Investigating the impacts of diurnal temperature and venlafaxine exposure in zebrafish

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

Increased intensity and frequency of heat waves is predicted to have detrimental impacts on aquatic organisms. Thermal variability coupled with exposure to xenobiotic stress could place significant strain on the thermal acclimatory capacity and the inflammatory response of some fish species. This thesis aimed to address how known contaminants from wastewater treatment plant (WWTP) effluent coupled with diurnal temperature cycling impact laboratory zebrafish innate immune function and heat shock responses (HSR). In Chapter 2, a pilot study was completed to determine if the coupled exposure to venlafaxine (VFX) and diurnal temperature impacted the inflammatory and HSR in zebrafish (*Danio rerio*). Zebrafish were exposed to three respective temperature patterns: constant 27°C, constant 37°C, and continuously fluctuating between 27-37°C with or without the addition of 1 µg/L of VFX for 96-hours. Following exposure, gill samples were extracted and quantified for immune cytokine, heat shock protein, and hif1α mRNA expression using qPCR. We determined that VFX impacts pro-inflammatory cytokine expression of *tnfα, il-1β,* and *il-8* and modulates a proinflammatory response in the gills when compared to the 27°C control. Gill mRNA increased in *hsp47, hsp70* and *hsp90* expression at diurnal temperatures.

In Chapter 3, we developed a robust tank design to increase experimental replicates and ran a two-week acclimation. Following the acclimation to respective experimental temperatures, we induced an acute heat stress and exposed zebrafish to VFX for 24 h to facilitate a multi-stressor environment. Here, we acclimated zebrafish to three respective temperatures: constant 25°C, constant 35°C and a fluctuating diurnal temperature between 25-35°C (12 h:12 h) for two-weeks. Following acclimation, zebrafish were exposed to an acute heat stress of 35°C and with or without 1 µg/L VFX for 24 h. We compared basal messenger RNA (mRNA) expression at 25°C to exposed zebrafish to quantify differences in several gene transcripts in the brain and gills, specifically examining innate immune cytokines, heat shock proteins, and key glycolytic and mitochondrial enzymes. No changes in cytokine expression were seen in the gills or brain. *Hsp47* and *hsp90* were upregulated in the gills compared to the control, but no changes were seen in *hsp70* expression. In the brain, *hsp70* and *90* were upregulated following acclimation to lower constant

temperature (25°C), but these impacts were attenuated at elevated and diurnal temperatures, suggesting that acclimation may influence the heat shock response in the brain. Our enzymatic analysis revealed that lactate dehydrogenase (LDH) and citrate synthase (CS) were upregulated in the gills, but VFX had no impacts on LDH and CS activity. Likewise, pyruvate kinase (PK) and CS were upregulated in the brain, but VFX did not impact this response. Here we demonstrate that VFX likely does not impact the proinflammatory and enzymatic response in zebrafish. In contrast, we demonstrated that VFX may dysregulate HSP expression, especially *hsp47* and *hsp90*, which may be detrimental to fish species living in watersheds that overlap with their upper thermal limit. Future studies should look at varying timepoints to assess the nature of the HSR to better understand the physiological changes necessary for thermal acclimation in the presence of multiple stressors.

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List of Abbreviations

WWTP—wastewater treatment plants

HSR—heat shock response

HSP—heat shock protein

UHI—urban heat island effect

CEC—contaminants of emerging concern

PCP—personal care products

VFX—venlafaxine

SNRI—serotonin norepinephrine reuptake inhibitor

TNFα—tumor necrosis factor-alpha

IL-1β—interleukin 1 beta

IL-8—interleukin 8

5-HT—serotonin

NE—norepinephrine

SMR—standard metabolic rate

RMR—routine metabolic rate

PAMP/DAMP—pathogen/damage associated molecular pattern

PRR—pattern recognition receptor

HIF1α—hypoxia-inducible factor 1-alpha

CS—citrate synthase

PK—pyruvate kinase

LDH—lactate dehydrogenase

DO—dissolved oxygen

Chapter 1 Introduction

1.1 Temperature

As global temperatures are predicted to rise, warming waters are expected to pose significant physiological stress on aquatic ectotherms (Åsheim et al., 2020; Morgan et al., 2019). Fish are poikilothermic and are therefore dependant on ambient water temperatures for thermal regulation. As such, numerous studies have explored how constant elevated temperatures elicit stress in teleost fish. Although water temperatures play a significant role in aquatic stress, there is little known to date regarding the impacts of fluctuating temperatures.

Climate change models predict a modest increase in global temperatures, with the frequency and intensity of heat waves expected to increase over the next century (NOAA, 2022; Bernhardt et al., 2020). Recent studies propose that temperature variability and daily temperature increases may more significantly impact species performance relative to mean increases in water temperatures proposed by climate change models (Kingsolver & Woods, 2015; Vasseur et al., 2014). Furthermore, considerations need to be made for seasonal variability. Recent studies suggest that during the summer, fish may have more robust hypoxic response, thermal tolerance, and metabolic rate when compared to the winter season (Love & Rees, 2002; Shultz et al., 2016; Tran & Johansen, 2023). Despite our recognition of the proposed impacts of thermal variability, relatively few laboratory studies have bridged the gap of understanding the impacts of diurnal temperatures perturbations, especially in the context of immunity. Previous studies have demonstrated that temperature fluctuations can negatively impact growth in a few fish species (Carveth et al., 2007; Imholt et al., 2011; Prakoso et al., 2021). Increases in standard metabolic rate (SMR) were noted in Atlantic salmon (*Salmo salar*) exposed to higher diel temperatures when compared to constant elevated temperatures (Enders & Boisclair, 2016). In contrast, a protective response on metabolism, heat shock, and hypoxia response was seen in fish reared under diel temperatures (Callaghan et al., 2016; de Alba et al., 2021; Ridgway & Scott, 2023). Taken together, the implications of diel temperatures are inconclusive, and

therefore represent a necessary area of research. This thesis aims to explore how diurnal thermal fluctuations impact zebrafish physiology.

Several abiotic factors are known to influence water temperature, such as stress imposed by anthropogenic effects. Alterations in vegetation density, caused by deforestation and clear cutting alter water temperatures due to decreased availability of shade (Hester & Doyle, 2011; Kalny et al., 2017). Amazonian streams near deforested areas were shown to be up to 6°C warmer than forested streams, resulting in smaller body size of fishes in these areas (Ilha et al., 2018). Perturbations in water flow caused by human interventions, such as irrigation and hydroelectric dams can increase the number of high heat events, particularly if dam water release is from surface waters (Caissie, 2006; Poole & Berman, 2001; Sinokrot et al., 1995). Likewise, the urban heat island (UHI) effect caused by urbanization can lead to changing temperature dynamics in watersheds within proximity (Mohajerani et al., 2017; Somers et al., 2013). Warmed water run-off from thunderstorms can elicit temperature variations from 5-15°C at baseflow due to UHI (Mohajerani et al., 2017; Somers et al., 2013). The thermal environment that a fish lives in directly impacts several aspects of physiology, with large consequences on species abundance, distribution, and population dynamics (Burel et al., 1996; Shaklee et al., 1977; Somero, 2005; Sunday et al., 2012). It is predicted that adverse impacts from anthropogenic stressors coupled with thermal variability will be problematic for fish species, especially those residing in shallow regions with minimal thermal buffering capacity (Morash et al., 2021). Likewise, fish that live in environments that overlap with their critical thermal maximum (CT_{max}) and/or those that have lower thermal acclimation capacity are the most likely to be impacted by warming waters (Madeira et al., 2012). Thus, it is foreseeable that changes in temperature, especially warming, will be one of the most prominent stressors over the next century for aquatic organisms.

1.2 Wastewater Treatment Plants (WWTPs)

Exposure to effluent from primary wastewater treatment plants (WWTPs) is a major contributor of xenobiotic waste within aquatic ecosystems (Fent et al., 2006; Halling-Sørensen et al., 1998). Although

there have been significant improvements made to wastewater treatment facilities, post-treated effluent still contains many contaminants of emerging concern (CECs) (Gauvreau et al., 2022; Hicks et al., 2017; Lajeunesse et al., 2011; Schultz et al., 2011). As a result, effluent exposure contributes to the distribution of environmental pollution through the release of pharmaceuticals, personal care products (PCPs), pesticides, and heavy metals into the aquatic ecosystem (Carey & Migliaccio, 2009; Metcalfe et al., 2010). Effluent exposure has been demonstrated to negatively impact a number of different fish species living within watersheds located near WWTPs (Dawe et al., 2024; Gauvreau et al., 2022; Hodgson et al., 2020; Lau et al., 2021; Luu et al., 2021; Mehdi et al., 2018, 2022).

Only 30-90% of pharmaceuticals are not metabolized fully, and are therefore excreted as a component of post-treated effluent (O'Flynn et al., 2021; OECD, 2019). Venlafaxine (VFX) is a selective norepinephrine reuptake inhibitor (SNRI) class antidepressant that is found at elevated concentrations in surface waters (Metcafe et al., 2010; Gauvreau et al., 2022). SNRIs, like VFX, are one of the primary pharmacotherapies recommended for the treatment of mental illnesses such as depression, anxiety-related disorders, and panic disorders. VFX works by blocking the reuptake of serotonin (5-HT) and norepinephrine (NE) by serotonergic and noradrenergic transporters located on the pre-synaptic cleft. Inhibition results in an increase of extracellular 5-HT and NE, and subsequent changes in brain chemistry (Blier, 2014; Coutens et al., 2022). Like mammals, serotonergic and noradrenergic receptor orthologs have been discovered in fish, and antidepressant exposure has been shown to impact neurotransmitter abundance and changes in gene expression (David et al., 2018; Gould et al., 2021; Melnyk-Lamont et al., 2014; Pei et al., 2016).

Several previous studies have demonstrated that VFX exposure can cause detrimental physiological changes including alternations in metabolism, behaviour, growth, reproduction, development, and heat shock responses in fish (Best et al., 2014; Bisesi et al., 2014; Ikert & Craig, 2020; Mehdi et al., 2019; Parrott & Metcalfe, 2018; Simmons et al., 2017; Thompson & Vijayan, 2020; Weber et al., 2023). However, only few of these studies have considered the impacts of VFX in the presence of multiple stressors (Ikert & Craig, 2020; Mehdi et al., 2019, 2022; Weber et al., 2023). For example, Medhi et al. (2019) saw significant

changes in routine metabolic rate (RMR), metabolically relevant enzymes, and muscle glucose levels in zebrafish, but only in the combined presence of both VFX and a temperature increase. Likewise, fathead minnows exposed to elevated temperature and wastewater effluent showed increased standard O_2 consumption rate and hematocrit, but these changes were not seen at lower temperatures (Mehdi et al., 2022). Weber et al., (2023) observed a dampened *hsp47* transcript response following CT_{max} and VFX exposure, suggesting thermal stress coupled with VFX exposure may negatively impact the heat shock response. These studies highlight how multiple stressor environments may be more indicative of how fish respond in the wild, where numerous factors are contributing to physiological changes. However, like previous studies, different physiological endpoints were measured under constant elevated temperatures and therefore do not reflect natural fluctuations in water temperatures that would be seen in nature (Fig. 1). Determining how fluctuating temperatures in combination with contaminant exposure impact fish is critical to comparing laboratory experimentation to field-based exposures, as this reflects the conditions fish may typically be exposed to in their natural habitats.

Considering the recent COVID-19 pandemic, increases in serotonergic and noradrenergic drugs have been reported in some countries; VFX consumption rate significantly increased from ~72.2 mg/1000 people/day in 2019 to ~135 mg/1000 people/day in Turkey in 2020 (Yavuz-Guzel et al., 2022). Likewise, Australia reported a 15% increase in dispensing of antidepressants in 2020 (Tscharke et al., 2022; Yavuz-Guzel et al., 2022).

A positive correlation exists between the rates of chronic diseases, such as mental health disorders, and the occurrence of natural disasters (O'Flynn et al., 2021). As climate change evolves at unprecedented rates, incidences of natural disasters are expected to rise (Smith et al., 2022). As such, it is plausible that antidepressant prescriptions could increase in coming years, leading to further physiological impacts in fish. Although many studies have addressed the impacts of environmental contaminants in fish, there still is a gap in knowledge of how multiple stressor environments impact fish physiology.

