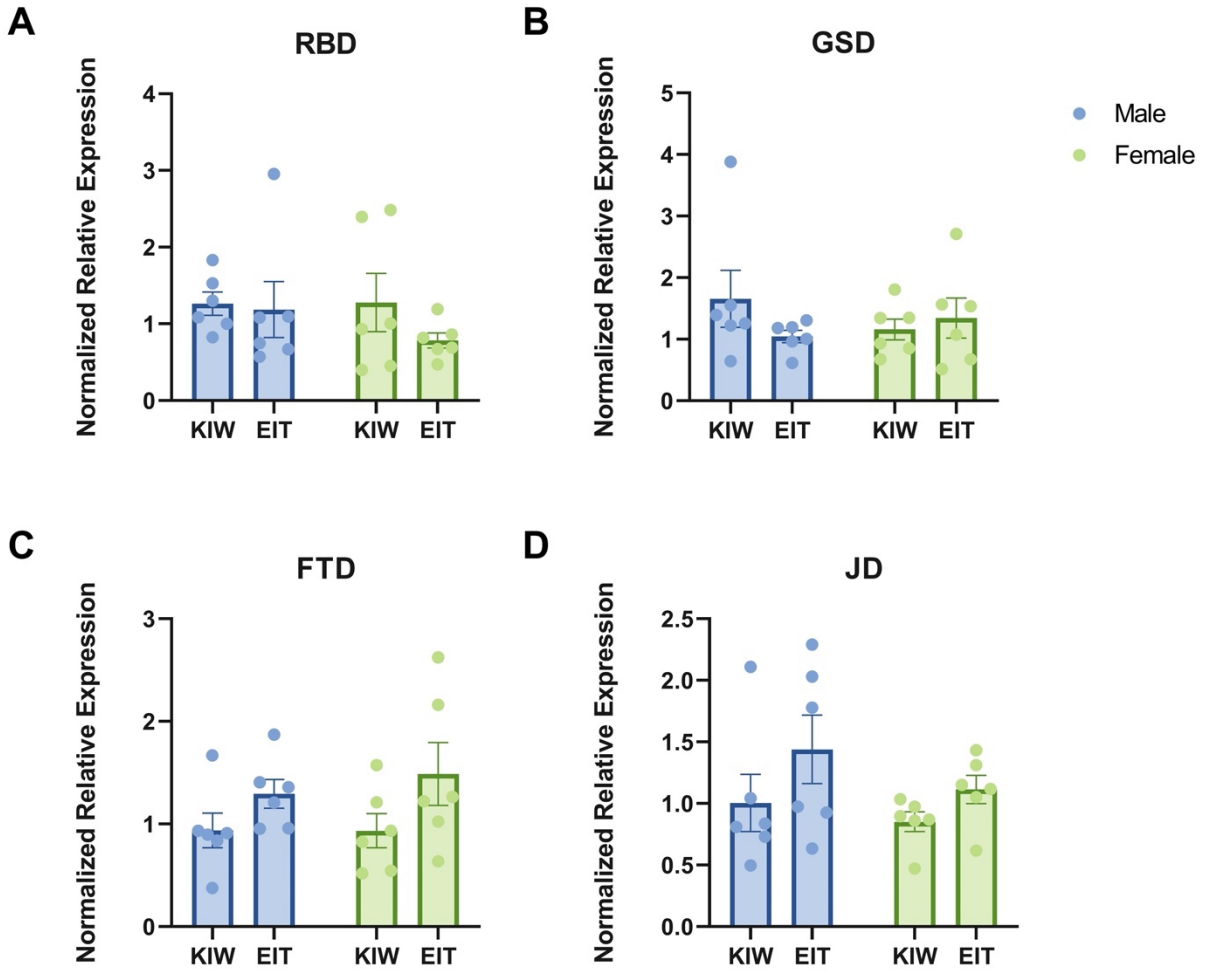

Figure 6: Normalized gene expression of interleukin 10 (*il-10*) measured in whole gill tissues of A) rainbow darters (RBD), B) greenside darters (GSD), C) fantail darters (FTD), and D) Johnny darters (JD) caught upstream (KIW) and downstream (EIT) of the Waterloo WWTP effluent outfall in October 2020. Data is presented as means ± SEM, compared using a Two-way ANOVA and post-hoc Tukey test ($p < 0.05$, $n = 12$ per site per species [6 males: 6 females]).

RBD (Fig. 7A), GSD (Fig. 7B), and FTD (Fig. 7C) all demonstrated no significant differences in *caspase 9* expression. JD displayed a significant sex effect for *caspase 9* ($F_{1, 20} = 5.492$, $p = 0.0296$) when analyzed by Two-way ANOVA however, no significant differences were found following a Tukey test (Fig. 7D).

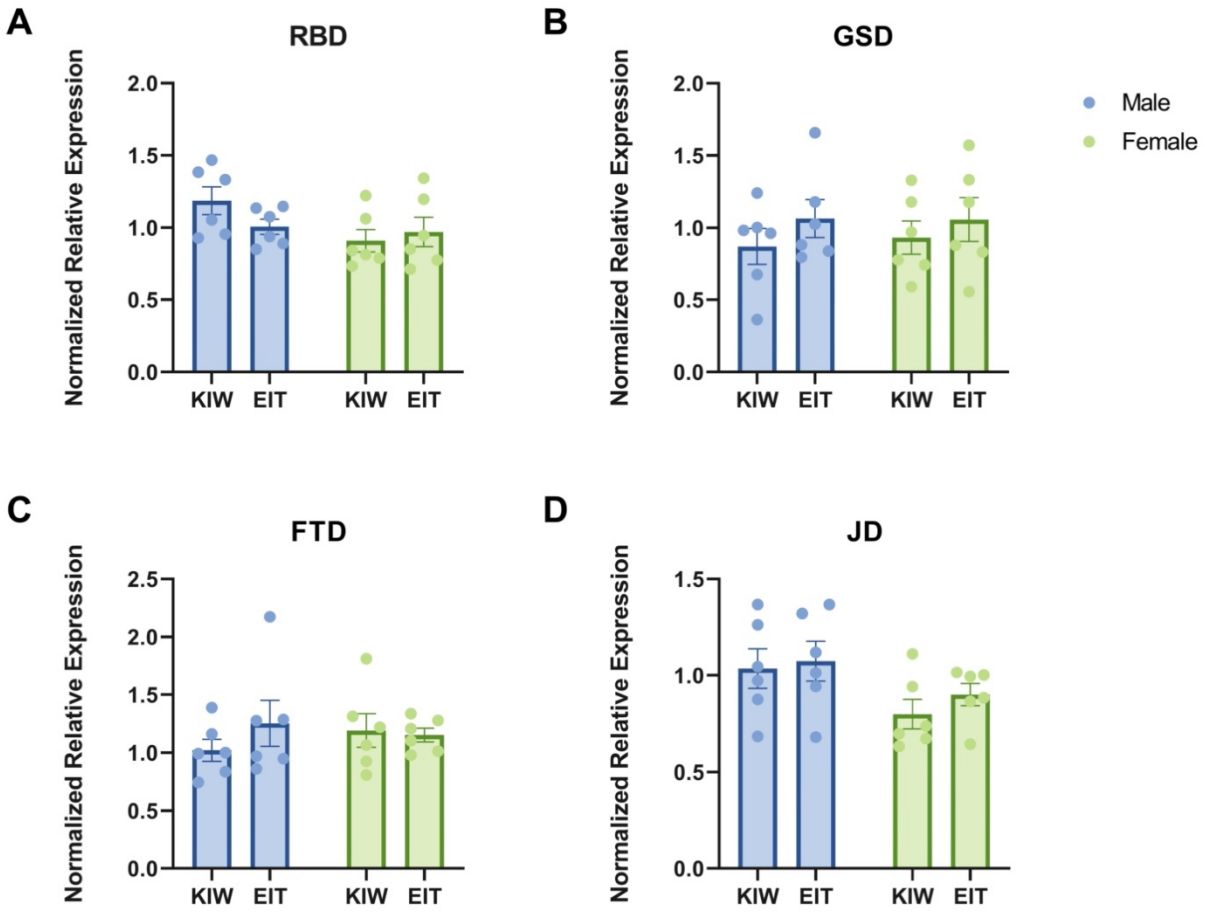

Figure 7: Normalized gene expression of *caspase 9* measured in whole gill tissues of A) rainbow darters (RBD), B) greenside darters (GSD), C) fantail darters (FTD), and D) Johnny darters (JD) caught upstream (KIW) and downstream (EIT) of the Waterloo WWTP effluent outfall in October 2020. Data is presented as means ± SEM, compared using a Two-way ANOVA and post-hoc Tukey test ($p < 0.05$, $n = 12$ per site per species [6 males: 6 females]).

2.4 Discussion

This study aimed to further investigate the potential impacts of effluent-associated stressors on the innate immune system of darters found downstream of the Waterloo WWTP by examining changes of innate cytokine transcript expressions in the gills. Following transcriptional analyses, significant site effects were found in several genes among varying species however, no major overall impacts were detected in darter gills. Johnny darters were the only species to display a

significant change, with decreased *il-1β* expression observed in females downstream of the WWTP.

Many current studies investigating the effects of effluent exposure on fish have centered mainly around RBD, as they are extremely abundant throughout the Grand River and have a high tendency to remain in effluent exposed environments (Brown et al., 2011; Fuzzen et al., 2016; Mehdi et al., 2018). Previous studies have demonstrated that downstream of the WWTP, RBD displayed significant increases in routine metabolic rates and greater pathologies in their gills but unfortunately, much is still unknown about the effects of effluent on other physiological aspects and other darter species, especially regarding their immunity (Mehdi et al., 2018; Hodgson et al., 2020). Currently, there is little to no information regarding the effects of municipal WWTP effluent exposure on gill immune responses of darters as, to our knowledge, this is one of the first studies to look at these effects. Although this study did not observe any major impacts on gill immunity following effluent exposure, gills of downstream JD females did display reduced transcript abundance of the pro-inflammatory cytokine *il-1β*. This reduction may suggest that JD gills have impaired immune responses due to effluent exposure as *il-1β* plays essential roles in early immune responses and inflammatory responses (Danabas et al., 2016; Zou & Secombes, 2016). While all species were collected downstream from the same effluent exposed environment, this significant reduction in only JD could potentially be attributed to their unique microhabitat. Environmental factors such as microhabitats in addition to others such as temperature, flow, and nutrient availability, may have important roles in altering gene expression across sites (Marjan et al., 2017). Compared to other species, JD prefer to remain in shallow, sandy areas of rivers and streams thus, it is possible that these areas downstream may be more exposed or affected than the other microhabitats (Krause et al., 2010). While this may provide a potential explanation for the

change in *il-1β* abundance, investigations of microhabitats factors would have to be further examined and so it cannot be assumed that the microhabitat of JD specifically, is the reason for the reduced *il-1β* abundance. Furthermore, several significant site effects were also seen in darters among some of the measured transcripts. These site effects could potentially be associated with higher contaminant exposure downstream from the WWTP. Analyzed samples of surface river water collected from downstream of the Waterloo WWTP in October 2020, displayed a greater presence of several pharmaceuticals and contaminants at high concentrations including carbamazepine (CBZ), diclofenac (DCF), and venlafaxine (VEN); compounds known to be difficult to biologically degrade and eliminate during wastewater treatment (Rúa-Gómez & Püttmann, 2012; Tran & Gin, 2017). As seen in previous studies (Metcalfe et al., 2010; Hodgson et al., 2020; Gauvreau et al., 2022), the highest concentration detected downstream was the antidepressant VEN. The exposure to high concentrations of VEN downstream may provide a possible explanation as to the effects observed in the abundance of several innate cytokine transcripts however, darters downstream are not only exposed to VEN but rather a mixture of pharmaceuticals and contaminants that can have synergistic or additive effects (Metcalfe et al., 2010; Hodgson et al., 2020). Thus, it cannot be said with certainty that the changes in transcript abundance in the gills and the site effects observed were a result of VEN exposure without further investigation.

In conclusion, effluent discharged from WWTPs is associated with multiple stressors and adverse effects threatening fish in the Grand River watershed (Carey & Migliaccio, 2009). High concentrations of pharmaceuticals and contaminants have also been observed in connection with effluents exposing fish to a complex mixture of toxicants that can have significant physiological impacts (Bahamonde et al., 2015; Mehdi et al., 2018; Hodgson et al., 2020). While this study did

not ultimately observe any significant effects of effluent exposure on innate cytokine transcript expression in darter gills, it highlights the need for further investigation of specific stressors, such as VEN. The high concentrations of VEN downstream may have negative effects on immunity that are concealed by confounding effects of other stressors associated with WWTP. As field work can pose challenges in obtaining fish, particularly during the COVID-19 pandemic where personnel numbers were restricted in Canada, a power analysis would be beneficial in future studies for within/between group comparisons to ensure appropriate numbers of fish are collected. Furthermore, this study focused on effluent impacts at the gill level as they are one of the first immune barriers to encounter environmental stressors. Future studies could examine additional immune tissues such as the spleen and kidney, which may have more robust effects.

Chapter 3

Effects of venlafaxine exposure on innate cytokine expression of darters (*Etheostoma* spp.) in the Grand River

3.1 Introduction

3.1.1 Pharmaceuticals in effluent

An inevitable consequence to the widespread use of PPCPs is their detected presence in surrounding aquatic environments (Overturf et al., 2015). PPCPs can be introduced to these environments through several routes such as urban and agricultural surface runoffs or, more commonly through discharged effluents from municipal WWTPs (Overturf et al., 2015; Tran et al., 2018). Many pharmaceuticals, in addition to chemicals used in personal care products, are now referred to as CECs due to their resilience to current wastewater treatment processes and their ability to act as physiological disruptors (Mennigen et al., 2011; Mehdi et al., 2018). WWTP effluents have become of particular interest and concern as they are a continuous source of CECs in aquatic environments (Tran et al., 2018). Although PPCPs are designed to produce therapeutic responses within a target species (i.e., humans), the specific processes in which they interact to alter physiological functions are highly conserved across other non-target vertebrates, especially those that share high homology with the target species (Overturf et al., 2015). As a result, non-target species, such as fish, can potentially become impacted when exposed to PPCPs in the environment. Chemical profiles of both effluents and effluent-receiving surface waters have been well studied, frequently detecting various contaminants at concentrations ranging from ng/L to µg/L and significant physiological impacts have already been observed in fish (Bahamonde et al., 2015; Mehdi et al., 2018; Hodgson et al., 2020).

While some of the Grand River watershed's thirty WWTPs have upgraded to advanced tertiary treatment processes that have reduced the concentrations of contaminants like estrogens and ibuprofen, some CECs still persist (Keegan A. Hicks et al., 2017; Tran & Gin, 2017). A compound's ability to be removed or degraded by treatment processes is heavily based on their

physiochemical properties, such as structure and solubility, and can be highly variable (Tran et al., 2018; Srikanthan, 2019). It is suggested that compounds with strong electron withdrawing groups, weak or no electron donating groups, and low octanol-water distribution coefficients (log D_{ow}) are much harder to biologically degrade and thus have poor elimination rates (Tran & Gin, 2017; Srikanthan, 2019). Compounds such as these include carbamazepine (CBZ); an anti-seizure medication, diclofenac (DCF); an anti-inflammatory drug, and venlafaxine (VEN); a common antidepressant (Rúa-Gómez & Püttmann, 2012; Tran & Gin, 2017).

3.1.2 Venlafaxine

VEN is a member of the serotonin-norepinephrine reuptake inhibitor (SNRI) family, the most common class of prescribed antidepressants used to treat illnesses such as depression, anxiety, and panic disorders (Melnyk-Lamont et al., 2014). The mechanism of action for VEN has been well studied in humans where it selectively inhibits the reuptake of serotonin and norepinephrine by presynaptic neurons in the brain (Melnyk-Lamont et al., 2014; Salahinejad et al., 2022). VEN is produced as a racemic mixture of 2 enantiomers; R-VEN inhibiting the reuptake of both serotonin and norepinephrine, and S-VEN more predominantly inhibiting the reuptake of serotonin (Gould et al., 2021; Hancu et al., 2021). VEN is mainly introduced to WWTPs through human excretion as the parent compound and its active metabolite, O-desmethyl venlafaxine (O-VEN), are excreted in urine (Metcalfe et al., 2010). Of the average human dose, approximately 5% is excreted as the parent compound, and 29% as O-VEN (Metcalfe et al., 2010). Within the surface waters of effluent receiving environments, VEN and O-VEN, are frequently detected, with previous studies observing concentrations ranging from 0.047 to 0.901 µg/L and 0.109 to 1.472 µg/L respectively (Metcalfe et al., 2010). In more recent studies, lower VEN and O-VEN concentrations have been detected ranging from 11.33 ng/L to 153.58 ng/L and 4.99 ng/L to 44.01 ng/L (Gauvreau et al.,

2022). VEN's affinity to water is likely due to the compounds physiochemical properties as it is moderately hydrophobic and unlikely to volatize in the atmosphere (Arlos et al., 2014). Although VEN's mechanism of action is less understood in non-target fish species, the organization of the teleost serotonergic system is similar to that of mammals and their receptors possess a high affinity for common antidepressants (Gould et al., 2021; Salahinejad et al., 2022). Therefore, it is important to gain further understanding of how antidepressants, such as VEN, will impact fish species.

3.1.3 Impacts of venlafaxine on fishes

VEN has been observed to have several behavioural impacts on various fish species. In larval fathead minnows (*Pimephales promelas*), exposure to 5 µg/L over five days resulted in slower escape responses (Painter et al., 2009). Furthermore, decreased brain serotonin levels and increased predation times were exhibited by hybrid striped bass (*Morone saxatilis x Morone chrysops*) exposed to increased concentrations of VEN (0-500 µg/L) for six days (Bisesi Jr et al., 2014). Rainbow trout (*Oncorhynchus mykiss*) also demonstrated increased norepinephrine, serotonin, and dopamine levels in the brain following VEN exposures of 0.2 and 1.0 µg/L over seven days (Melnyk-Lamont et al., 2014). Additionally, transcript levels of genes associated with stress and appetite were also elevated in rainbow trout, with fish consuming less food and displaying higher plasma cortisol levels (Melnyk-Lamont et al., 2014). In conjunction with its numerous behavioural effects, VEN has also been shown to have other physiological effects as well. Reduced survival was observed in fathead minnows exposed for 21 days to VEN concentrations of 305 and 1104 ng/L (Schultz et al., 2011). Moreover, routine metabolic rates of zebrafish (*Danio rerio*) were significantly increased in those exposed to 1.0 µg/L VEN and a water temperature increase of 5ºC (Mehdi et al., 2019). Catalase was also elevated only in zebrafish exposed to a temperature stressor compared to those exposed to multi-stressors, indicating a

potential detrimental impact of VEN on anti-oxidant defence mechanisms (Mehdi et al., 2019). Furthermore, significant reductions in zebrafish embryo production were also observed following a six week VEN exposure of 10 µg/L (Galus et al., 2013). Overall, these studies are indicative that aquatic exposures to VEN can have significant behavioural and physiological impacts on fish that may ultimately affect their populations. As VEN continues to be released and detected in effluent receiving environments of the Grand River watershed, it is important to assess its potential impacts on native fish species such as darters. Despite the increasing knowledge on the effects of VEN, the potential impact on integral aspects such as fish immunity is still poorly understood.

3.1.4 Objectives and hypotheses

The objectives of this study were to investigate the impacts of a specific effluent-associated stressor, pharmaceutical exposure, on the innate immune systems of darters in the Grand River since aquatic exposures to VEN, a prominently detected pharmaceutical, have been demonstrated to have several behavioural and physiological impacts in fishes. If other systems and processes of fish are affected by VEN exposure, it is logical to assume that the immune system can be potentially impacted as well. Furthermore, while the effect of VEN on the immune system is not well understood in fish, VEN has been observed to have anti-inflammatory effects in mammals (Vollmar et al., 2008; Hajhashemi et al., 2015). If these effects have been seen in mammalian species, it is important to investigate whether these same effects occur in fish. It is hypothesized that darters exposed to 1.0 µg/L of VEN for 96-hours in a laboratory setting would have impaired transcript expressions of key innate cytokines in the gills as this is the first innate immune barrier exposed to pharmaceutical contamination.

3.2 Materials and methods

3.2.1 Fish collection

In November 2021, male and female RBD, GSD, FTD, and JD were collected from a single site in the Grand River, Ontario, Canada. The site was located further upstream (West Montrose; WMR) of the Waterloo municipal WWTP than the previously used sites and served as another 'clean' reference site (Fig. 8). The collection site was selected for the prospect of decreased contaminant presence due to its greater distance from the Waterloo WWTP effluent outfall. It was also selected based upon its accessibility for sampling. Similarly, fish were collected using the same backpack electrofishing (Smith Root) methods, stunning and collecting fish in a zig-zag pattern across the river before placing them in aerated buckets of river water. Fish smaller than 4.0 cm were not collected as they were considered as immature. Fish were then transported to the Waterloo Aquatic Threats in Environmental Research (WATER) facility at the University of Waterloo where they were housed in 10 L acrylic tanks in an Aquatic Habitats (AHAB) unit. System water was maintained at 13 ºC, comparable to the Grand River temperature at the time of collection and fish were housed under a 12hr:12hr light-dark cycle while being fed once daily. Food consisted of San Francisco Bay Brand frozen bloodworms (Newark, USA) and feeding was maintained before and throughout the exposure. PVC housing structures were also added to each tank for habitat enrichment, providing a place for fish to hide underneath or sit on top. Fish were acclimated to the lab setting for two weeks allowing for maximum contaminant depuration before experimentation began. All animal use and handling protocols followed the Canadian Council of Animal Care guidelines and were approved by the University of Waterloo's Animal Care Committee (AUPP #40315).