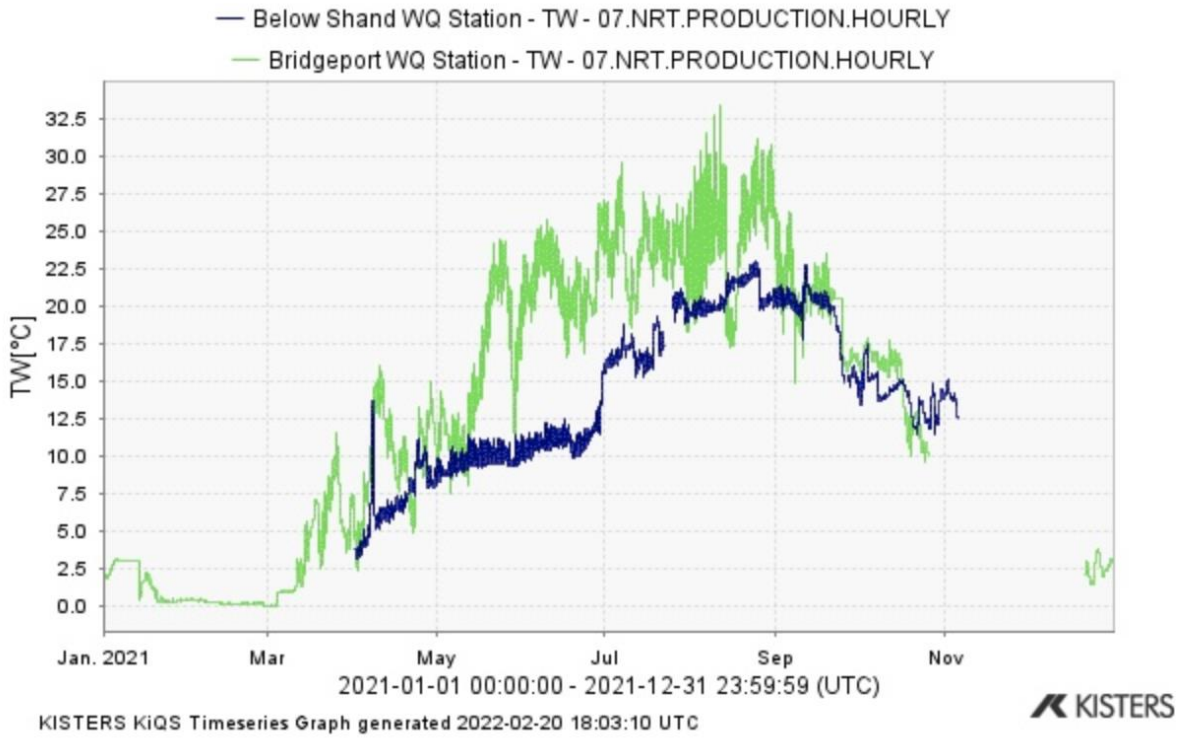

Figure 0.1 2021 monthly temperature readings in the Grand River watershed.

Water temperatures in the Grand River, located in southwestern Ontario, Canada for the months of January to December of 2021 (https://data.grandriver.ca/downloads-monitoring.html). The blue line represents the Shand Dam, and green is Bridgeport station.

1.3 The Immune System

The vertebrate immune system can be divided into two specialized subcomponents: the innate and adaptive immune systems. The innate immune system is considered the first line of defense against infection and damage, while the adaptive response is specialized and involved in long-term immunological memory (Secombes et al., 2001; Smith et al., 2019; Uribe et al., 2011). Since fish lack a specialized lymphatic system, the adaptive immune response is typically slower and less robust than the innate immune response (Elumalai et al., 2019; Semple & Dixon, 2020). For this reason, the adaptive immune response was not a focus of this study due to the short time frame of exposure to VFX (96 h or 24 h; Ch 2 & 3, respectively).

This thesis focuses on the occurrence of inflammation in the gills and the brain. The gills are integral for the maintenance of osmotic balance and gas exchange and play an important role as a physical and biochemical barrier against pathogens (Smith et al., 2019). Additionally, the gills are the primary site of entry for many pollutants and pathogens; thus, the gills are a major contributor to immune and metabolic homeostasis in teleosts (Collet, 2014; Jiao et al., 2019). Exploring how diurnal temperatures and antidepressant exposures impact the innate immune system and inflammation in the gill tissue of fish provides insight into whether the combined effects of multiple stressors damage the structural integrity of the gills and elicit an immune-related response.

The brain is not considered a primary immunological organ. However, monoamine compounds, such as 5-HT and NE, can pass through the blood-brain barrier of fish (Khan & Deschaux, 1997). In mammalian models, 5-HT can regulate inflammation by interacting with 5-HT receptors on immune cells and proinflammatory cytokines have been implicated in the occurrence of neuroinflammation (Herr et al., 2017; Roumier et al., 2019; Vezzani et al., 2019). In fish, there is evidence that 5-HT impacts immune cell proliferation and responses in rainbow trout and bluegill sunfish (Duffy-Whritenour & Zelikoff, 2008; Ferriere et al., 1996, 1999). Conversely, in Nile tilapia, few serotonergic biomarkers were found to be present on immune cells (Li et al., 2022). These conflicting results highlight the gap in knowledge

surrounding our understanding of the link between antidepressant exposure and CNS-mediated immunity in fish.

1.3.1 The Innate Immune Response

The innate immune system is responsible for the recognition of non-self-molecules and can be divided into various subcomponents, including physical barriers, cellular and humoral constituents (Magnadóttir, 2006). Following infection or damage, recognition is modulated by the germline-encoded pattern recognition receptors (PRRs), which bind to pathogen-associated or damage-associated molecular patterns (PAMPs; DAMPs) on the cellular surfaces (Alinejad, 2020). These highly conserved molecules include polysaccharides, lipopolysaccharides (LPS), peptidoglycans, bacterial DNA, and double-stranded RNA and are considered foreign to the host (Lieschke & Trede, 2009; Magnadóttir, 2006). Recognition of these molecules is regulated by type I transmembrane proteins, known as, toll-like receptors (TLRs), which initiate the expression of phagocytes, such as macrophages, lymphocytes, granulocytes, and other immune cells (L. Zhu et al., 2013). This leads to the secretion of cytokines and chemokines, which are necessary for immunomodulation and recruitment of immune cells to the infected or damaged tissue (Commins et al., 2010; L. Zhu et al., 2013).

Cytokines are a family of small glycoproteins that regulate inflammation, cellular movements, and immune responses (Semple and Dixon, 2020). A few important fish pro-inflammatory cytokines include *il-1β*, and *tnfα*, which play a role in propagating an inflammatory response by mitigating tissue damage caused by infection and/or injury (Commins et al., 2010; Semple & Dixon, 2020). *Il-8* is a chemokine that modulates chemotaxis by recruiting more macrophages to a site of infection (Commins et al., 2010). Preliminary evidence from our lab suggests that VFX induces a proinflammatory response through the upregulation of *il-1β*, *il-8* and *tnfα* in the zebrafish gills (Dawe and Craig, unpublished data). Additionally, VFX exposure causes dysregulation of immune transcripts *il-1β, il-6,* and *caspase9*, and this response is different between sexes, location, and species of darters (*Etheostomatinae*; Dawe et al., 2024)

One of the most important innate immune cells are macrophages, which are equipped with PRRs and can recognize and bind to PAMPs, initiating phagocytosis (Semple and Dixon, 2020). Like in mammalian models, two distinct populations of macrophages exist in teleosts: M1—leads to the production of proinflammatory cytokines, tnfα and the production of reactive oxygen and nitrogen species; and M2—supports the induction of anti-inflammatory cytokines, such as il-10 (Bavia et al., 2022; Hodgkinson et al., 2015). Other important cells include neutrophils, basophils, and eosinophils, which are armed with enzymes and peptides that support the immune response (Semple and Dixon, 2020). Monocytes are distributed throughout the bloodstream and support teleost immunity by contributing to the inflammatory response and homeostasis (Semple & Dixon, 2020).

Like mammals, teleosts possess non-specific cytotoxic cells which modulate pathogen destruction through the necrotic or apoptotic pathway (Abram et al., 2017). Necrosis leads to an inflammatory response, while apoptotic cellular destruction is propagated by the production of reactive oxygen species (ROS) as an oxidative burst or lysosomes present within phagocytotic cells (Yang et al., 2013). When a pathogen is engulfed, destruction is modulated by the production of reactive oxygen intermediates, which are catalyzed to produce hydrogen peroxide, hydroxyl radicals, and hypochlorous acid (Semple and Dixon, 2020). However, this pathway is not strongly impacted by temperature and therefore will not be the focus of this thesis (Semple and Dixon, 2020).

In teleosts, the complement system bridges the gap between the innate and adaptive immune systems (Bavia et al., 2022). It is composed of circulating proteins and those bound to the cell surface, and include three primary activating pathways: the classical, alternative, and lectin (Bavia et al., 2022). The classical pathway is modulated through the binding of plasma membrane-bound protein C1 to pathogen-bound antibodies, like immunoglobin M (IgM) (Zhang & Cui, 2014). The alternative pathway acts independently of antibodies and is activated through the direct binding of viruses, bacteria, fungi, or host cells, such as tumour cells (Bavia et al., 2022). Last, the lectin pathway is initiated through the binding of collectins and ficolins to carbohydrate residues present on the microbial surface (Bavia et al., 2022). Collectively, the complement pathways lead to the downstream activation of innate and adaptive immune

responses, including opsonization, cellular respiratory burst, clearing apoptotic and necrotic cell debris, and inflammatory responses (Bavia et al., 2022; Boshra et al., 2006; Zhang & Cui, 2014).

1.4 Heat Shock Proteins

The impacts of temperature changes on fish physiology have been extensively studied in recent years. Temperature and light are important external factors that play a role in circadian rhythm (López-Olmeda et al., 2009). Heat stress can cause stunted growth, reproductive complications and even death in teleosts (Mohanty et al., 2018). Heat shock proteins (HSPs) are responsible for modulating the folding of proteins and play a critical role in the organism's response to stress (Madeira et al., 2020; Mohanty et al., 2018). Furthermore, HSPs are regarded as useful biomarkers for analyzing thermal stress in fish (Tedeschi et al., 2015). The heat shock response demonstrates high acclimation plasticity following repeated thermal stress, further implicating their importance in an organisms ability to respond to temperature perturbations (Mitra et al., 2018; Sessions et al., 2021). Several studies have implicated HSPs in the physiological responses seen following contaminant exposure (Guo et al., 2018; Luu et al., 2021; Weber et al., 2023). Luu et al., (2021) provide some evidence that VFX may be responsible for the expression of *hsp70* and *hsp90* in the liver tissue of zebrafish. It is known that heat stress also induces the expression of these HSPs, thus we expect HSP expression may be impacted by diurnal temperature fluctuations.

HSPs contribute to the immune response by acting as intracellular signalling molecules to induce the expression of proinflammatory cytokines and adhesins (Roberts et al., 2010). Particularly, HSP70, is responsible for the regulation of the mitochondrial-dependent, death-receptor-mediated, and apoptosis pathways when teleosts are subject to thermal stress (Mohanty et al., 2018). Several cytokines can be induced by HSPs, including tnfα, il-1β, and -12 (Xie et al., 2015). In channel catfish, *Flavobacterium columnare* infection resulted in the upregulation of *hsp90* mRNA in the gills, suggesting that *hsp90* may play a role in disease defence against bacterial infection (Xie et al., 2015). Similarly, He et al., (2016) demonstrated that following pathogenic infection with *Vibrio alginolyticus*, *hsp70* and *hsp 90* mRNA was upregulated in the liver of juvenile *Larimichthys crocea (L. crocea)*. Investigating how xenobiotic exposure

and temperature changes exacerbate pathologically induced expression of HSPs will provide insight into how environmentally relevant stressors may impact HSP-induced immune function.