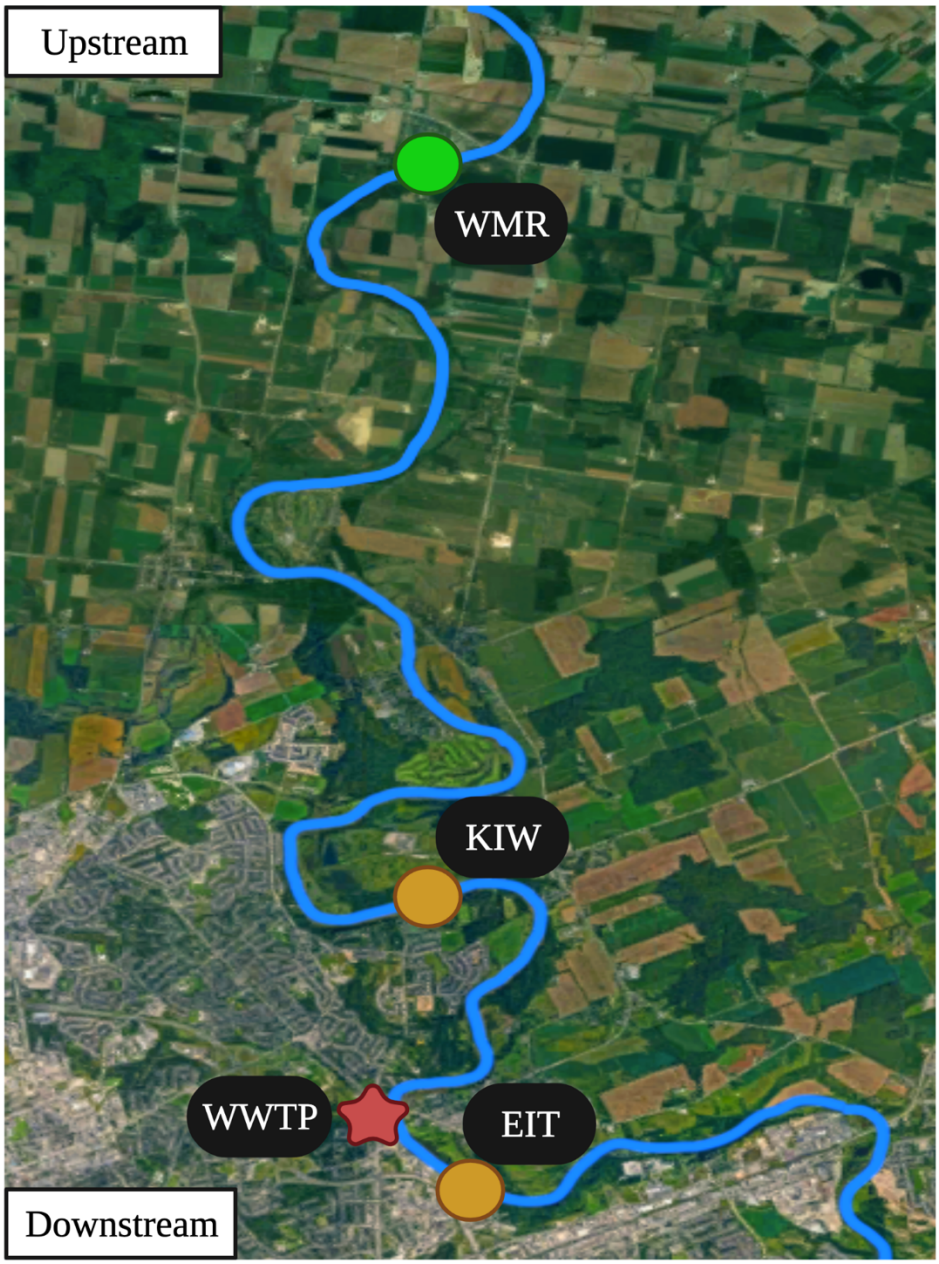

Figure 8: Map of Grand River sampling locations used in this study. Yellow markers indicate general locations of previously used collection sites; green indicates the general location of the site used to capture darters; red indicates the general location of effluent outfall from the Waterloo municipal WWTP. WMR, West Montrose is the further upstream 'clean' collection site, KIW, Kiwanis is the previously used upstream 'clean' site and EIT, Economic Insurance Trailway is the previously used downstream 'contaminated' site (Google Maps; Map data ©2022, CNES/Airbus, Landsat, Copernicus, Maxar Technologies).

3.2.2 Water quality

While at the collection site, water quality measurements (temperature, pH, DO, conductivity) were recorded using a YSI Professional Plus multimeter (Yellow Springs, USA). In addition, grab samples of surface river water were also collected in triplicate (near, center, and far bank of the river, or within the effluent plume of downstream sites) for pharmaceutical and contaminant analysis using the techniques outlined by (Fuzzen et al., 2016). Samples were preserved (1 g/L sodium azide; 50 mg/L ascorbic acid) in 500 mL amber glass bottles and stored at 4 °C until extraction. Extraction methods and analyses of water samples are detailed by (Tanna et al., 2013). Pharmaceuticals and contaminants were analyzed using solid phase extraction followed by liquid chromatography and tandem mass spectrometry (LC-MS/MS) using an Agilent 1200 HPLC (Mississauga, Canada) coupled to an Applied Biosystems 3200 QTRAPmass spectrometer (ABSciex, Concord, Canada). Using the same methods as outlined above, water quality measurements and grab samples were also collected from the previously used sampling sites, KIW and EIT, to further compare pharmaceutical and contaminant concentrations at reference sites near the Waterloo WWTP effluent outfall.

3.2.3 Exposure design

Male and female darters of each species were exposed to an environmentally relevant concentration of VEN (1.0 µg/L) over an exposure period of 96 hours. 14 fish (apart from GSD *'Control'* group with 13) with relatively equal numbers of males and females (~7 males: 7 females), were placed in 12 L glass aquaria containing the same system water described above. 2 tanks were used per species and fish were separated into one of two treatment groups: (1) 0 µg/L VEN and (2) 1.0 µg/L VEN, serving as '*Control'* and '*Exposed'* treatments respectively. Due to difficulties of sexing some darter species by external characteristics, male and female ratios varied

between treatment groups, the numbers of which can be seen in Table 4. Proper aeration and cooling were supplied to each tank using air stones and a surrounding circulating tank. A chiller and 2 air pumps were placed into the circulating tank, creating a cold-water current around all experimental tanks maintaining them at 13 ºC. Additionally, non-transparent dividers were placed between each experimental tank to remove any interactions between fish in adjacent tanks (Fig. 9). Fish acclimated to experimental tanks for 3 days prior to the start of the exposure and continued to be fed once daily. During the acclimation, each tank was also supplied with a back-hanging filtration unit that was removed after the acclimation period and prior to the start of the exposure. Fish in selected '*Exposed*' treatment groups were then dosed with 1.0 µg/L VEN (Millipore-Sigma-Aldrich) using 200 µL VEN aliquots that were made in advance. During the 96-hour exposure, daily 50% water changes were performed 1 hour after feeding to remove uneaten food, waste, and nitrogenous products. Daily VEN dosing was also performed after water changes occurred. At timepoints of 0, 24, 48, and 96-hours, 100 mL water samples were collected from each experimental tank 1 hour after the addition of the daily VEN dose to ensure VEN concentrations were maintained. Samples were preserved (1 g/L sodium azide; 50 mg/L ascorbic acid) in 125 mL amber glass bottles and stored at -20 °C until further extraction and analysis. Once the 96-hour exposure was completed fish lengths ($\pm$ 0.1cm) and weights ($\pm$ 0.001g) were recorded prior to tissue collection in which fish were stunned and euthanized before gill tissues were collected in cryotubes. Tissues were immediately snap-frozen in liquid nitrogen and stored at -80ºC for further molecular analysis. During dissection, sex was recorded after fish were verified internally, either by the presence of testes or ovaries respectively. For health status of sampled fish, please refer to Appendix A, Table A2.

Table 4: Male and female darter ratios of exposure treatment groups. '*Control'* treatment groups were exposed to 0 µg/L VEN while '*Exposed'* treatment groups were exposed to 1.0 µg/L VEN. Ratios were determined during darter tissue collection, verifying sex internally by either the presence of testes or ovaries.

Species	'Control' treatment group	'Exposed' treatment group
RBD	7 males 7 females	7 males 7 females
GSD	7 males 6 females	6 males 8 females
FTD	10 males 4 females	9 males 5 females
JD	8 males 6 females	8 males 6 females

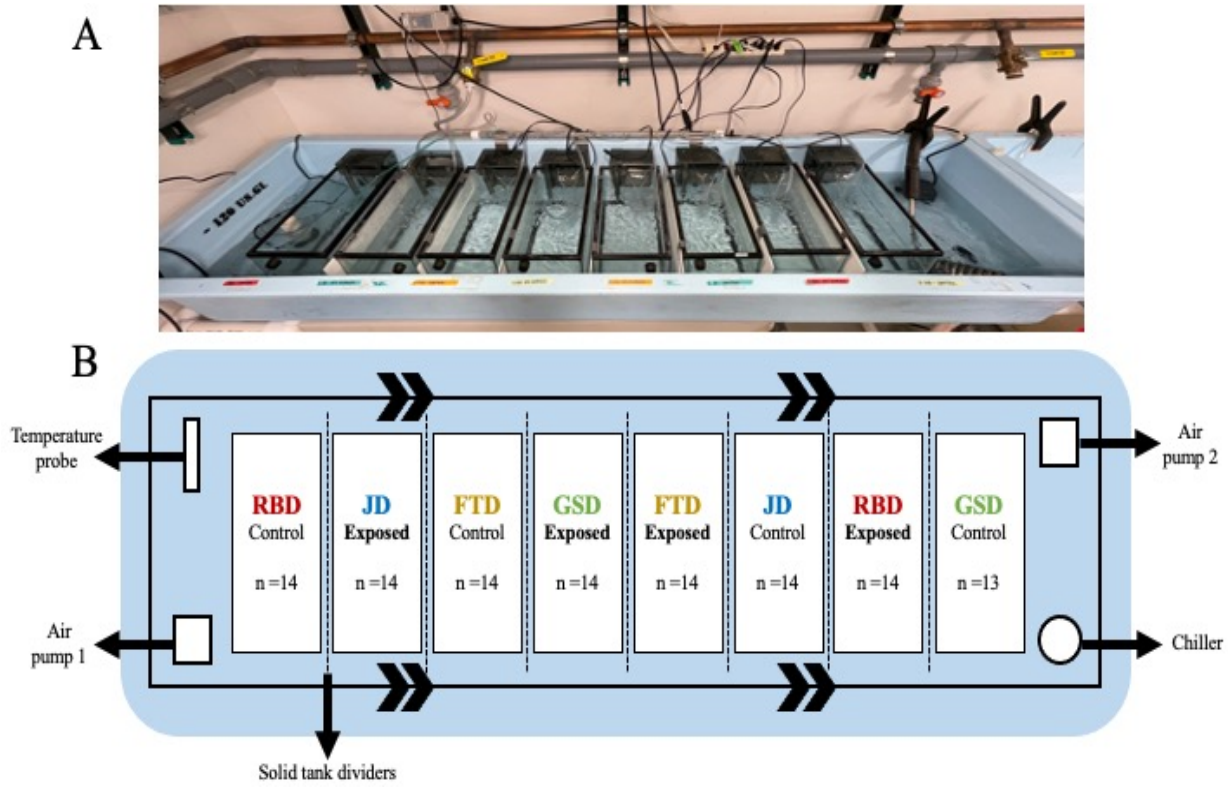

Figure 9: A) Experimental setup and B) schematic of acute venlafaxine (VEN) exposure.