1.5 Hypoxia-Inducible Factors

Aquatic organisms are particularly vulnerable to hypoxia, due to changes in oxygen availability caused by climactic warming, inefficient water turnover, daily rhythmic cycles, and seasonal variability (Diaz & Rosenberg, 2008; Domenici et al., 2017; Jenny et al., 2016; Pelster & Egg, 2018). Fish possess robust physiological mechanisms aimed at maintaining metabolic homeostasis during routine bouts of hypoxia, including alternations in behaviour, gill morphology, ventilation, O_2 binding affinity, and ATP production (Borowiec et al., 2016, 2018; Borowiec & Scott, 2021; Domenici et al., 2017; Heinrichs-Caldas et al., 2023; Richards, 2009; Scott et al., 2017; Sollid et al., 2003; Weber et al., 2016). Hypoxia-inducible factor alpha (Hif1α) is known to be a key modulator of the hypoxic response, playing a role in oxygen sensing, and metabolic alterations (Choudhry & Harris, 2018). Under low oxygen conditions, Hifs can stimulate the expression of genes and proteins involved in glycolysis and inhibit oxygen-dependent pathways as a means of maintaining metabolic homeostasis (Fuhrmann & Brüne, 2017). This process is stimulated by the stabilization and upregulation of Hif1α-subunits and subsequent dimerization with Hif1β-unit to induce the expression of hypoxia-related genes (Burtscher et al., 2021). In contrast to mammalian models, fish Hif mRNAs provide reliable analysis of hypoxic-signaling and are considered biomarkers for hypoxia-induced stress (Pelster & Egg, 2018). Additionally, immune cells often encounter hypoxic environments and thus Hifs play an active role in regulating inflammation and aspects of innate immunity (Taylor & Colgan, 2017). More specifically, Hifs play a role in neutrophil proliferation, inflammatory gene expression, and participate in the metabolic shift to glycolysis during an immune response (Kelly & O'Neill, 2015; Palazon et al., 2014) The current thesis assesses the impacts of acute elevated and diurnal temperatures, close to the thermal maximum of zebrafish (Morgan et al., 2019). Thus, it is expected that lower dissolved oxygen concentrations at these temperatures, possibly leading to alterations in Hif mRNA expression.

1.6 Enzymatic Activity and Energy Metabolism

As discussed above, fish are well-equipped with robust physiological mechanisms to support cellular homeostasis and ATP supply. However, several ecologically relevant stressors have been shown to dysregulate metabolism, ultimately having repercussions on metabolic function. A few key enzymes can be used to understand and monitor metabolic alterations. Citrate synthase (CS) is considered a biomarker for oxidative stress due to its role in the citric acid cycle and ATP production (Zak et al., 2017). Likewise, glycolytic enzymes, pyruvate kinase (PK) and lactate dehydrogenase (LDH) can be indicators environmental stressors such as thermal stress, pollution, and immune stress (Alves et al., 2022; McClelland et al., 2006; Mehdi et al., 2018).

Differences in environmental temperatures can induce numerous alterations in metabolic function at molecular, cellular, and whole-body levels to sustain the energy requirements needed for proper physiologic functioning (Sandblom et al., 2014; Schulte, 2015). Specifically, acclimation responses to deviations in temperature can include changes to enzyme activity, through regulatory responses like changes in substrate concentration and relative enzyme quantities, or metabolic reorganization through shifts in pathway importance and reliance (Shaklee et al., 1977; Somero, 1978). Water temperature has been shown to alter energy metabolism, including changes in metabolic enzymes, metabolic rate, and glycolytic activity in fish (Eme et al., 2018; Firth et al., 2023; Lau et al., 2021; McClelland et al., 2006; Mehdi et al., 2018, 2019). Temperature alone can cause adverse impacts on metabolism, but few studies have addressed the impacts of diel temperatures on metabolic activity.

Environmental pollutants have also been shown to impact fish metabolic responses. Increases in temperature can also increase the toxicity of environmental pollutants (Little et al., 2020; Little & Seebacher, 2015). Previous studies have demonstrated changes in metabolic, enzymatic, and glucose metabolism in the presence of various environmental contaminants (Best et al., 2014; Craig et al., 2007; Duan et al., 2018; Gagnon & Holdway, 1999; Melvin et al., 2013; Mennigen et al., 2010; Ransberry et al., 2016). A recent study found that effluent exposure can impact the antioxidant response in the brains of

four darter species (Gauvreau et al., 2022). The brain has a limited ability for anerobic metabolism and upper thermal tolerance limits have been suggested to be connected to temperature-mediated brain dysfunction (Andreassen et al., 2022). Given that contaminant exposure may impact oxidative stress responses in the brain (Gauvreau et al., 2022), we aimed to assess the enzymatic impacts in the presence of contaminants and diurnal temperature exposure. Likewise, gills are integral to oxygen uptake and metabolism, thus this thesis aims to investigate how multiple stressor environments impact gill enzymatic activity.

1.7 Study Species

The zebrafish (*Danio rerio)* is a tropical freshwater fish native to the Ganges River and its tributaries located in Northern India (Teame et al., 2019). The benefits of using zebrafish include the short generation time, external fertilization, and the large number of eggs produced during reproduction (Briggs, 2002). The zebrafish is considered a model organism when examining development, immunity, behaviour, genetics, and physiology (Teame et al., 2019). Previous studies in zebrafish demonstrate that RMR, microRNA expression and transcriptional expression increase in response to increased temperature and VFX exposure (Bennoit & Craig, 2020; Ikert & Craig, 2020; Mehdi et al., 2019). Moreover, Bennoit and Craig (2020) demonstrated that heat-killed *Vibrio anguillarum* and exposure to a temperature increase (+5.5°C) resulted in a 30% increase in RMR. Similarly, a 5°C temperature increase and exposure to 1ug/L VFX elicited the downregulation of miR-22b-3p and miR-301a microRNAs in the zebrafish gonad tissue (Ikert & Craig, 2020). These laboratory studies suggest that to some degree VFX impacts metabolically relevant transcripts and increased temperature in part may enhance this response. Using diurnal temperatures changes may mimic more natural water temperature changes seen in the wild. Furthermore, the approach of combining wastewater contaminants with diurnal temperature fluctuations has yet to be explored and represents a new direction in understanding multiple stressors impacts on fish physiology.

1.8 Research Objectives

1.8.1 Objectives

The objective of this thesis was to determine if VFX exposure altered relevant immune transcripts and assess how static and fluctuating temperatures may have impacted this response. In Chapter 2, a pilot study was performed to characterize the inflammatory response of zebrafish gill tissue, following a 96 h exposure to static and diurnal temperature fluctuations with or without 1 μg/L of VFX. Following this, a more robust experimental protocol was designed for Chapter 3, and fish were acclimated to respective static and diurnal temperatures for two-weeks. The two-week acclimation was followed by a 24 h heat exposure with or without 1 μg/L of VFX to determine if inflammatory cytokines and metabolically relevant enzymes were differentially regulated in the presence of multiple stressors. Overall, our goal was to assess a novel avenue of research pertaining to climate change stressors and contaminant exposure and their impacts on fish immunity and physiology.

1.8.2 Hypothesis

Previous studies have demonstrated that VFX impacts a range of different physiological functions, therefore it was hypothesized that VFX-exposed zebrafish will have increased cytokine and heat shock protein transcript expression. Furthermore, fish exposed to diurnal temperatures will have different enzymatic and gene expression responses when compared to zebrafish exposed to static conditions. We suspect an exacerbated cytokine response in fish exposed to VFX and higher temperatures, as this would corroborate previous studies. Lastly, HIF expression will be elevated at high and diurnal temperatures, and that VFX-treated fish will exhibit increased HIF levels relative to control fish.

1.8.3 Thesis Format

The subsequent chapters of this thesis are divided into distinct methods and results (Chapters 2, and 3) followed by a discussion (Chapter 4) and synthesis of the data. In Chapter 2, I performed a

preliminary pilot experiment to build upon our initial evidence that VFX can cause inflammation but added in an additional temperature stress by investigating the impacts of diurnal temperature fluctuations. Briefly I exposed zebrafish to either static or fluctuating temperatures with or without 1 μg/L of VFX for 96 h. I assessed molecular endpoints pertaining to the inflammatory and heat shock response in the gills, which helped inform our experimental design for Chapter 3. In Chapter 3, I redesigned my experimental approach into a randomized tank-based design for increased statistical power and used state-of-the-art equipment to regulate diurnal temperature fluctuations (Loligo, Denmark). Additionally, I exposed zebrafish to either static or diurnal fluctuating temperatures for two-weeks, then exposed fish to 24 h of a heat stress with or without 1 μg/L VFX. I assessed molecular endpoints of exposure in the gills and brain, which included relative abundances of immune, and heat shock transcripts. I further assessed enzymatic activity of key metabolic markers to determine if glycolytic and mitochondrial metabolism was impacted by multiple stressor environments. Taken together, this thesis aims to determine how fluctuating diurnal temperatures and VFX exposure impact zebrafish immune response and metabolism.

Chapter 2 Pilot Study

1.9 Methods

1.9.1 Zebrafish Care

Adult wild-type pet store zebrafish of mixed sex were obtained from Big Al's Canada (Kitchener, Waterloo). One-hundred mixed-sex fish were housed in 30 L glass aquaria (stocking density (SD) =3.33 fish/L). Fish tanks were supplied with a bubbler connected to O_2 supply, Aquaclear 10/20 Power Filter with filter foam and activated charcoal media, and a heater. The fish were held at 27°C, pH of 7.5, on a 12 h:12 h light: dark cycle, and fed ground Gemma Micro Zebrafish Diet 300 (Skretting, Westbrook, Maine, USA) once daily until exposure period. All experiments performed in this study were reviewed and approved by the Canadian Council of Animal Care and were fulfilled under the guides set by the University of Waterloo Animal Care Committee (AUP# 40989).

1.9.2 Experimental Design

Three 15 L tanks were used and supplied with an Aquaclear 20 filter, filter sponge and activated charcoal media, bubbling oxygen, temperature/ dissolved oxygen (DO) probes, and stainless-steel heat exchanger. A 1686A Peltier Heat Pump system (Qubit Biology, Kingston, ON) was connected to an Eheim Universal 600 Water Pump (120V; Deizisau, Germany), which was connected to the stainless-steel heat exchanger placed in each aquarium via clear plastic tubing (Fig 2.1). A BK Precision DC Regulated power supply (1686 Amp (A)) and the A447 Digital Thermometer (Qubit Biology, Kingston, ON) were connected to the 1686A Peltier Heat Pump System (Fig 2.1). When the BK Precision power supply is set to a low voltage, water is drawn in through the condenser then the evaporator causing cooling of the system. Alternatively, at a high voltage, water drawn up through the evaporator then passed through a condenser, causing heating of the system. Additionally, an A447 Digital Thermometer (Qubit Biology, Kingston, ON) with a temperature probe was connected to the 1686A Peltier Heat Pump to control and monitor each respective temperature. Additional temperature/DO probes were placed in each tank and connected to a

computer to record the temperature and DO content using LoggerPro Software application over 96 h (Fig 2.1). Two additional 30 L tanks were used as reservoir tanks for experimental water changes, one set to 27°C and 37°C.

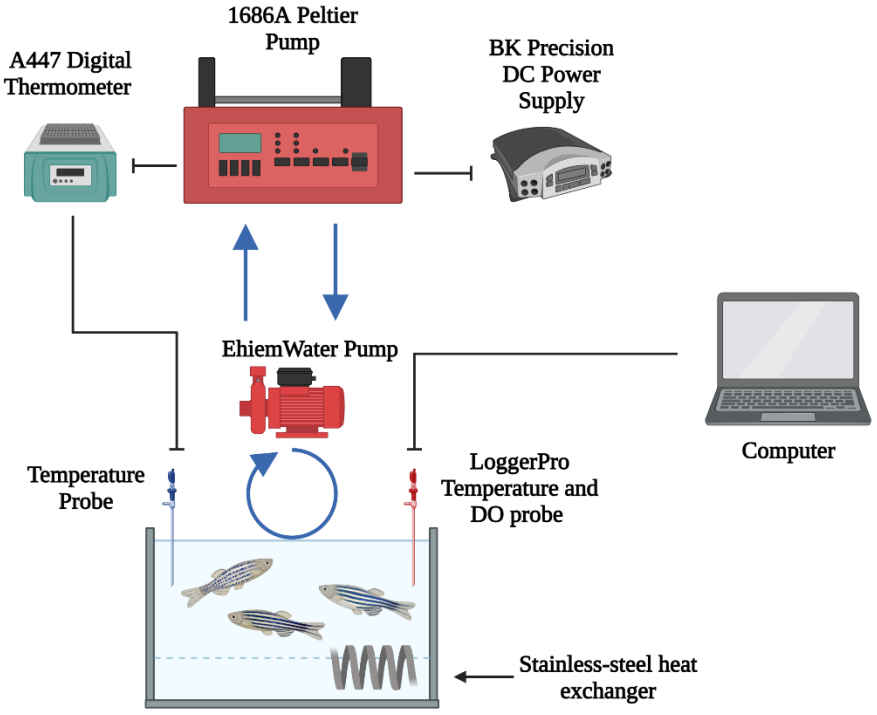

Figure 0.1. Schematic of individual in-lab tank design for 96-hour exposure.