3.2.4 Water chemistry

Water samples (100 mL) were collected at timepoints of 0-, 24-, 48-, and 96-h from each tank and analyzed to ensure VEN concentrations were maintained throughout the exposure experiment. Water samples were collected 1 hour after daily VEN dosing and preserved (1 g/L sodium azide; 50 mg/L ascorbic acid) in 125 mL amber glass bottles that were stored at -20 °C. Samples collected at 0 hours (with the exception of JD *'exposed'*), FTD *'control'* (24-h), GSD *'control'* (96-h), JD *'control'* and *'exposed'* (96-h) broke during storage and were therefore excluded from the analysis. VEN concentrations were quantified using extraction methods and analyses described by (Tanna et al., 2013). Samples were analyzed using solid phase extraction followed by LC-MS/MS using an Agilent 1200 HPLC (Mississauga, Canada) coupled to an Applied Biosystems 3200 QTRAPmass spectrometer (ABSciex, Concord, Canada).

3.2.5 Molecular analysis

Whole gill tissues (n = 14, ~ 7 males: 7 females) per treatment per species were used for RNA extraction using Qiagen miRNAeasy kits (Hilden, Germany). Samples were homogenized using an OMNI TH handheld tissue homogenizer (Kennesaw, USA) and the protocol was followed according to manufacturer guidelines. RNA quantification and purity (280:260 μm and 260:230 μm) was determined using as SpectraMax 190 from Molecular Devices (San Jose, USA) and the SpectraDrop Abs DNA Quant protocol. Extracted RNA was then converted into cDNA using Qiagen QuantiTect Reverse Transcription kits (Hilden, Germany). The total quantity of template RNA per reaction tube was adjusted to contain 500 ng and the cDNA synthesis protocol was followed according to manufacturer guidelines. The resulting cDNA was then diluted 5x with RNA free water. The previously developed and tested primers (Table 1) used in Chapter 2 were used again in this study.

After cDNA synthesis, RT-qPCR analysis was conducted using Bio-rad CFX Maestro software linked to a Bio-rad CFX96 Touch Thermal Cycler (Hercules, USA) and SSoAdvanced Universal SYBR green (Hercules, USA) was used to stain DNA for analysis. Following manufacturer guidelines for a 10 µl reaction volume: 1 µl of nuclease-free water, 1 µl of forward primer, 1 µl of reverse primer, 5 µl of SYBR green, and 2 µl of sample cDNA were used in each reaction. Each reaction was performed in duplicate using Bio-rad low-profile, hard-shell plates covered with optically clear, Bio-rad Microseal B Adhesive seals (Hercules, USA). The thermal cycling conditions used for PCR were: 30 s polymerase activation at 95 °C, 10 s denaturation at 95 °C, followed by a 15 s annealing and extension phase at a primer's optimal temperature. The annealing and extension phase were cycled for a total of 40 cycles. Following amplification, a melt curve was determined between 65 and 95 °C with recordings taken every 0.5 °C, to verify the amplification of only one product.

3.2.6 Statistical analysis

All statistical analyses were completed using Prism 8 software from Graphpad (San Diego, USA). Within each species, Two-way ANOVAs and post-hoc Tukey tests were used to determine any significance in innate cytokine transcripts between sexes and sites. Statistical differences were determined using an alpha (α) value of 0.05; p-values less than α were considered significant. Fold changes in transcript abundance were calculated as B/A (B = VEN exposed, A = control) and D/C (D = control females, C = control males). Data is presented as the mean $\pm$ SEM.

3.3 Results

3.3.1 Water quality

Water quality measurements collected from the Grand River in November 2021 are recorded in Table 5. Means were calculated using three measurements from each collection site.

There was increase in water temperature of 2 °C and conductivity of 246.1 µS/cm. There was also decrease in DO of 0.16 mg/L.

Table 5: Water quality measurements of the Grand River collection site further upstream (WMR) of the Waterloo WWTP and the previously used upstream (KIW) and downstream sites in November 2021. Samples were taken from the near, center, and far bank of the river or within the effluent plume of downstream sites and values are presented as means (n = 3 per site).

Collection Site	Water temperature (°C)	DO (mg/L)	Conductivity (µS/cm)
WMR	7.67	12.53	406.57
EIT	9.67	12.37	652.67

Moreover, grab samples of surface river water from the further upstream collection site, and the previously used sample sites were analyzed for 24 pharmaceuticals and contaminants (Table 6). The least number of pharmaceuticals and contaminants screened for were observed furthest upstream from the Waterloo WWTP at WMR. Further upstream, 14 out of the 24 pharmaceuticals and contaminants were detected compared to 17 out of 24 upstream at KIW and 20 out of 24 detected downstream at EIT. Between upstream sites, concentrations of naproxen, P-hydroxy atorvastatin, desmethyl-venlafaxine, fluoxetine, triclosan, triclocarban, sulfamethazine, carbamazepine, atrazine, and monensin increased from WMR to KIW while concentrations of diclofenac, atorvastatin, O-hydroxy atorvastatin, gemfibrozil, venlafaxine, sulfamethoxazole, and caffeine decreased. Downstream, concentrations of naproxen, diclofenac, ibuprofen, atorvastatin, P-hydroxy atorvastatin, O-hydroxy atorvastatin, gemfibrozil, venlafaxine, desmethyl-venlafaxine, fluoxetine, norfluoxetine, triclosan, triclocarban, sulfamethazine, trimethoprim, carbamazepine, and acetaminophen increased compared to both upstream sites. Sulfamethoxazole, atrazine, and caffeine concentrations however, decreased downstream compared to upstream. The most

prevalent concentrations detected were venlafaxine, 119.66 ng/L, and desmethyl-venlafaxine, 101.85 ng/L, downstream of the WWTP at EIT.

Table 6: Comparison of pharmaceutical and contaminant concentrations detected at upstream (WMR / KIW) and downstream (EIT) collection sites in November 2021. Three samples were collected at each site from the near, center, and far bank of the river or within the effluent plume of downstream sites. Values are presented as means (n = 3 per site).

Classification	Pharmaceutical or contaminant	WMR (ng/L)	KIW (ng/L)	EIT (ng/L)
NSAIDs	Naproxen	0.00	3.08	7.83
	Diclofenac	10.75	8.58	73.83
	Ibuprofen	0.00	0.00	12.89
Cholesterol lowering	Atorvastatin	0.65	0.54	2.69
	P-hydroxy atorvastatin	1.06	1.10	4.66
	O-hydroxy atorvastatin	1.09	1.01	5.08
	Gemfibrozil	10.75	8.58	73.83
Antidepressants	Venlafaxine	9.59	6.48	119.66
	Desmethyl-venlafaxine	3.46	16.69	101.85
	Fluoxetine	0.22	0.24	4.15
	Norfluoxetine	0.00	0.00	0.48
Antibacterials	Triclosan	1.62	1.66	4.14
	Triclocarban	0.00	0.23	0.37
	Sulfamethoxazole	20.41	14.85	11.15
	Sulfamethazine	12.13	12.53	46.59
	Trimethoprim	0.00	0.00	12.95
Antiepileptics	Carbamazepine	4.09	4.56	27.93
	11,12-epoxide carbamazepine	0.00	0.00	0.00
Herbicide	Atrazine	14.87	20.27	16.50
Pain relievers	Acetaminophen	0.00	0.00	4.21
	Oxycodone	0.00	0.00	0.00
Stimulant	Caffeine	30.27	9.18	13.67
Antibiotics	Lincomycin	0.00	0.00	0.00
	Monensin	0.00	0.42	0.00

3.3.2 Water chemistry

Water samples collected from each experimental exposure tank at timepoints of 0-, 24-, 48-, and 96-h were used to measure the concentration of VEN and ensure its concentration was maintained throughout the exposure. Of the analyzed samples, no VEN was detected in any of the *'control'* tanks. VEN was detected at concentrations higher than the expected 1.0 µg/L in samples collected between 0-h and 48-h, and detected at expected concentrations in samples collected at 96-h.

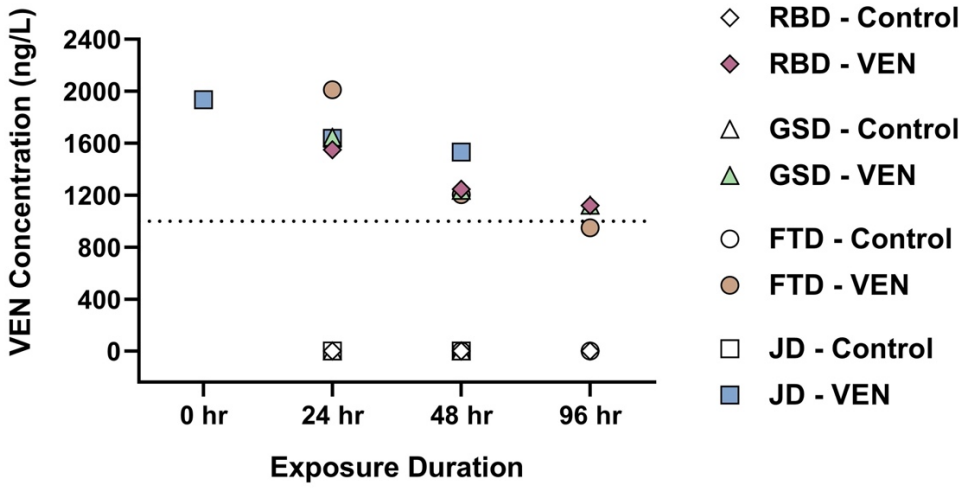

Figure 10: Venlafaxine (VEN) concentrations (ng/L) of each treatment tank across the exposure period.