Adult, male and female zebrafish were exposed to different thermal stressors with or without the addition of VFX (1μg/L) for 96 h. A maximum of three tanks could be run simultaneously, so the experiment was adapted to be carried out in two separate trials over two weeks. Week one consisted of setting up control and temperature tanks, while week two consisted of VFX exposure tanks. Seventy-two zebrafish were divided evenly (n=12 per treatment group; stocking density= 0.8 fish/L) and exposed to one of six treatments: **Week One:** i) 0 μg/L VFX at 27°C (*27°C Control*), ii) 0 μg/L VFX at 37°C (*37°C Temp.)*, *iii)* 0 μg/L VFX with a diurnal fluctuation from 27-37°C *(*12 h:12 h; *27-37°C Temp*.); **Week Two:** iv) 1 μg/L VFX at 27°C (*27° VFX*), v) 1 μg/L at 37°C (*37°C VFX*), and vi) 1 μg/L with a diurnal fluctuation from 27-37°C (12 h:12 h; *27-37°C VFX;* Table 2.1). Experimental treatments will be referred to as *Control, Temp.,* and *Temp.+VFX*. Each day pH, nitrite, nitrate, and ammonium levels were measured one hour after feeding using the Freshwater Master Test Kit (API, Chalfont, Pennsylvania, USA). Note that fish in 27-37°C groups were sampled at 27°C. Fish were fed Gemma Micro Zebrafish Diet 300 (Skretting, Westbrook, Maine, USA) once daily at 8AM until satisfied, and feeding was ceased 24 h prior to euthanasia.

Week One: Twelve zebrafish (26 Males: 9 Females; 36 total) were placed in three 15 L glass aquaria. To ensure stress response was not from handling, fish in all experimental tanks were housed in respective glass tanks for 72 h at 27°C prior to exposure. On *Day 0* at 8AM, LoggerPro software was launched to begin recording experimental data and all filter media was removed; only the O_2 bubbler, empty filter, circulating pump, and heat exchanger remained in each tank to maintain oxygen supply and water turbidity. Temperatures were manually maintained by changing the voltage on the BK Precision DC Regulated Power Supply (Fig 2.1). The temperature setpoint was controlled by the A447 Digital Thermometer (Fig. 2.1), and the *Control* was set to 27°C at 2.5V (V=volts) and the *37°C Temp.* group was set to 37°C (7.5V) for the duration of the exposure. To facilitate fluctuations for the *27-37°C Temp*. group, at 8AM the temperature setpoint was changed to 37°C at 7.5V for daily warming, and at 8PM was set to 27°C at 2.5V for nightly cooling (Fig 2.1). These manual temperature changes were repeated daily at 8AM/PM to maintain consistent temperature cycling. Temperature fluctuations were chosen to mimic

natural warming and cooling cycles that may be seen during the day and at night in the wild. It should be noted that the chosen temperature range (a diurnal fluctuation of 10°C) is more likely to be representative of summer diurnal temperature fluctuations in temperate regions as opposed to wintertime water temperatures.

A 50% water change was performed each day 1 h after feeding on *Days 1-3* to reduce nitrogenous and organic waste. To minimize changes in water temperatures in experimental tanks during water changes, two reservoir tanks, set to 27°C and 37°C, were used to refill experimental tanks. One mortality was recorded in the *27-37°C Temp.* group as the fish was missing during dissections, but no body was found in the tank or equipment and cause and time of death was unknown. At 96 h fish were euthanized via overdose of MS-222 (0.5 g/L; Millipore-Sigma-Aldrich, Oakville, ON, Canada). The length and weights of fish were recorded, and gill tissues were sampled, flash frozen in liquid nitrogen, and stored in the -80°C for molecular analysis. Glass aquaria and equipment were bleach washed and reset for *Week Two* exposure.

Week Two: Twelve male zebrafish were placed in each 15 L glass aquaria at 27°C to acclimate for 72 h (36 fish total; SD=0.8 fish/L). To facilitate a multiple stressor environment, the same procedure outlined above was followed, with one deviation. Zebrafish were exposed to 1 μg/L of VFX following a similar protocol as outlined by Mehdi et al. (2019). Briefly, VFX aliquots were made by diluting racemic VFX in nuclease-free water and stored in the -20°C freezer (Millipore-Sigma-Aldrich, Oakville, ON, Canada). On *Day 0*, 75 μL of thawed racemic VFX (1 μg/L) was added to all *Temp.+VFX* tanks. On *Days 1-3*, a 50% water change was performed one hour after feeding, followed by the re-addition of 35 μL of VFX stock solution (50%) to maintain aim concentration of 1 μg/L of VFX. VFX concentration was chosen based on previous studies which show that VFX can be found at 1 μg/L in surface waters and can impact transcript expression (Weber et al., 2023; Gauvreau et al., 2022; Metcafe et al., 2010). Two mortalities were recorded, one in the *37°C VFX* group on *Day 3* and one in the *27-37°C VFX* group. The latter was discovered to be missing during dissections and no body was recovered, thus time and cause of death is unknown.

Table 0.1 Experimental treatment groups.

Treatment groups and respective temperatures and concentrations of VFX were added in week one and week two of the 96 h exposure (Y=yes and N=no).

Group	Temperature	VFX (Y/N)
	Week One	
Control	27	N
Temp.	37	N
Temp.	27-37	N
	Week Two	
Temp.+VFX	27	Y
Temp.+VFX	37	Y
Temp.+VFX	27-37	Y

1.1.1 Temperature Analysis

To determine the rate of change in temperature for the 27-37°C groups, temperature data was isolated by time points that correspond to cooling and heating cycles. Heating occurred at times 0-11, 24-35, 48-59, and 72-83 h, while cooling occurred during times 12-23, 36-47,60-71, and 84-96 h, respectively. The slope of the line was determined for each timeframe using $m_n(t)=(y_2-y_1)/(x_2-x_1)$, where y represents the temperature in Celsius, x represents the time in hours, and $m_n(t)$ represents the slope at some time (t). The average slope was then calculated for heating and cooling independently. It should be noted this is an approximate rate of change, as no line of best fit could be calculated for the sinusoidal curve.

1.1.2 Molecular Analysis

Frozen gill tissue was used for RNA extractions using TRIzol reagent (T3934, Sigma-Aldrich) as outlined in Craig et al. (2013). For 100 mg of gill tissue, samples were homogenized with 1 mL TRIzol reagent using the OMNI Tissue Homogenizer TH (OMNI International, Kenneaw, USA) for 30 s. Following a five-minute room temperature (RT) incubation, 0.5 mL of chloroform was added, and samples were shaken vigorously for 15 s to combine. Samples were incubated at RT for 3 min and spun down for 15 min using the Eppendorf® Centrifuge 5810/5810R (12000xg, 4°C; Eppendorf Canada Ltd., Mississauga, ON). Samples were precipitated with 1 mL of 100% isopropyl alcohol and spun down centrifuged for 10 min (12000xg at 4°C). Next, 1 mL of 75% ice-cold EtOH was added, vortexed for 15 s and centrifuged at 7500xg at 4ºC for 5 min and repeated once more. EtOH was decanted, then pulse spun for 10-15 s before carefully vacuuming the remaining EtOH from the sample using the ISS-Integrated Suction System (Oroborous Instruments, 20810-02). The samples were air-dried on ice for 5 min and reconstituted with 20 µL of nuclease-free water and incubated for 10 min at 55ºC. RNA concentration and purity (280:260µm and 260:230µm) was tested in duplicates using the SpectraMax 190 Molecular Device (San Jose, California) following the SpectraDrop Abs DNA Quant protocol prior to RNA sample storage in the -80ºC freezer.

cDNA analysis was completed using the Qiagen QuantiTech Reverse Transcription Kit (205311; Hilden, North-Rhine Westphalia, Germany) and instructions were followed to manufactures guidelines, with no deviations. RNA was thawed on ice and using the SpectraMax 190 Molecular Devices (San Francisco, California) 2 µL of thawed sample was loaded in triplicates to determine the average concentration of each sample. To mitigate variability between samples, samples were adjusted to 500 ng and samples that exceeded 500 ng/L were diluted. To dilute samples, 10 µL of sample was added to a new microfuge tube, diluted 10x number of times with nuclease-free water (W4502-1L; Sigma-Aldrich) and subtracted 10 µL to account for sample amount (dilution= 10x-10= 10(x-1); x represents the number of dilutions necessary for <500 ng/L). Samples were incubated using the Bio-Rad T100 Thermal Cycler (Bio-Rad Laboratories Inc.) and placed on ice after. Following incubation, samples were reconstituted with 80 µL of nuclease-free water (W4502-1L; Sigma-Aldrich). A 5-point dilution curve was made with the pooled RNA sample. A 4x dilution was prepared by adding 15 uL of pooled sample cDNA to 45 uL of nuclease-free water (W4502-1L; Sigma-Aldrich). cDNA samples and dilution series were stored at -20°C for RT-qPCR.

Primers were designed using NCBI Primer-Blast and primers were set to span exon to exon. Primers and accession numbers are reported in Table 2.2. New primers were ordered, and variable amounts of nuclease-free water were added as outlined by manufacture for a primer concentration of 100 µg/L. Reconstituted primers were aliquoted by added 30 µL of primer to 570 µL of nuclease-free water to create 5 mM forward and reverse primers for RT-qPCR analysis.

The expressions of *il-1β, il-8, tnfα, hsp47, hsp70*, *hsp90, hif1α,* and *hifαβ* were quantified via RT-qPCR analysis using the SsoAdvanced Universal SYBR Green Supermix Kit (1725271; Bio-Rad Laboratories Inc.). Samples were prepared with 1 µL of 5 µM forward and reverse primer, 1 µL of nuclease-free water, 5 µL of SYBR Green, and 2 µL of diluted cDNA. qPCR plates were incubated for 30 s at 95°C, denatured for 10 s at 95°C, and annealed for 20 s at 60°C or 57°C (Table 2.2). Denaturing and annealing cycles repeated 39 more times till completion. For some primers, proper annealing did not occur at 60ºC,

therefore a temperature gradient was performed for these primers—namely, IL-1β and IL-8. Plates were prepared as outlined above, and using the Bio-Rad CFX96 Touch Thermal Cycler (Bio-Rad Laboratories Inc.; Hercules, California, USA) the following temperatures were tested: 65°C, 64.5°C, 63.3°C, 61.9°C, 59°C, 57°C, 55.9°C and 55°C. It was determined that the best annealing temperature for IL-1β and IL-8 was 57°C (Table 2.2). The melt curve was checked to ensure that no alternative splicing of mRNA transcripts occurred, efficiencies were accepted between 90-110%, and R^2 scores were reviewed (R^2>0.97). Housekeeping genes *rps18, β-actin,* and *ef1α* were used as they were determined to be stable and previous studies have shown these transcripts are ubiquitously expressed in eukaryotic cells (McCurley & Callard, 2008). Gene expression was normalized to housekeeping genes and fold changes were calculated using the geometric mean of internal reference genes (Livak & Schmittgen, 2001; Vandesompele et al., 2002). For each primer, the sequence, efficiency, amplicon size, annealing temperature, M score and accession number are reported in Table 2.2.

Table 0.2. mRNA primers of gene targets used for RT-qPCR.

Primer	Sequence (5'-3')	Efficiency (%)	Amplicon (bp)	*Annealing Temp. (°C)*	M Score	Accession No.
rps18	**F:** GAGGTTGAGAGGGTGGTGAC	96.7	111	60	0.6566	NM_173234
	R: AAGGACCTGGCTGTATTTCCC					
ef1α	**F:** CAAGGAAGTCAGCGCATACA	107.8	107	60	0.6566	NM_131263
	R: TCTTCCATCCCTTGAACCAG					
β -actin	**F:** TCCATTGTTGGACGACCCAG	107.8	80	60	0.8914	NM_131031.2
	R: TGGGCCTCATCTCCCACATA					
il-1β	**F:** TGGACTTCGCAGCACAAAATG	105.4	150	57	N/A	NM_212844.2
	R: GTTCACTTCACGCTCTTGGATG					
il-8	**F:** GCTGTCGCTGCATTGAAACA	95.0	118	57	N/A	XM_009306855.3
	R: GTTGTCATCAAGGTGGCAATGA					
tnfα	**F:** CCATGCAGTGATGCGCTTTT	96.1	76	60	N/A	NM_212859.2
	R: CGTGCAGATTGAGCGGATTG					
hsp47	**F:** AGTGAGCATGGAAGTCAGCC	98.9	146	60	N/A	NM_131204.2
	R: AGGAGGAGGCATGGAAGACA					
hsp70	**F:** AAAGCACTGAGGGACGCTAA	95.1	127	60	N/A	AF_210640.1
	R: TGTTCAGTTCTCTGCCGTTG					
hsp90	**F:** CACGATCATGGCGATAAGTG	95.5	100	60	N/A	BC134081
	R: ACAGCGGTTTGGTTTTTGTTC					
hif1α	**F:** CGTGCAGCAGAAAAACAAAA	101.9	103	60	N/A	NM_001308559.1
	R: CTTGAGTAGCTTCGGGTGG					

1.9.3 VFX Analysis

To confirm VFX concentrations throughout exposure period, 100 mL glass amber water bottles were used for water samples and were taken 1 h post-addition of VFX from each tank. These bottles were stored in the -20°C freezer for analysis via mass spectrometry. It should be noted that *Day 2* bottles for all *VFX* groups broke while in storage and were removed from analysis (Fig 2.3). Sample analysis was conducted using a previously developed protocol (Arlos et al., 2015; Medhi et al., 2021). Briefly, samples were analyzed with Aglient Bond Elute Plexa (6 cc, 500mg) using solid phase extraction cartridges (Arlos et al., 2015). venlafaxine samples were adjusted to pH 2 using methanol and water, then eluted with methanol, evaporated, and reconstituted with methanol. Samples were analyzed via liquid chromatography and tandem mass spectrometry (LC-MS/MS) with Agilent 1260 HPLC with 6460 QQQ and Aglient Jet Stream source using a flow rate of 0.3 mL/min (Mississauga, ON; Mehdi et al., 2021). Sample recoveries were reported to be between 70-120%

1.9.4 Statistics

Data was analyzed using GraphPad Prism 9.5.1 Software for statistical testing. Two-way ANOVAs were performed, and results were analyzed via Tukey post-hoc test was used to determine individual differences between treatment groups. Assumptions of ANOVA were met by assessing normality using the Shapiro-Wilk's or the Anderson-Darling normality test, variation of parameters was assessed using an F-test, and the Spearman test for heteroskedasticity. The p-values for the ANOVA were compared to the 5% alpha value (α=0.05), and p was deemed significant if $p<\alpha$. Data that failed to meet ANOVA assumptions were log or square root transformed and reassessed using two-way ANOVA. Data that failed ANOVA assumptions following transformation were assessed using the Mann-Whitney non-parametric test. The Holm-Sidak correction was applied to non-parametric assumptions to control for pairwise error, which reports the multiplicity adjusted p-value (Wright, 1992). To determine if there were differences in temperature between *Control*/*Temp.*, and *Temp.+VFX* tanks, a two-tailed paired t-test was used, and normality was assessed using the Shapiro-Wilks test. ANOVA figures are presented as untransformed for

visual clarity; figures and data are presented as the mean± standard error of the mean (SEM). Mann-Whitney U non-parametric data are presented as untransformed and in a boxplot with the median value.

1.10 Results

1.10.1 Body Length and Weight

The average body length of zebrafish was 3.6±0.2 cm in the *Control*, 3.5±0.01 cm in the *Temp.* groups, and 3.6±0.04 cm in the *Temp.+VFX* groups. A significant difference in body length was seen among the *Control* and *Temp.* groups, as *Temp.* fish were smaller ($F(1,2) = 6.179$, $P=0.0445$). The average body weight was determined to be 347.5±78.4 mg in the *Control*, 229.4±44.8 mg in the *Temp.* groups, and 291.7±44.7 mg in the *Temp.+VFX* groups. A significant difference in body weight was seen in the *Control* and *Temp* groups, as *Temp.* fish were lighter ($F(1,2) = 11.46$, $p=0.008$) and the *Control* and *Temp.+VFX* groups ($F(1,3) =14.10$, $p=0.0044$). No differences were observed in the *Temp.* and *Temp+VFX* groups.

1.10.2 Water Chemistry

Water temperatures were monitored and logged continuously each minute with LoggerPro software during 96 h exposure. No significant differences in temperature were seen between *Control/Temp.,* and *Temp.+VFX* tanks (Two-Tailed Paired T-Test; $t=1.327$, $df=2$, $p=0.3157$). An average temperature of 27.1±0.1°C for *27°C Control*, 35.4±0.8°C for *37°C*, and 31.6±4.4°C for *27-37°C Temp* across the 96 h period (Fig 2.2A). As the temperature was fluctuating for the *27-37°C Temp.* group, a maximum temperature of 37.2°C and a minimum temperature of 27.1°C was observed (Fig 2.2). For the venlafaxine groups, the average temperatures were 27.1±0.06°C for *27°C Temp.+VFX*, 34.6±1.2°C for *35°C Temp.+VFX*, and 31.4±4.2°C for *27-37°C Temp.+VFX*. A maximum temperature of 37.3°C and a minimum temperature of 27.0°C was recorded for the *27-37°C Temp.+VFX* group (Fig 2.2B).

For the *27-37°C Temp.* group, the average rate of change in heating was calculated to be 0.81°C/h ($y=0.81x+28.1$), ultimately reaching 37°C in ~6 h each day. Conversely, the rate of change in cooling was on average -0.73°C/h ($y=-0.73+28.2$), reaching 27°C in ~4 h. For the 27-37°C Temp.+VFX group the

average rate of change in heating was 0.898°C/ h (y=0.90x+27.1) reaching 37°C in ~7 h. The rate of change in cooling was calculated to be -0.88°C/h (y=-0.88x+27.1) reaching 27°C in ~4 h each day. These differences in heating/cooling times may be explained by the ambient temperature of the aquatic's facility, which is ~18-20°C. The temperature probe for the *35°C VFX* tank had communication errors with LoggerPro Software application starting Day 2, thus a glass temperature rod was used to confirm temperatures daily.

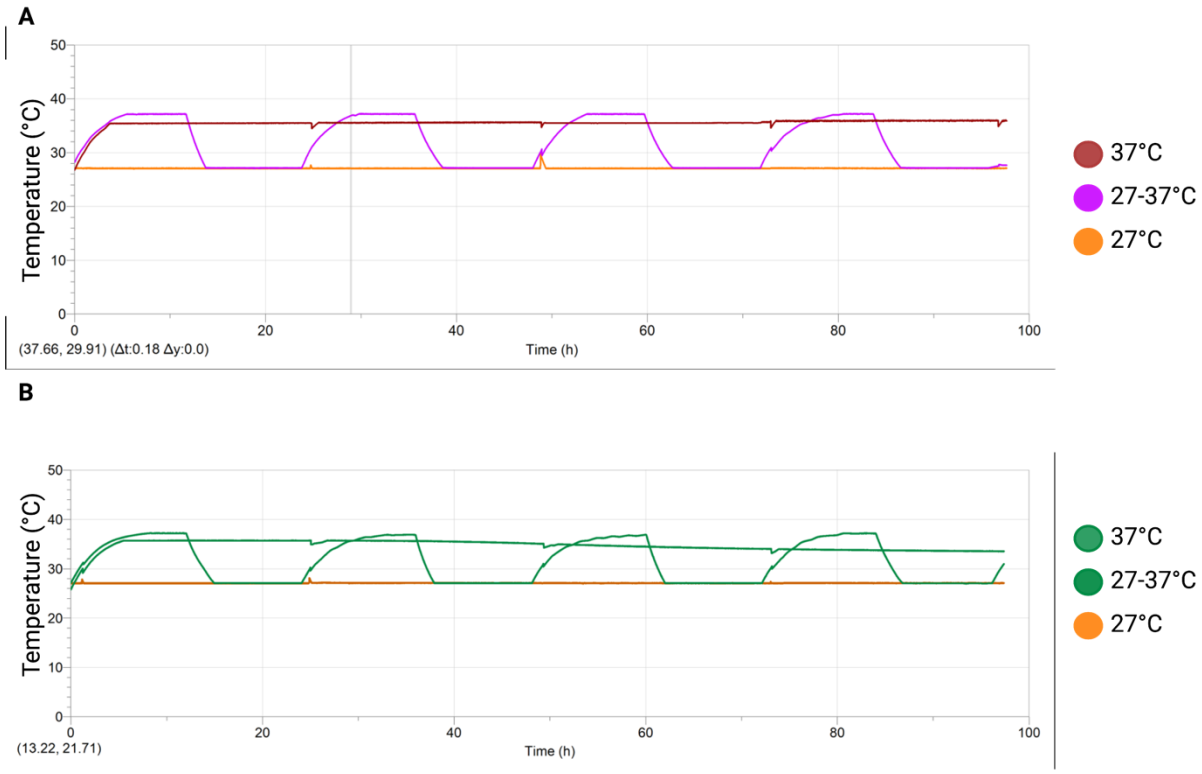

Figure 0.2 Recorded temperature curve for experimental tanks over 96-hour exposure.

A) Temperatures from control tanks over 96-h. B) Temperature from VFX tanks over 96 h. Note that temperature probe for 37°C VFX tank (B) stopped communicating correct temperatures; a manual glass rod was used starting Day 2 to confirm tank remained at 37°C.

Venlafaxine concentrations were higher than expected for all experimental tanks, where the average concentration across all treatments was 1643.5±225.8 ng/L. The average VFX concentration of each tank was determined. The VFX tanks were reported to have an average concentration of 1560.7±191.9 ng/L for *27°C* Temp.+*VFX*; 1952.1±436.1 ng/L for *37°C Temp.+VFX*; and 1417.8±148.9 ng/L for *27-37°C Temp.+VFX* (Fig 2.3).

1.10.3 VFX differentially modulates cytokine responses in the presence of multiple stressors

The activity of three cytokines, *il-1β, tnfα,* and *il-8* were measured following the 96-hour exposure to determine if temperature and VFX exposure impacted the pro-inflammatory response in zebrafish gills (Fig 2.4). A significant decrease in *il-1β* expression was observed in the *27-37°C Temp.+VFX group* relative to the *Control* (Tukey HSD, $p<0.0001$), which may suggest that *il-1β* expression is suppressed in the presence of VFX, but only under diurnal fluctuations. No significant difference was observed in other treatment groups when compared to the *Control* (Tukey HSD, $p>0.05$). *Il-1β* was downregulated in the *27-37°C Temp.+VFX* group relative to the *27-37°C Temp.* group (Tukey HSD, $p<0.0001$). *Il-1β* was significantly different between temperature exposure (Two-way ANOVA, $F_{2,41}=19.11$, $p<0.0001$), VFX treatment (Two-Way ANOVA, $F_{1,41}=11.02$, $p=0.0019$), and the interaction between temperature and VFX treatment (Two-way ANOVA, $F_{2,41}=10.91$, $p=0.0002$).

No significant difference was seen in *il-8* expression in the *37° Temp* and *27-37°C Temp.* groups compared to the *Control* (Tukey HSD, $p>0.05$). Interestingly, *Il-8* increased ~4.1-fold in the *27°C Temp.+VFX* (Tukey HSD, $p=0.0015$), ~2.9-fold in the *37°C Temp.+VFX* (Tukey HSD, $p=0.0358$), and ~3.7-fold in the *27-37°C Temp.+VFX* (Tukey HSD, $p=0.0196$) groups relative to the *Control*. It is also worth noting that *il-8* expression was significantly upregulated in *27-37°C Temp.+VFX* group when compared to its temperature counterpart (Tukey HSD, $p<0.0209$; Fig 2.4B). *Il-8* was significantly different between treatment groups (Two-way ANOVA, $F_{1,42}=28.71$, $p<0.0001$), but no temperature (Two-way ANOVA, $F_{2,42}=0.3723$) or interactive (Two-way ANOVA, $F_{2,42}=1.696$, $p=0.1957$) impacts were noted (Fig 2.4B).

Tnfα was not significantly different in the *37°C Temp.* and the *27-37°C Temp.+VFX* groups relative to the Control (Mann-Whitney U=30.00, p>0.05; Mann-Whitney U=14.00, p>0.05; Fig 2.4C). A significant difference in *tnfα* expression was seen across treatment groups, as tnfα expression was increased ~14.4-fold in the *27°C Temp.+VFX* (Mann-Whitney U=5.00, p=0.002953), ~39.5-fold in the *37°C Temp.+VFX* (Mann-Whitney U=0.00, p=0.000466), and ~30.9-fold in the *27-37°C Temp.+VFX* group (Mann-Whitney U=3.00, p=0.002174) relative to the *Control* (Fig 2.4C). Comparing between temperature groups, *37°C Temp.+VFX* expression was elevated compared to the *37°C Temp.* group (Mann-Whitney U=3.00, p=0.002174), but not within the 27-37°C groups (Mann-Whitney U=27.00, p>0.05); Fig 2.4C). ANOVA statistics are reported in Appendix Table 6.3.