3.3.3 Molecular analysis

Whole gill tissues of RBD, GSD, FTD, and JD collected after an acute (96-hour) VEN exposure in November 2020 were used to measure the RNA abundance of innate cytokine transcripts (*il-1β, il-6, il-8, il-10*) and *caspase 9*. RBD demonstrated no significant differences in *il-1β* expression (Fig. 11A). A significant interaction effect for *il-1β* ($F_{1, 23} = 7.679$, $p = 0.0109$) was displayed by GSD when analyzed by Two-way ANOVA but, no significant differences were

demonstrated when analyzed by a Tukey test (Fig. 11B). FTD also demonstrated no significant differences in *il-1β (*Fig. 11C). JD displayed a significant sex effect for *il-1β* ($F_{1, 24} = 4.651$, $p = 0.0413$) when analyzed by Two-way ANOVA however, when analyzed by a Tukey test, no significant differences were demonstrated (Fig. 11D*)*.

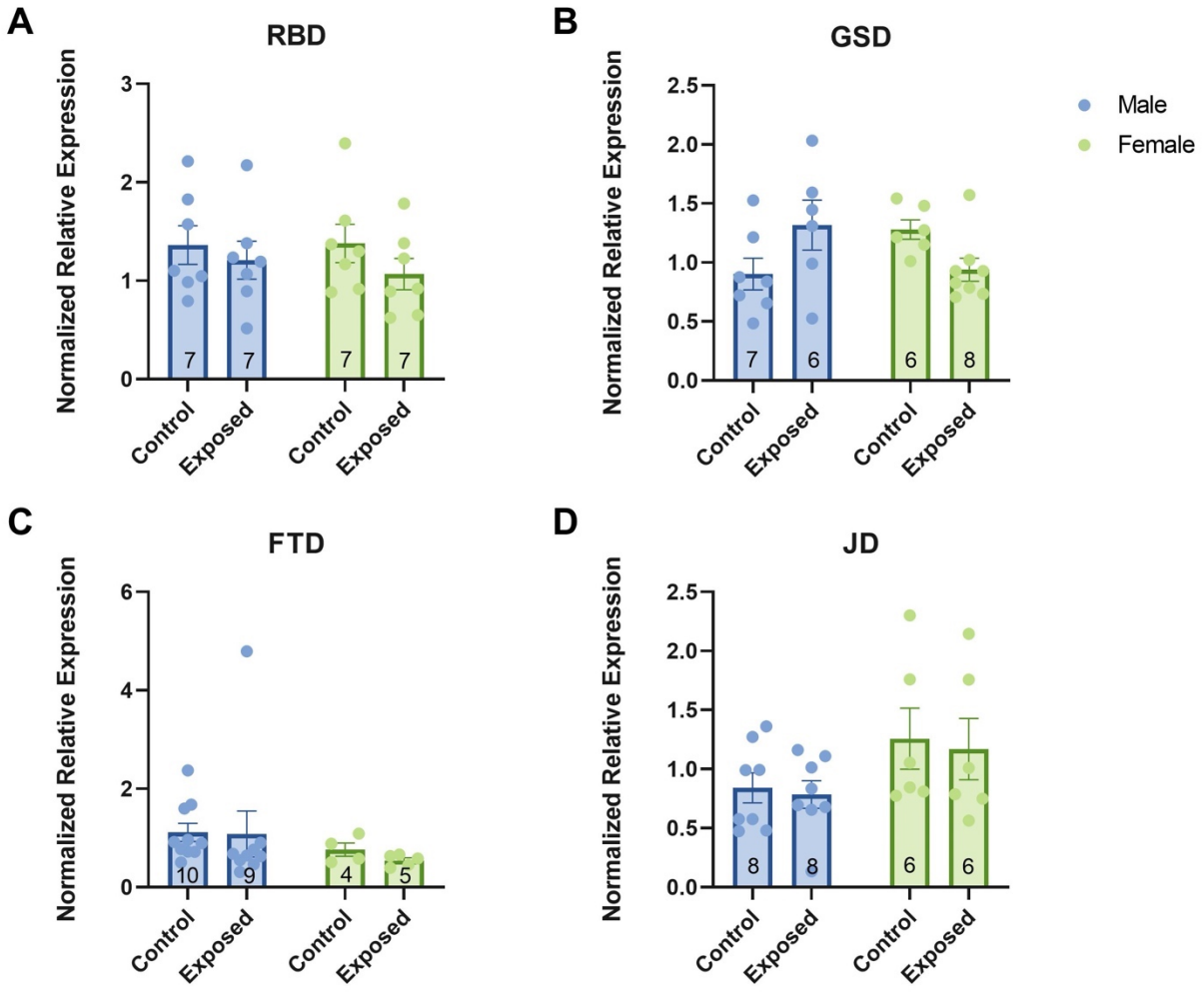

Figure 11: Normalized gene expression of interleukin 1β (*il-1β*) measured in whole gill tissues of A) rainbow darters (RBD), B) greenside darters (GSD), C) fantail darters (FTD), and D) Johnny darters (JD) exposed to 1.0 µg/L VEN for 96-hours in November 2021. Data is presented as means ± SEM, compared using a Two-way ANOVA and post-hoc Tukey test ($p < 0.05$, $n = 14$ per treatment per species [exception being GSD 'control' with 13]). Number of males and females in each treatment are represented by the number in each bar.

A significant treatment effect for *il-6* ($F_{1, 24} = 10.39$, $p = 0.0036$) was demonstrated by RBD analyzed by Two-way ANOVA and a Tukey test displayed a significant increase in VEN exposed females compared to control females by 1.77- fold (Fig. 12A). The other species, GSD (Fig. 12B), FTD (Fig. 12C), and JD (Fig. 12D), demonstrated no significant differences in *il-6* expression.

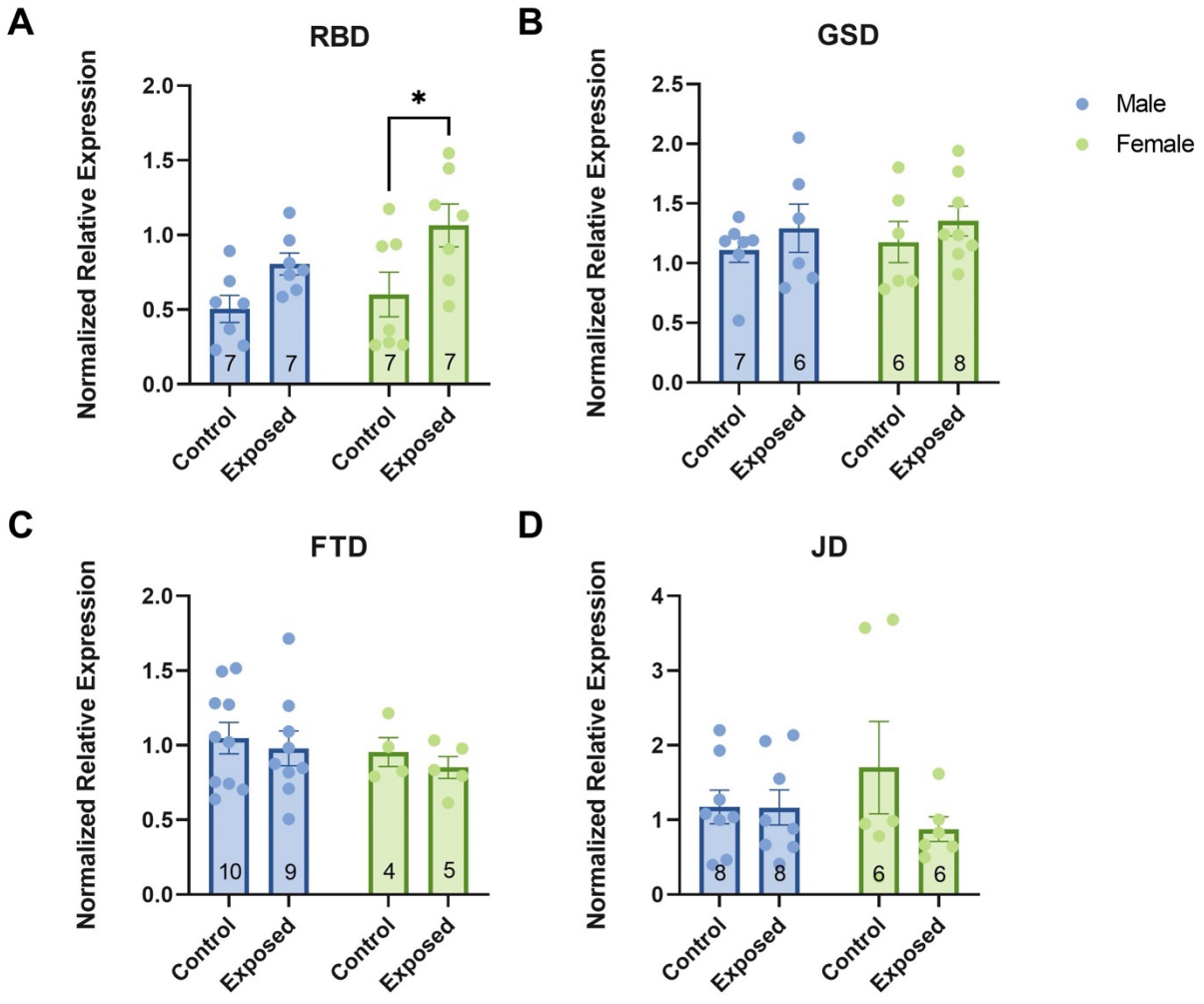

Figure 12: Normalized gene expression of interleukin 6 (*il-6*) measured in whole gill tissues of A) rainbow darters (RBD), B) greenside darters (GSD), C) fantail darters (FTD), and D) Johnny darters (JD) exposed to 1.0 µg/L VEN for 96-hours in November 2021. Data is presented as means ± SEM, compared using a Two-way ANOVA and post-hoc Tukey test (* = $p < 0.05$, n = 14 per treatment per species [exception being GSD 'control' with 13]). Number of males and females in each treatment are represented by the number in each bar.

RBD displayed a significant sex effect for *il-8* ($F_{1, 24} = 12.13$, $p = 0.0019$) when analyzed by Two-way AONVA and a Tukey test displayed a significant decrease ($p = 0.0085$) in control females compared to control males by a 0.18-fold change (Fig. 13A). Additionally, RBD also displayed a significant treatment effect ($F_{1, 24} = 4.496$, $p = 0.0445$) however, no significant differences were displayed by a Tukey test (Fig. 13A). GSD (Fig. 13B), FTD (Fig. 13C), and JD (Fig. 13D) displayed no significant differences in *il-8* expression.