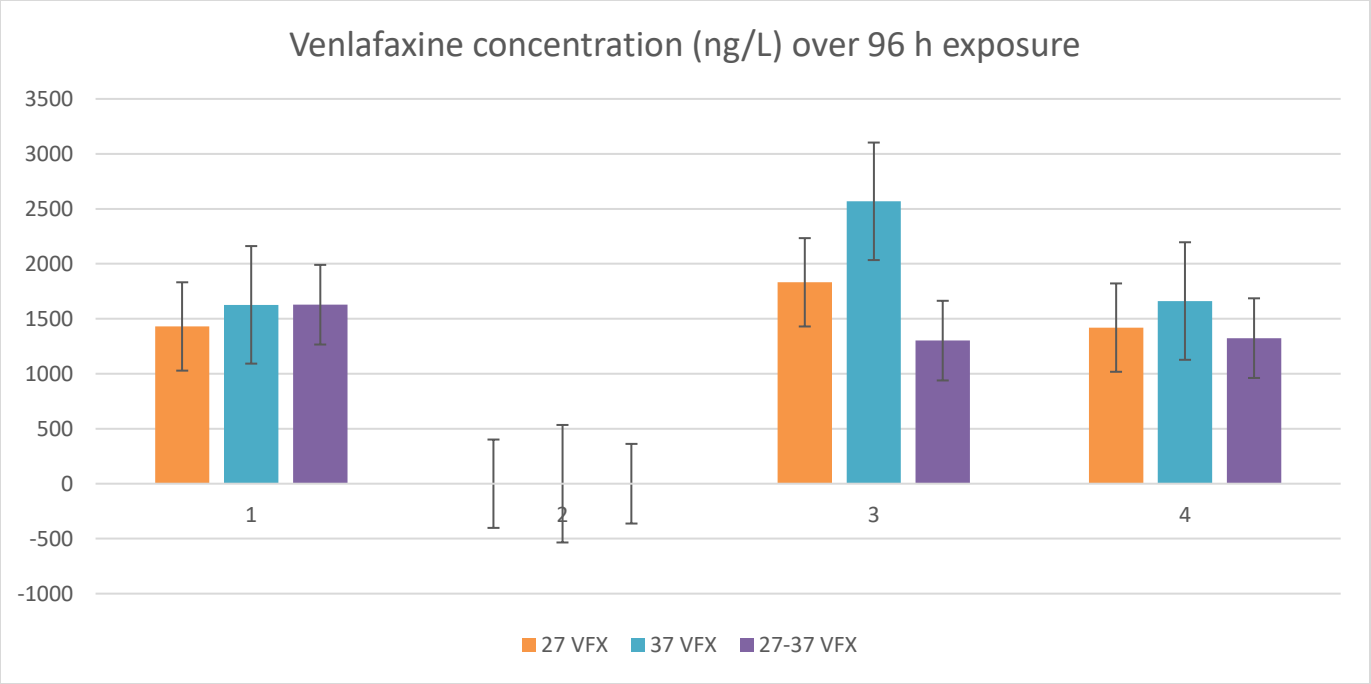

Figure 0.3 VFX concentrations (ng/L) over 96 h exposure.

Venlafaxine concentrations were determined for Days 0-3. Note that day 2 samples broke in the -20ºC freezer and were removed from analysis.

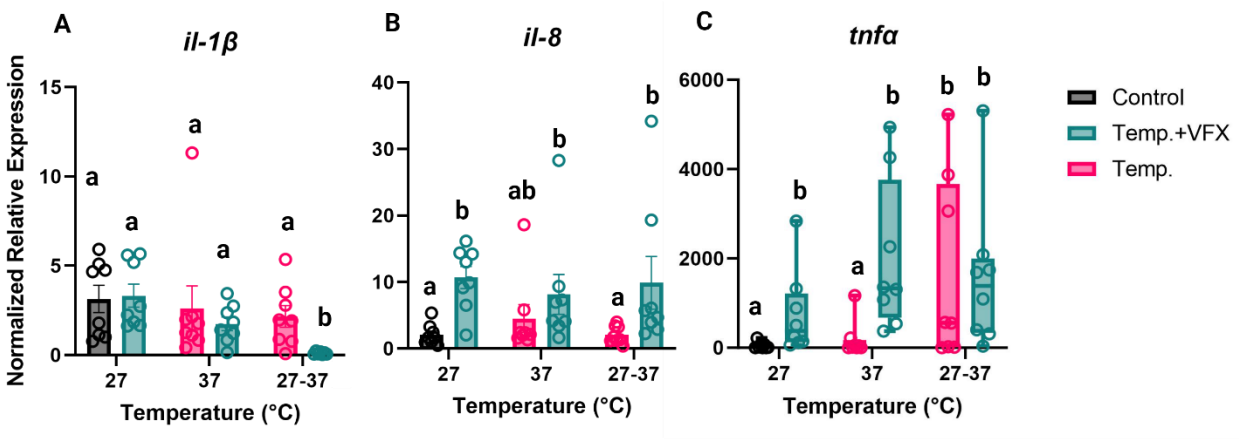

Figure 0.4 Normalized relative expression of cytokines in zebrafish after 96 h exposure.

Zebrafish expression (n=8 fish per treatment group) following 96 h exposure of (A) il-1β, and (B) tnfα, and (C) il-8 in the gills. Il-1β and il-8 data were sqrt and log transformed, respectively. Two-way ANOVAs were performed following Tukey post hoc test; tnfα was analyzed via Mann-Whitney non-parametric test with the Holm-Sidak correction (α=0.05) and presented as a boxplot with the median. Bars that have different letters indicate significant results.

1.10.4 VFX upregulates HSP expression in the presence of multiple stressors

The expression of three heat shock proteins, *hsp47, hsp70* and *hsp90* was assessed to determine the impacts of multiple stressors on the heat shock response in zebrafish gills (Fig 2.6). Compared to the *Control*, *hsp47* was upregulated ~2.9-fold in the *37°C Temp.* group (Tukey HSD, p=0.0092), but no significant changes were seen in the *27-37°C Temp.* group (Tukey HSD, p>0.05; Fig 2.6A). In the presence of VFX, *hsp47* expression increased ~9.6-fold in the *37°C Temp.+VFX* group (Tukey HSD, p<0.0001) and ~2.2-fold in the *27-37°C Temp.+VFX* group (Tukey HSD, p=0.0449), but no significant changes were seen at 27°C (Tukey HSD, p>0.05). Interestingly, there were no significant difference observed within temperature groups at 27°C (Tukey HSD, p>0.05) or at 37°C (Tukey HSD, p>0.05), but within our 27-37°C groups, there was an observed difference when VFX was introduced as a parameter (Tukey HSD, p=0.0007). *Hsp47* was significantly different across temperature (Two-way ANOVA, $F_{2,42}$=26.09. p<0.0001), treatment groups (Two-way ANOVA, $F_{1,42}$=20.45, p<0.0001), and a significant interactive impact was seen between temperature and treatment (Two-way ANOVA, $F_{2,42}$=3.624, 0.0353; Fig 2.6A).

Hsp70 expression in the gills was not significantly impacted in either *Temp.* group when compared to the *Control* (Tukey HSD, p>0.05; Fig 2.6B). Alternatively, in the presence of VFX, *hsp70* was upregulated ~33.9-fold in the *37°C Temp.+VFX* group (Tukey HSD, p<0.0001) and 21.4-fold in the *27-37°C Temp.+VFX* group when compared to the *Control* (Tukey HSD, p<0.0001). No differences were seen in the *27°C Temp.+VFX* group compared to the *Control* (Tukey HSD, p>0.05). Additionally, *hsp70* expression was significantly different between the *27-37°C Temp-27-37°C Temp.+VFX* group (Tukey HSD, p=0.0154), but these differences were not seen at constant temperatures (Tukey HSD, p>0.05; Fig 2.6B). *Hsp70* expression was significantly impacted between temperature groups (Two-way ANOVA, $F_{2,39}$=16.22, p<0.0001), and treatment (Two-way ANOVA, $F_{1,39}$=0.0001), but no interaction between the two parameters was seen (Two-way ANOVA, $F_{2,39}$=1.722, p>0.05; Fig 2.6B).

Compared to the *Control*, *hsp90* expression was significantly upregulated ~13.7-fold in the *37°C Temp.* group (Mann-Whitney U=0.000, p=0.000311), but this response was attenuated at 27-37°C (Mann-

Whitney U=18.00, p>0.05; Fig 2.6C). Likewise, *hsp90* increased in all *Temp.+VFX* groups, ~4.8-fold at 27°C (Mann-Whitney U=4.000, p=0.001865), ~34.6-fold at 37°C (Mann-Whitney U=0.000, p=0.000466), and ~18.3-fold at 27-37°C when compared to the *Control* (Mann-Whitney U=0.000, p=0.000466). Between experimental temperature groups, a difference was seen between *37°C Temp.-37°C Temp.+VFX* (Mann-Whitney U=8.00, p=0.010412), and *27-37°C Temp.-27-37°C Temp.+VFX* groups (Mann-Whitney U=0.000, p=0.000311; Fig 2.6C). ANOVA statistics are reported in Appendix Table 6.3.

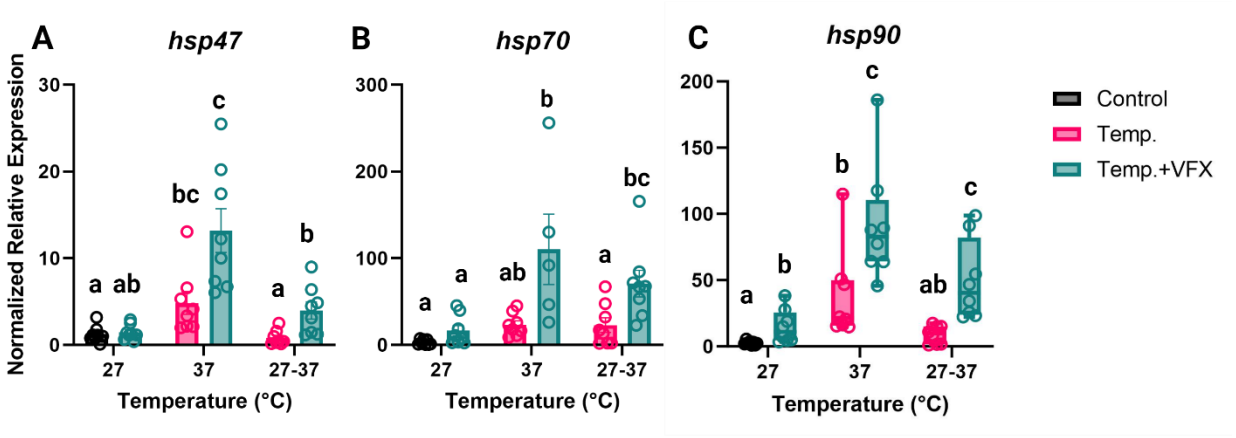

Figure 0.5 Normalized relative expression of HSPs in zebrafish following 96 h exposure.

Adult mixed-sex zebrafish gills following 96 h exposure of (a) *hsp47*, (b) *hsp70*, and (c) *hsp90* (n=8 fish/group). *Hsp47* and *hsp70* data was log and sqrt transformed, respectively, and analyzed via a two-way ANOVA followed by Tukey post hoc test. *Hsp90* was analyzed via Mann-Whitney U non-parametric test, followed by the Holm-Sidak correction (α=0.05) and presented as a boxplot with the median. Different lettering indicates significant result.

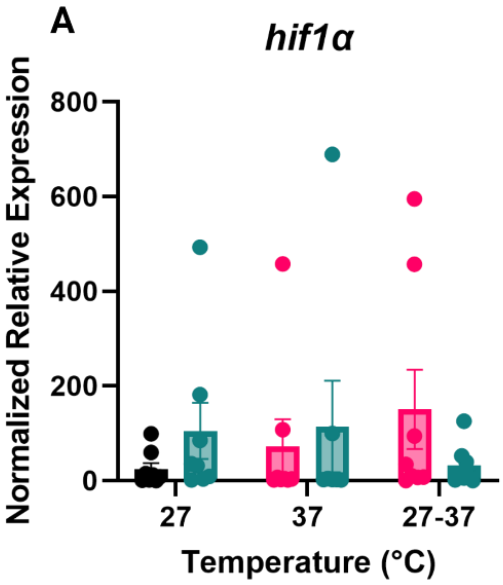

Figure 0.6 Normalized relative expression of Hif1-alpha.

Adult mixed-sex zebrafish gills (n=8 fish/ group) of (a) *hif1α* following 96 h exposure. Data were log transformed and analyzed via a two-way ANOVA followed by Tukey post hoc test ($p<0.05$).

Chapter 3 Acclimation Study

1.11 Methods

1.11.1 Zebrafish Care

Male and female wild-type pet store adult zebrafish were ordered from Big Al's Canada (Kitchener, Waterloo). Fish were held in recirculating Z-HAB system (Pentair Aquatic Eco-System Inc., Apopka, Florida, USA) at 27°C, pH of 7.5, conductivity of ~670 µS, on a 12 h:12 h light-dark cycle. The system was supplied with water from a purification system, which is subject to UV sterilization, chemical and biological filtration, and reverse osmosis. Tanks were stocked with 15 to 30 fish (SD= 1.25 and 2.5 fish/L) and all fish in the system were fed ground Gemma Zebrafish Micro Diet 300 (Skretting, Westbrook, Maine, USA) until satiety. All experiments performed in this study were reviewed and approved by the Canadian Council of Animal Care and were fulfilled under the guides set by the University of Waterloo Animal Care Committee (AUP# 40989).

1.11.2 Acclimation Study Design

This experiment was broken up into two parts: a two-week acclimation followed by a 24 h heat stress with or without 1 µg/L of VFX. The first two weeks tanks were set to one of three temperatures based on fig 3.4 tank outline: 25ºC, 35ºC, and fluctuating between 25-35ºC (Fig. 3.1). These two weeks were used to allow the zebrafish to acclimate to respective temperatures. Following the two-week acclimation, fish in all experimental groups (excluding control)— 25ºC Temp., 25ºC Temp+VFX, 35ºC Temp., 35ºC Temp+VFX, 25-35ºC Temp., and 25-35ºC Temp+VFX were elevated to 35ºC for 24 h (Fig. 3.1). Additionally, Temp.+VFX groups received a 1 µg/L dose of VFX during the 24 h period (Fig. 3.1). At 24 h, fish were euthanized via MS-222 and gill and brain samples were harvested.

To control the temperature and dissolved oxygen content within the system, the Loligo Core OMNIctrl System (Loligo Systems, Viborg, Denmark) was used. The Loligo Core OMNIctrl system was

set up to manufacturers guidelines, with one deviation. Water pumps were screwed into place outside of the tanks as opposed to placement inside the tank (Fig 3.2). The computer was connected to a WTW Oxi 3310 Instrument, which collected data from the WTW Oxi 3310 temperature and DO probe (Loligo Systems, Viborg, Denmark; Fig 3.2, 3.3). The computer receives information about the temperature and DO of each tank and relays directions to the Bluetooth connected WTW Power X4 power bar (Loligo Systems, Viborg, Denmark). The power bar is equipped with two temperature modules, one O_2 module, and one N_2 module. For this experiment, N_2 was not used. The temperature modules connect to one hot and one cold water pump, which are switched on/off automatically to maintain each temperature set-point (Fig 3.2). When the temperature module is turned on, water exits the tank, passes through the water pump, and into a stainless-steel heat exchanger (not shown) inside the reservoir tank. Water is warmed/cooled as it passes through the warm or cool reservoir tank and returns to the respective tank. A second temperature probe and A477 Digital Thermometer (Qubit, Kingston, ON) was used to track the temperature of the reservoir tank. Three aquarium heaters set to 96°F were used to heat the warm reservoir and a commercial grade chiller was used to cool the cold reservoir (Fig 3.2). All tanks were insulated with Owens Corning Pink Next generation fiberglass insulation on the exterior to maintain high heat requirements, which were cut to size and adhered with duct tape (Fig 3.3).

The OMNIctrl application was loaded on the computer and a custom protocol was developed using the OMNIctrl protocol designer (Main Menu>Experiment>Settings). For the two-week acclimation, *25°C* and *35°C* groups were set to *"Manual"*, and the temperatures were set to 25°C and 35°C, respectively. The *"Step-Time*" was set to 8:00:00 (HH:MM:SS) for the 35°C groups, which allowed the tanks to gradually warm to 35°C over 8 h before staying at 35ºC for the duration of the exposure. To facilitate a fluctuating environment (Main Menu>Protocol Designer), "*Step Wise*" protocol was chosen, and the *"Min"* was set to 25°C and the *"Max"* was set to 35°C. The rate of change in temperature was set to ±0.833°C/h (*"Step Height"*) which was calculated by dividing the difference in temperature over 12 h (10°C/12 h= 0.833°C/ h). Lastly, the *"Step-Time"* was set to 12:00:00 (HH:MM:SS), resulting in the system increasing or

decreasing in temperature every 12 h. The DO content for all tanks was set to 8 mg O_2/L but was subject to change as water temperatures changed.

When experimentation started, *"Start Logging"* was clicked to begin. Data was collected every second over the duration of the acclimation. Once two-weeks passed (14 d), logging was stopped, and protocol designer was launched again. The "*Stairs"* protocol was chosen, and each experimental tank (excluding *25ºC Control*) was set to 35°C. At 8AM 25-35°C groups were at 25°C, so the 25°C and 25-35°C groups were set to warm 0.833°C/ 10 min, ultimately reaching 35°C in 2 h (*"Step Height"* = 2:00:00) for an acute heat shock. Tanks in the 35°C groups remained at 35°C. Data was logged for 24 hours each second for the duration of the exposure. Note that trial 1, tank 2-5 (*25°C Temp.*) did not heat to 35°C (no heating occurred) and therefore these fish were excluded from analysis thus, brain samples have a reduced n of 6 per treatment group, while gill tissues were only used from trial 2 and 3.

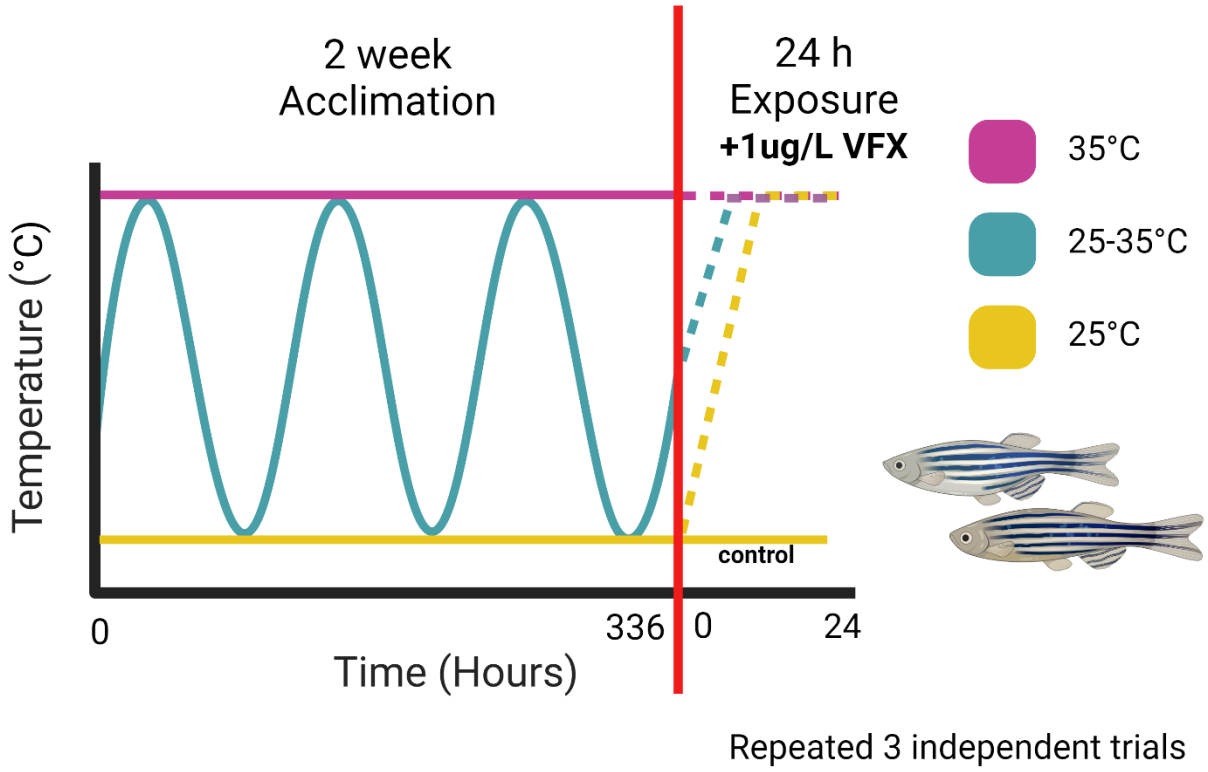

Figure 0.1 Acclimation study exposure design over 15 d trial.

Zebrafish were acclimated for two-weeks at respective temperatures, followed by a 24 h heat stress with or without 1 µg/L of VFX.

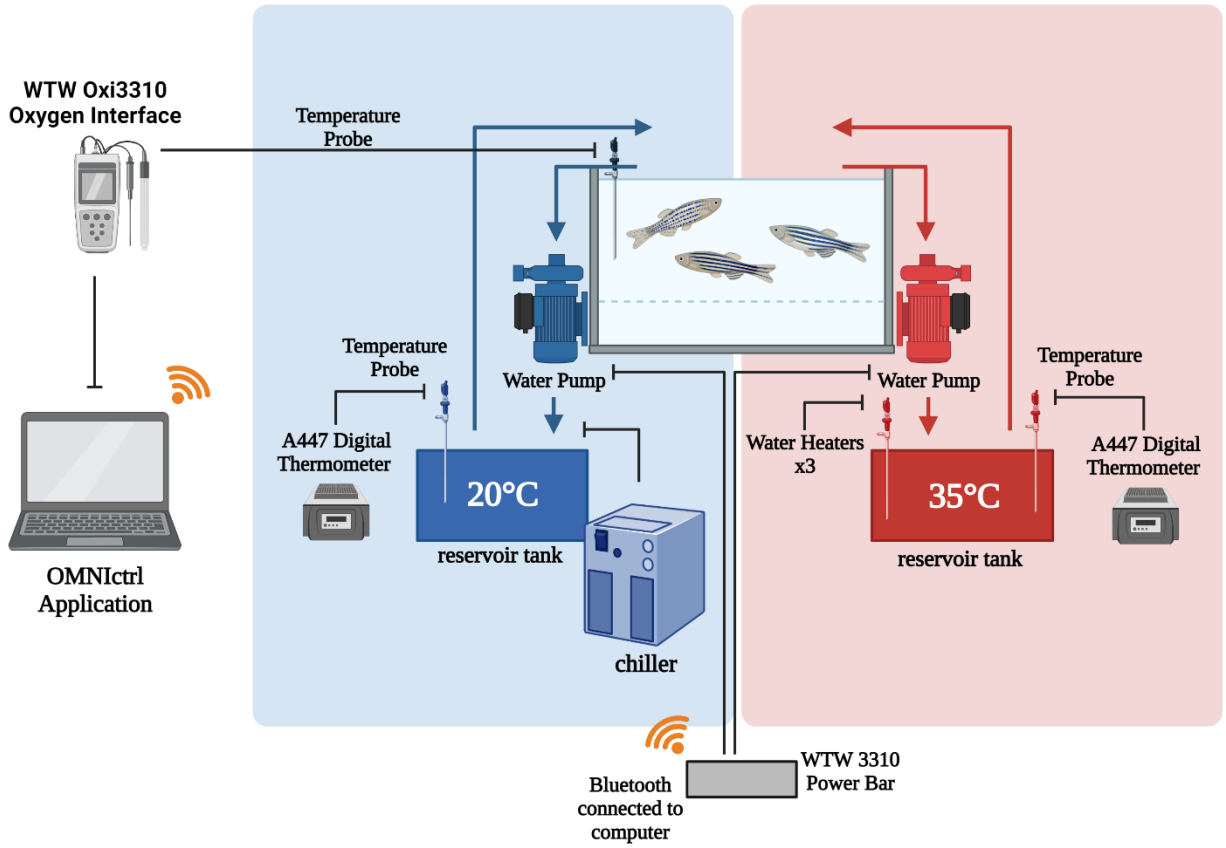

Figure 0.2 Acclimation study in-lab tank design over 15 d exposure.

Loligo WTW CellOx 325 oxygen probes were used to determine the oxygen content and temperature of each tank. Information relayed back to the computer via the WTW Oxi3310 interface would determine when the circulating pump would be turned on leading to heating/cooling of respective tank.