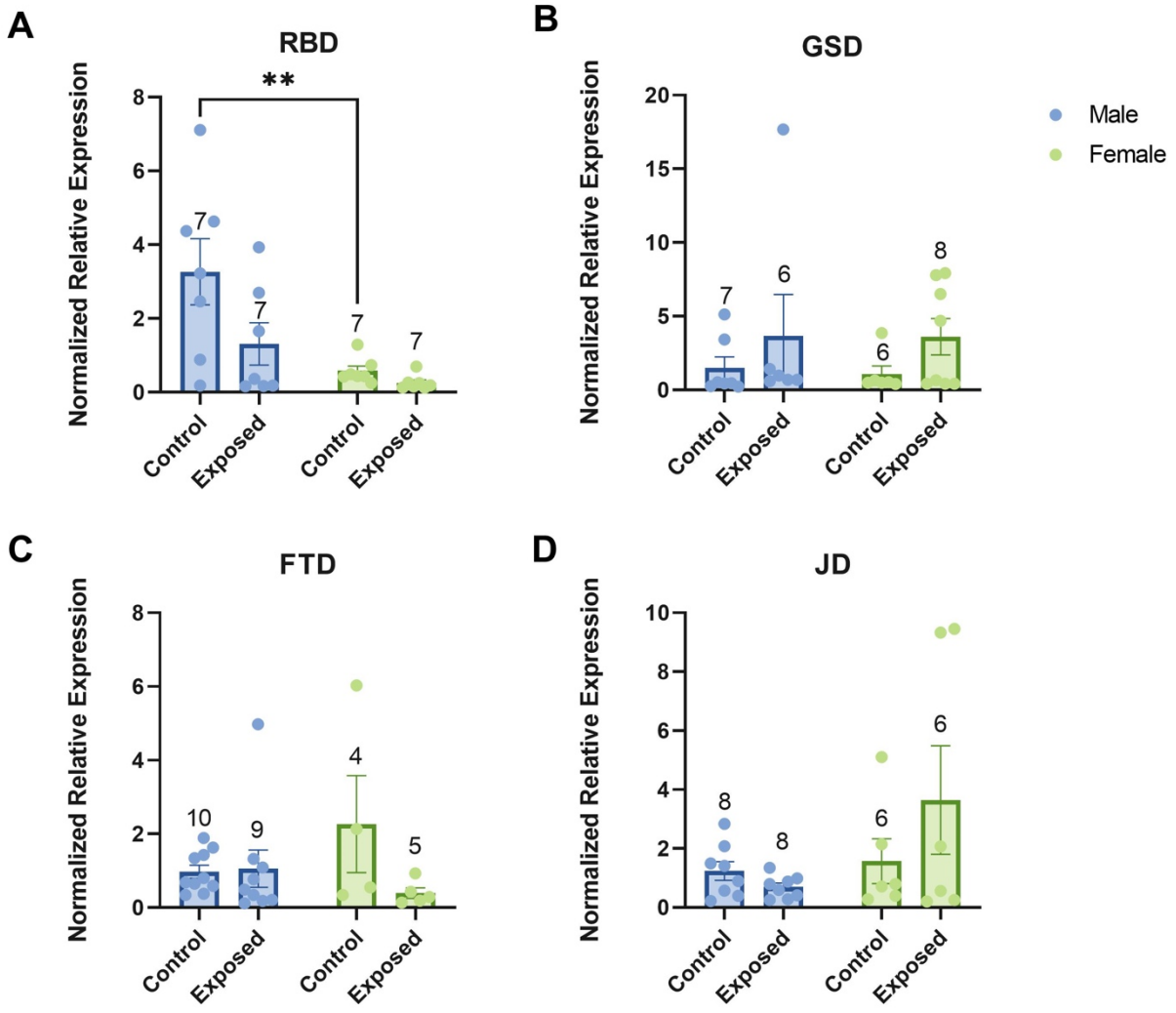

Figure 13: Normalized gene expression of interleukin 8 (*il-8*) measured in whole gill tissues of A) rainbow darters (RBD), B) greenside darters (GSD), C) fantail darters (FTD), and D) Johnny darters (JD) exposed to 1.0 µg/L VEN for 96-hours in November 2021. Data is presented as means ± SEM, compared using a Two-way ANOVA and post-hoc Tukey test (** = $p < 0.01$, n = 14 per treatment per species [exception being GSD 'control' with 13]). Number of males and females in each treatment are represented by the number above each bar.

RBD displayed a nearly significant sex effect for *il-10* ($F_{1, 24} = 4.177$, $p = 0.0521$) when analyzed by Two-way ANOVA however, no significance was found following a Tukey test (Fig. 14A). No significant differences for *il-10* were found in GSD (Fig. 14B), FTD (Fig. 14C), or JD (Fig. 14D) following analyses by Two-way ANOVA or a Tukey test.

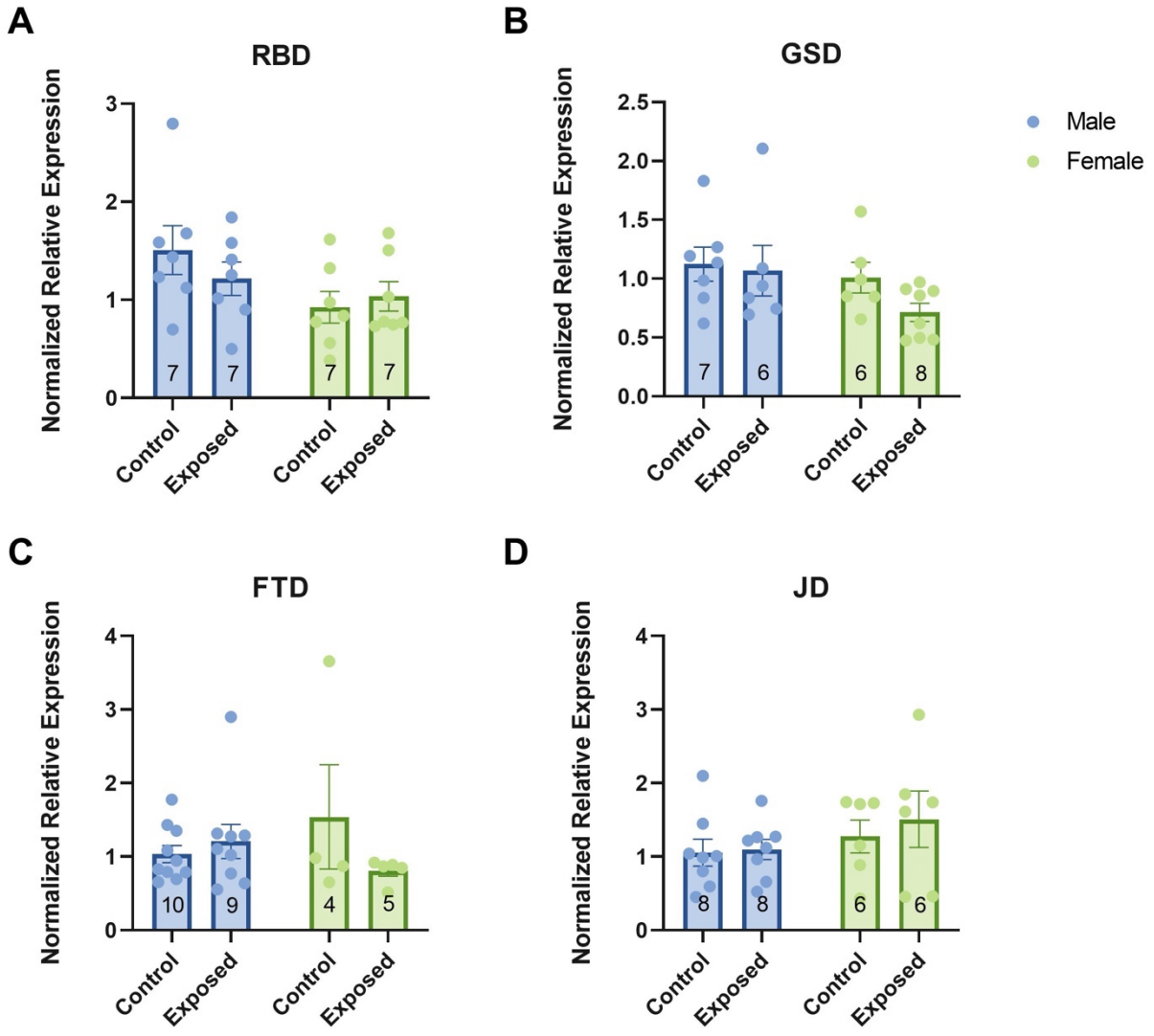

Figure 14: Normalized gene expression of interleukin 10 (*il-10*) measured in whole gill tissues of A) rainbow darters (RBD), B) greenside darters (GSD), C) fantail darters (FTD), and D) Johnny darters (JD) exposed to 1.0 µg/L VEN for 96-hours in November 2021. Data is presented as means ± SEM, compared using a Two-way ANOVA and post-hoc Tukey test ($p < 0.05$, $n = 14$ per treatment per species [exception being GSD 'control' with 13]). Number of males and females in each treatment are represented by the number in each bar.

No significant differences for *caspase 9* were found in RBD (Fig. 15A). GSD displayed a significant treatment effect for *caspase 9* when analyzed by Two-way ANOVA (Fig. 15B). Following a Tukey test, GSD also displayed a significant ($p = 0.0423$) decrease in VEN exposed females compared to control females by a 0.59-fold change (Fig. 15B). A significant treatment

effect for *caspase 9* ($F_{1, 24}$ = 12.60, p = 0.0016) was demonstrated by FTD following Two-way ANOVA and a Tukey test displayed a significant (p = 0.0334) decrease in VEN exposed males compared to control males by a 0.71-fold change (Fig. 15C). JD demonstrated no significant differences in *caspase 9* expression (Fig. 15D).

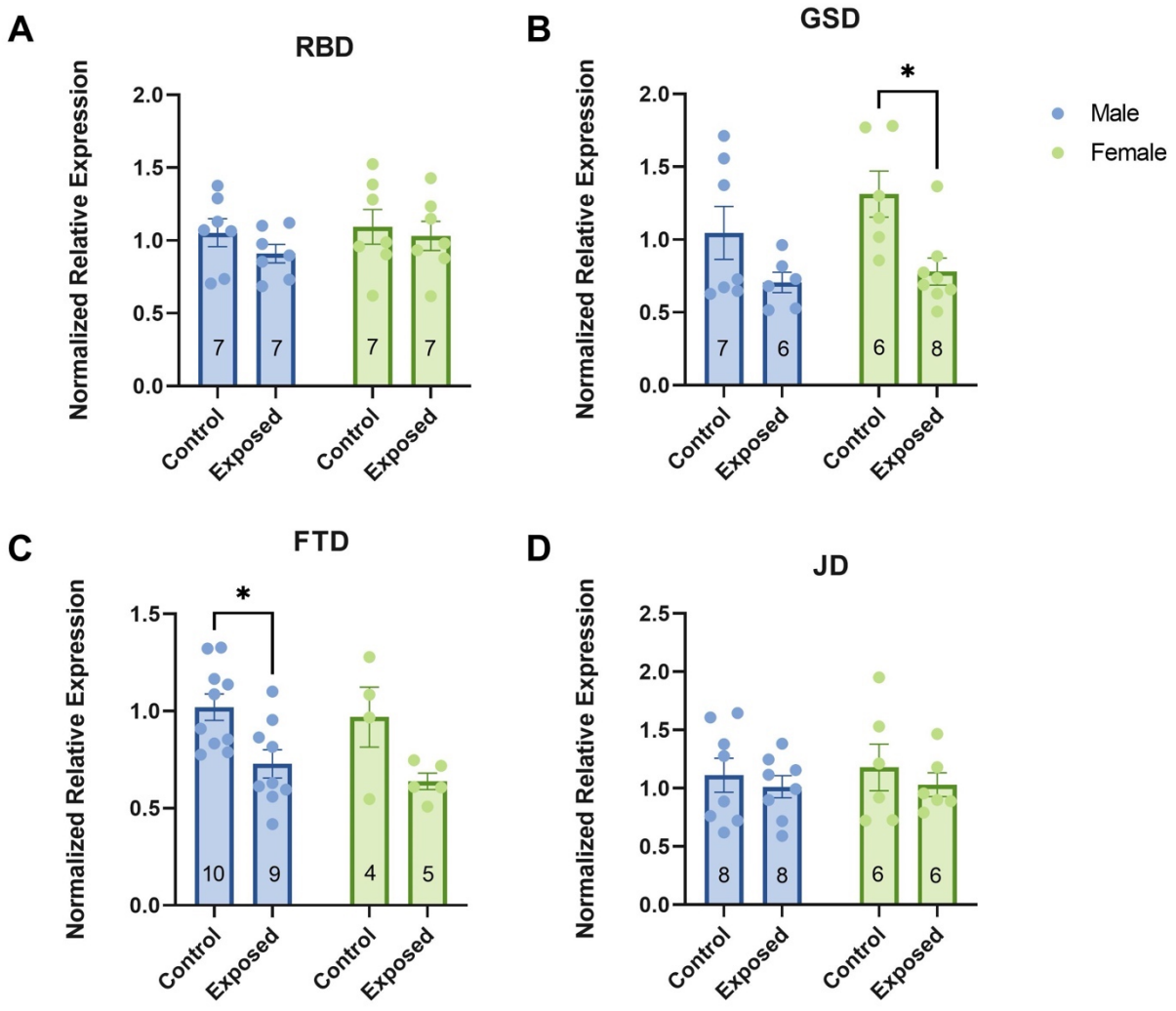

Figure 15: Normalized gene expression of *caspase 9* measured in whole gill tissues of A) rainbow darters (RBD), B) greenside darters (GSD), C) fantail darters (FTD), and D) Johnny darters (JD) exposed to 1.0 µg/L VEN for 96-hours in November 2021. Data is presented as means ± SEM, compared using a Two-way ANOVA and post-hoc Tukey test (* = p < 0.05, n = 14 per treatment per species [exception being GSD 'control' with 13]). Number of males and females in each treatment are represented by the number in each bar.

3.4 Discussion

This study aimed to investigate the specific stressor of pharmaceutical exposure on innate immune system of darters relatively naïve to contaminants by measuring the abundance of innate cytokine transcripts in the gills following an acute exposure to VEN. Subsequent transcriptional analyses revealed moderate impacts of VEN on cytokine expression within the gills. RBD displayed increased *il-6* expression in VEN-exposed females compared to control females and decreased *il-8* in control females compared to control males. Furthermore, VEN-exposed GSD females and VEN-exposed FTD males also demonstrated decreased expressions of *caspase 9* when compared to their control counterparts.

Current studies of pharmaceutical exposure, specifically VEN, on fish have centered around investigating its effects on behavioural and physiological aspects however, the effects of VEN on immunity is not well understood. Although the impact of VEN on the innate immune responses of gills is less understood, there has been some studies examining the effects of other contaminants on the gills such as non-steroidal anti-inflammatory drugs (NSAIDs). In a study by (Hoeger et al., 2005), exposures to 0.5, 5 and 50 µg/L of DCF for 7, 14 and 21 days were carried out on brown trout (*Salmo trutta* f. *fario*). It was observed through immunohistological analyses that trout demonstrated an increase of granulocytes within the primary gill filaments suggesting the presence of inflammatory processes in response to damage of the vascular endothelium (Hoeger et al., 2005). Another study examined the effects of ibuprofen exposure on various immune parameters in juvenile grass carp (*Ctenopharyngodon idella*) (Zhang et al., 2021). Fish exposed to environmentally relevant concentrations of ibuprofen (4.8, 48.0 and 480.0 ng/L) for 14 days, demonstrated decreased serum lysozyme activity and increased expression of the pro-inflammatory cytokines *il-1β* and *tumor necrosis factor alpha* in the gills (Zhang et al., 2021).

Aside from the gills, other immune tissues have also demonstrated impacts on innate immune responses following exposures to insecticide contaminants. Chinook salmon (*Oncorhynchus tshawytscha*) exposed to 1.2, 7.3 and 81mg/L of chlorpyrifos and 0.01, 0.1 and 1mg/L of esfenvalerate, displayed elevated transcription of *il-1β* and *transforming growth factor β* in the kidney (Eder et al., 2009). In this study, VEN exposure was observed to impact *il-6* and *caspase 9* abundance. RBD females demonstrated an increase in the pro-inflammatory cytokine *il-6*, suggesting the presence of an inflammatory response occurring within the gills due to VEN exposure. Additionally, GSD females and FTD males demonstrated decreases in caspase 9, suggesting that cytokine signaling may be impaired. It is important to note that live immune responses are likely to generate large fold increases in cytokine expressions approaching 100- to 1000-fold, depending on the severity of the response as they are essential in regulating and signaling additional immune cells to clear any infection or subsequent damage (Commins et al., 2010; Zou & Secombes, 2016). The fold changes observed in this study were minimal, approximately 2-fold, and therefore, the significant differences observed may not be biologically significant and are not necessarily indicative of an immune response occurring due to VEN.

In conclusion, VEN is frequently detected as the contaminant with one of or the highest concentration downstream in effluent exposed environments (Metcalfe et al., 2010; Hodgson et al., 2020; Gauvreau et al., 2022). Furthermore, fish exposed to VEN have been observed to display significant behavioural and physiological impacts (Painter et al., 2009; Schultz et al., 2011; Melnyk-Lamont et al., 2014; Mehdi et al., 2019). While the findings of this study do not directly indicate an effect of VEN on the immune response, they highlight the evidence of altered innate cytokine expressions in the gills of darters exposed to environmentally relevant concentrations of VEN. Unfortunately, interpretation of these results has limitations due to the experimental design

of this study as there was no true replication. This study took a preliminary approach due to the lack of knowledge around the effects of VEN on the immune system and the challenges presented by field work. Field work can present challenges in obtaining fish and equal sexes, particularly during the COVID-19 pandemic and due to sexing difficulties of species through external characteristics. Therefore, there were not enough fish available to have replicate tanks for each species and for each treatment, which are needed to limit possible tank effects on the obtained results. Future studies should conduct a power analysis to ensure that the appropriate number of fish are captured for the use of replicate tanks and for within/between group comparisons. Furthermore, sampling at several timepoints throughout an exposure would also be beneficial as it would allow for observations of time related changes in gene expression following an exposure to a stressor. Additionally, examining other immune tissues such as the spleen and kidney or conducting a subsequent pathogen challenge can also provide more information of the potential effects of VEN on different immune tissues within the body and on live immune responses.

Chapter 4

General Discussion

4.1 Conclusions

The primary aims of this thesis were to examine the impact of WWTP effluent and pharmaceutical exposure on the immune system of darters (*Etheostoma* spp.) from the Grand River by investigating important innate cytokines through transcriptional analyses. Cytokines are important biomarkers of immune responses as they are produced rapidly by activated immune cells and the effectiveness of the immune system relies heavily on their signaling capabilities (Alejo & Tafalla, 2011). The studies conducted in chapter 2 and chapter 3 of this thesis demonstrated results pertaining to innate cytokine gene expression of darters following effluent exposure and VEN exposure respectively, as well as results between species and sexes. Comparisons between species were not examined as they may be difficult to compare due to species-specific responses and tolerances and would also require much larger sample sizes than those used in these studies. Chapter 2 reported no major evidence of effluent effects on the abundance of innate cytokine transcripts in darters living downstream of the Waterloo WWTP. Although the high concentration of VEN downstream provided a possible explanation of the minor effects observed, a variety of other pharmaceuticals and contaminants were also detected that may have synergistic or additive effects under chronic exposure (Metcalfe et al., 2010; Hodgson et al., 2020). Thus, the minor effects observed cannot be directly related to pharmaceutical exposure without further investigation. Chapter 3 aimed to further that investigation and provided some evidence of the role VEN exposure may have on innate responses of darter species facing frequent exposure in downstream effluent environments. While moderate effects were observed in some species, live immune responses would generate large changes in cytokine expression and since the changes observed were minimal, they may not be indicative of a significant biological effect of an active immune response occurring within the gills of exposed darters.

4.2 Recommendations and future work

Overall, there are several avenues that could further be explored from the findings of this thesis. For instance, the studies carried out in chapters 2 and 3 only provided evidence of changes in innate cytokine expression in darter gills following effluent or pharmaceutical exposure. Further analysis of cytokine abundance following these exposures would be beneficial in other immune tissues such as the spleen and kidney; two additional organs of the immune system that may have more robust effects. Moreover, there are additional endpoints other than cytokine expression that could be measured to provide a greater understanding of the effects of effluent and pharmaceutical exposure on the innate immune response. Phagocytic cells, such as macrophages and neutrophils, are key participants in innate immune responses as they rapidly remove foreign material and produce considerable amounts of cytokines (Grayfer et al., 2018; Mokhtar & Abdelhafez, 2021). Additional studies could examine the number of these phagocytic leukocytes in the blood or immune tissues, along with measuring phagocytic and respiratory burst activity. Enzymatic analyses could also be used to measure lysozyme, an innate antimicrobial enzyme present in neutrophils, macrophages, and mucus (Smith et al., 2019).

There are also several other experiments that can be used in future studies to further investigate the impact of effluent and pharmaceutical exposure on the innate immune responses of darter species. Firstly, a caging experiment could be conducted to further investigate the effects of effluent exposure on the innate immune system of darters. Similar to chapter 3, darters could be collected from WMR, a clean upstream site and naïve to heavy effluent exposure. Upon collection some could then be caged at the collection site in WMR whereas another group could be transported and caged downstream at EIT in the WWTP effluent outfall. After an acute exposure, darters from both sites could be sampled to measure and examine different endpoints, such as the

ones used in this thesis or mentioned above, in key immune tissues. While chapter 2 of this thesis did not observe any impact of effluent exposure on the gills of darters, an experiment such as this would allow researchers to gain a better understanding of how effluent exposure may impact the innate immune responses of fish that are not as accustomed to effluent exposure unlike those that have lived downstream for their entire lifetime. Another experiment could explore the effects of varying concentrations of VEN on the innate immune system. An extension of the experiment conducted in chapter 3 of this thesis could again expose wild-caught darters, naïve to contaminant exposure, to increasing VEN concentrations for either an acute or chronic exposure period. Following exposure, innate cytokine expression along with other endpoints as mentioned previously, could be measured in multiple immune tissues to further investigate the impacts of VEN on the innate immune system. Concentrations of VEN vary between sampling years in downstream effluent-exposed environments as seen in the studies of this thesis and in previous studies (Metcalfe et al., 2010; Hodgson et al., 2020; Gauvreau et al., 2022). As VEN concentrations continue to change, further examining its impact at various concentrations would be imperative for our understanding on how increasing concentrations in the wild may affect the immunity of darters and other local fish populations. Another extension of the previously suggested experiment could also include a pathogen challenge. Following exposure to increasing VEN concentration, darters could be exposed to a bacterial pathogen through either an intraperitoneal injection or other method (i.e., bath exposure). While monitoring incidences of morbidity and mortality, immune tissues of darters could then be sampled at selected time points post infection, to investigate any impact on immune-regulatory genes during an immune response needed situation. As aquatic animals, darters are continuously exposed to their outside environment and it has been shown that there is an abundance of pathogens present in RBD exposed to wastewater effluent (Lobb et al.,

2020). Since many pathogens can infect and kill fishes within days, it important to understand how factors, such as pharmaceutical exposure, will impact their innate immunity which is vital in combating pathogenic infections.

4.3 Significance and impact of research

In summary, Fig. 16 displays the knowledge that was known before this thesis, and how this thesis contributed knowledge of additional effects of effluent and pharmaceutical exposure on the gill immunity of local fish. Examining these effects on the immunity of fish species in the Grand River watershed is critical for several reasons. The watershed currently serves a large population of over 1 million people however, due to urbanization, that population is expected to increase (Hagan et al., 2020). Consequently, significant population growth will produce more wastewater that will ultimately result in greater amounts of WWTP effluent being discharged into the watershed (Hagan et al., 2020). Furthermore, the unexpected circumstances and duration of the COVID-19 pandemic have negatively impacted the mental health of many individuals and since antidepressants are common therapeutic treatments for stress and anxiety, concentrations in aquatic environments are expected to increase in the coming years (Khan et al., 2020; Castillo-Zacarías et al., 2021; Gould et al., 2021). Overall, it is important to further investigate the impacts of effluent and pharmaceutical exposure on immunity as the immune system of teleost fishes is complex and ultimately vital in their defence against toxicants and antigenic threats. While this thesis did not indicate significant biological effects of effluent and VEN exposure on innate immune responses in the gills, it does provide a novel avenue of study illustrating the impact that these stressors may have on other fundamental immune responses of local fish populations in the Grand River watershed.

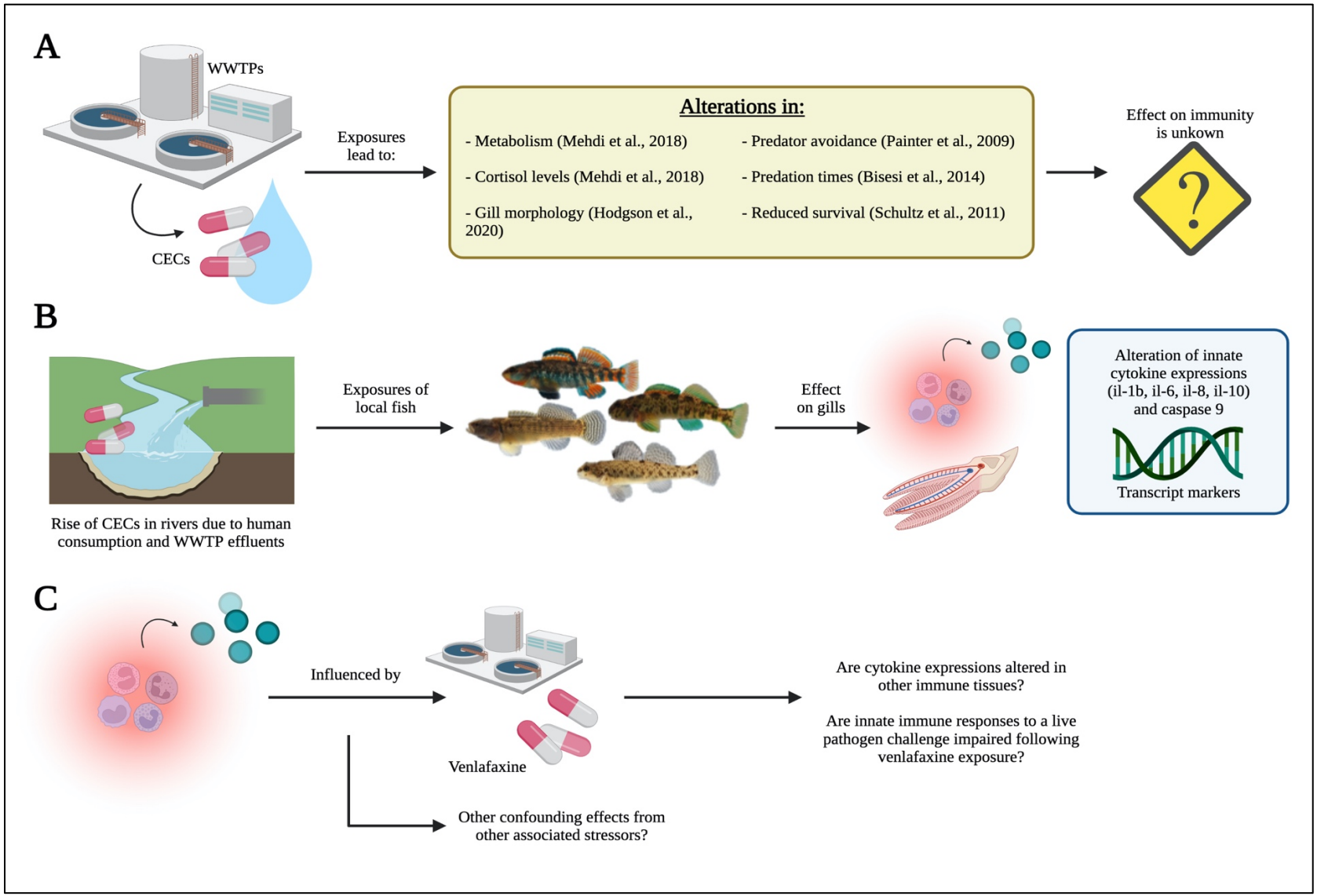

Figure 16: Summary diagram of A) what was known prior to this thesis, B) what this thesis investigated, and C) conclusions and suggested future directions.

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Appendix

Table A1: Morphological indices of rainbow darters (RBD), greenside darters (GSD), fantail darters (FTD), and Johnny darters (JD) collected from upstream (KIW) and downstream (EIT) of the Waterloo WWTP in October 2020. Data is derived from (Gauvreau et al., 2022) as the same fish were collected and analyzed. 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/length3] × 100) are presented in the table and separated based on sex and collection site. Significant differences between upstream and downstream fish were determined by a Welch T-test and are represented by an asterisk (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). One JD male collected from the upstream site was removed as an outlier. Values are presented as mean ± S.E.M.

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

Table A2: Morphological indices of rainbow darters (RBD), greenside darters (GSD), fantail darters (FTD), and Johnny darters (JD) collected further upstream (WMR) of the Waterloo WWTP in November 2021. Measurements of total length (cm), total mass (g), HSI (hepatosomatic index = [liver mass/body mass] × 100), and K (Fulton's condition factor = [body mass/length3] × 100) are presented in the table and separated based on sex and experimental treatment. Values are presented as mean ± S.E.M.

RBD	*Males*		*Females*	
Measurement	Control $n = 7$	VEN exposed $n = 7$	Control $n = 7$	VEN exposed $n = 7$
Total length (cm)	5.24 ± 0.185	5.59 ± 0.184	5.35 ± 0.172	5.67 ± 0.152
Total mass (g)	1.74 ± 0.208	2.20 ± 0.186	1.82 ± 0.243	2.12 ± 0.191
HSI	1.13 ± 0.135	0.82 ± 0.139	1.68 ± 0.143	1.60 ± 0.168
K	1.17 ± 0.034	1.24 ± 0.026	1.15 ± 0.055	1.14 ± 0.027
GSD	*Males*		*Females*	
Measurement	Control $n = 7$	VEN exposed $n = 6$	Control $n = 6$	VEN exposed $n = 8$
Total length (cm)	8.1 ± 0.318	8.25 ± 0.278	7.17 ± 0.131	8.2 ± 0.177
Total mass (g)	6.03 ± 0.624	6.15 ± 0.8	3.92 ± 0.195	5.99 ± 0.359
HSI	1.06 ± 0.098	0.96 ± 0.11	1.37 ± 0.157	1.43 ± 0.085
K	1.11 ± 0.023	1.06 ± 0.037	1.06 ± 0.013	1.08 ± 0.017
FTD	*Males*		*Females*	
Measurement	Control $n = 10$	VEN exposed $n = 9$	Control $n = 4$	VEN exposed $n = 5$
Total length (cm)	5.87 ± 0.161	5.66 ± 0.128	5.08 ± 0.103	5.24 ± 0.214
Total mass (g)	1.84 ± 0.158	1.67 ± 0.092	1.34 ± 0.097	1.34 ± 0.179
HSI	1.08 ± 0.083	0.71 ± 0.115	1.08 ± 0.081	0.99 ± 0.157
K	0.89 ± 0.016	0.92 ± 0.038	1.02 ± 0.017	0.91 ± 0.025
JD	*Males*		*Females*	
Measurement	Control $n = 8$	VEN exposed $n = 8$	Control $n = 6$	VEN exposed $n = 6$
Total length (cm)	4.84 ± 0.288	5.14 ± 0.145	5.2 ± 0.222	5.95 ± 0.262
Total mass (g)	0.99 ± 0.259	1.08 ± 0.073	1.14 ± 0.162	1.80 ± 0.265
HSI	0.59 ± 0.104	0.83 ± 0.144	1.49 ± 0.243	1.36 ± 0.089
K	0.78 ± 0.023	0.80 ± 0.038	0.79 ± 0.02	0.82 ± 0.028