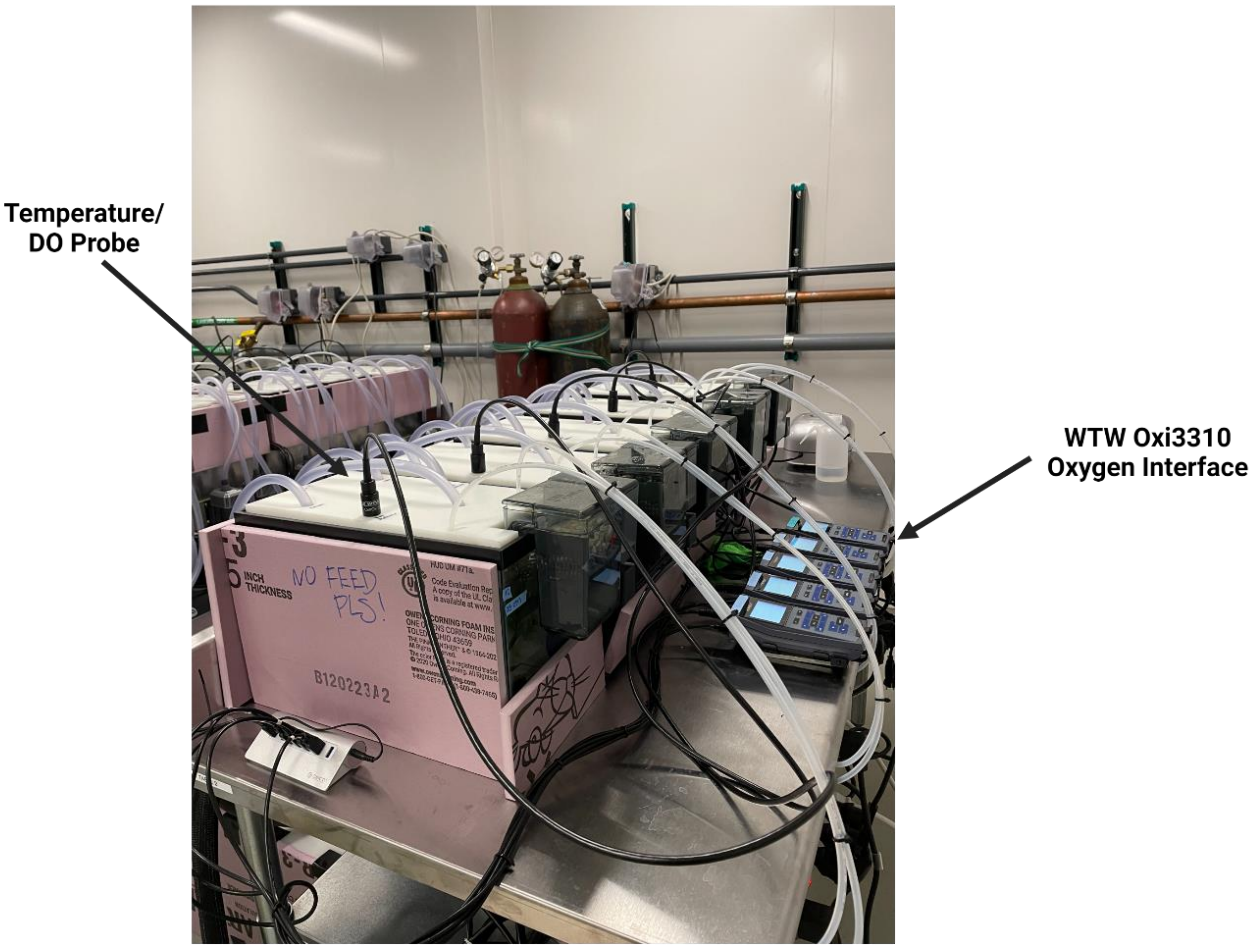

Figure 0.3 In-lab tank set-up for 15 d acclimation exposure.

To account for tank replicates experimentation was carried out in three independent trials (n=84 fish/trial; Fig 3.4). Two additional tank repeats (*35°C and 25-35°C Temp.+VFX*) were added to trial three as a mistake was made during trial two and filter media was not removed from the filters prior to VFX addition (Fig 3.4). To provide adequate acclimation time, zebrafish were placed in one of three temperature environments for two-weeks: 25°C, 35°C, and fluctuating diurnal cycle of 25-35°C (12 h:12 h). Two-weeks was chosen as an acclimation period as previous studies have reported similar timeframes for laboratory acclimations (Morgan et al., 2019; Zhou et al., 2019). Tanks fluctuating from 25-35°C were calculated to increase/decrease in temperature by 0.833°C/h for 12 h. Additionally, a plastic plant was provided to each tank for appropriate hiding space. Following the two-week acclimation period, fish in experimental tanks were exposed to an acute heat stress of 35°C with or without 1 μg/L VFX for 24 h at 8AM. Zebrafish were exposed to one of seven experimental treatments: *25°C Control, 25°C Temp., 35°C Temp., 25-35°C Temp., 25°C Temp.+VFX, 35°C Temp.+VFX,* and *25-35°C Temp.+VFX* (Table 3.1, Fig. 3.4; *25°C Control* tanks were not temperature or VFX exposed). It should be noted that *Empty* tanks were filled and set-up the same as experimental tanks to control for noise discrepancies (Fig 3.4). The heat-stress was rapidly induced over 2 h, where fish experienced a 0.833°C increase in temperature per 10 min. Following 24 h acute heat stress/VFX exposure, fish were euthanized using the MS-222 (0.5g/L; Millipore-Sigma-Aldrich, Mississauga, Ontario, Canada) protocol. Gill and brain tissue were extracted, flash frozen in liquid nitrogen in individual cryovials and stored in the -80°C for experimentation.

Several issues arose with the Loligo Core OMNIctrl System (Loligo Systems, Viborg, Denmark). A few times throughout the acclimation period for trial 1 and trial 2, the Loligo application would spontaneously quit, causing WTW 3310 power bars to stop Bluetooth communication with the computer (Fig 3.1). This led to tanks being temporarily overheated or underheated, resulting in periods of time where temperatures varied from desired temperatures. Alternatively, this would cause complete shutdown of the system, resulting in water circulation ceasing and data logging to be halted. There were no consistent timepoints or reasons as to why the application would quit. It was speculated to possibly be due to a

transition to the emergency power kicking in throughout the night or issues with Wi-Fi connections. Updates and other 3rd party downloads were tried to resolve the issue, but neither were successful.

Another common issue was the Oxi 3310 meter reporting the incorrect temperature. Although the connection was USB wired, the meter would read the incorrect temperature or not reflect the setpoint provided by the computer. Probes were re-calibrated following shutdowns to ensure probe solutions were at the appropriate level and that probe malfunction was not the reason for the shutdown. Often, the only resolve for this issue was shutting down the program and restarting logging. For these reasons, some timepoints and temperatures may be missing from analysis, thus there are temperature fluctuations that may not be accounted for over the course of the two-week acclimation. Additionally, due to complications with troubleshooting, data files from week 1 trial 1 acclimation were corrupted, thus temperature and DO is missing several days of acclimation data, ultimately impacting average temperature and DO reporting (Table 3.3; Table 6.6).

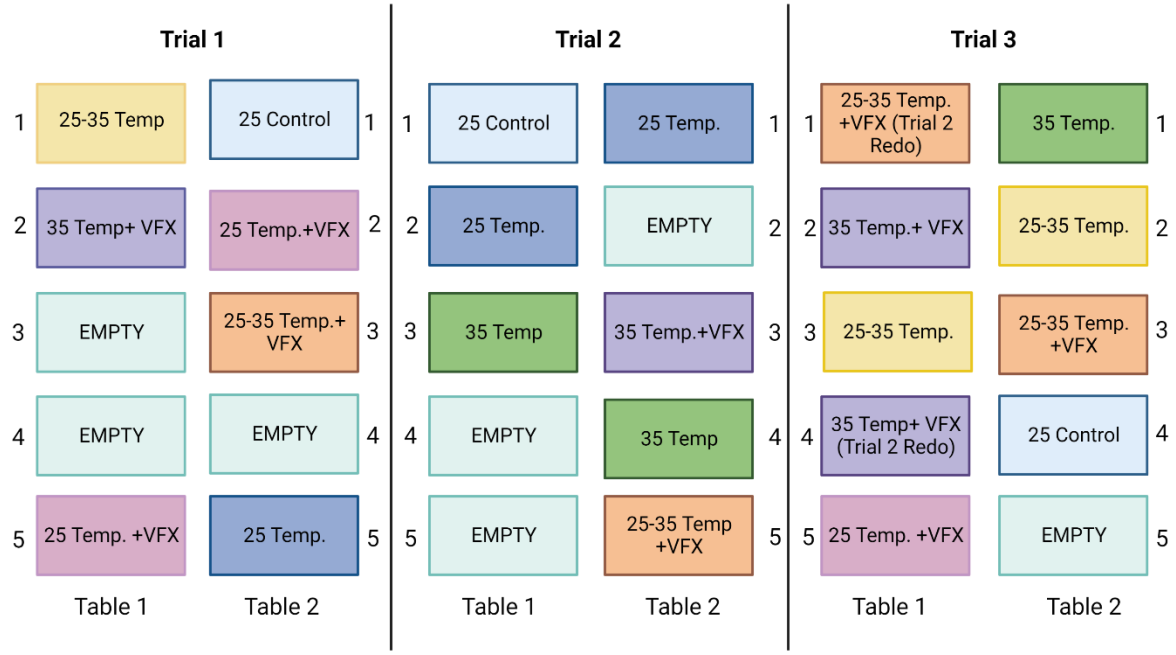

Figure 0.4 Randomized tank design and groups for 15 d exposure.

A seating chart website was used to randomly assign experimental groups to respective tanks, apart from a *25ºC Control* group included in each trial (https://www.randomnamepicker.net/seating-chart). Different colours represent each experimental group (n=3 tank replicates per treatment).

Table 0.1 Experimental groups and treatments.

Experimental groups with respective treatment, where Y=yes and N=no.

Group	Acclimation Temperature	Heat Stress (35ºC) – Y/N	VFX—Y/N
25ºC Control	*25ºC*	N	N
25ºC Temp.	*25ºC*	Y	N
25ºC Temp.+VFX	*25ºC*	Y	Y
35ºC Temp.	*35ºC*	Y	N
35ºC Temp.+VFX	*35ºC*	Y	Y
25-35ºC Temp.	*25-35ºC*	Y	N
25-35ºC Temp.+VFX	*25-35ºC*	Y	Y

1.11.3 Molecular Analysis

Originally, RNA extractions were carried out using 1 mL of TRIzol (Millipore-Sigma-Aldrich, Mississauga, Ontario, Canada) per 100 mg of gill and brain tissues (Craig et al., 2013). However, due to low RNA sample concentrations (<5 ng/µL) determined using the Qubit Fluorometric Quantification Device (Thermo Fisher Scientific Inc., Mississauga, Ontario, Canada), the Qiagen miRNeasy Mini Kit (Hilden, North-Rhine Westphalia, Germany) was used, with one deviation. The 20 uL sample was eluted twice through the column to increase RNA concentration in the final sample. A pooled sample was created with 1 µL of each sample and all RNA extracts were stored in the -80°C freezer for molecular analysis. It should be noted that when feasible, the gill and brain tissue used for analysis was taken from the same fish. Unfortunately, due to low RNA abundance in some tissues, tissues from the same fish could not always be used and therefore gill, or brain tissues were taken from another fish.

cDNA analysis was completed using the Qiagen QuantiTech Reverse Transcription Kit (Hilden, North-Rhine Westphalia, Germany) and instructions were followed to manufactures guidelines. Gill samples were prepared with 500 ng of RNA, while brain samples were prepared with 375 ng of RNA as RNA concentrations for the brain were lower. For analysis a 5-point dilution curve was made with the pooled cDNA sample. A 4x dilution was prepared by adding 15 uL of pooled sample to 45 uL of nuclease-free water. These were stored in the -20ºC for RT-qPCR (see *2.1.3 Molecular Analysis* for detailed methods).

The expressions of *il-1β, il-8, tnfα, hsp47, hsp70, hsp90, and hif1α* were quantified using the SsoAdvanced Universal SYBR Green Supermix Kit (1725271; Bio-Rad Laboratories Inc.). The same protocol as outlined in *Ch. 2.1.4* was followed for RT-qPCR and temperature gradients were ran for primers that did not anneal at 60°C. Primer sequences, efficiency, amplicon, annealing temperature, M score, and accession numbers are reported in Table 3.1.

Table 0.2 mRNA primers for RT-qPCR analysis.

Forward (F) and reverse (R) primers are listed 5' to 3', and brain and gill efficiencies are reported for each gene. Housekeeping genes for the gills were *18s* rRNA, *rps18*, and *ef1α*, while for the brain *18s* rRNA, *rps18*, and *β-actin* were used, and relative M scores are included below. Note that brain and gill annealing temperatures were 60°C unless otherwise specified.

Primer	Sequence (5'-3')	Efficiency (%)	Amplicon Size (bp)	M Score	*Annealing Temp.(°C)*	Accession No.
18s rRNA	**F:** ATGGCCGTTCTTAGTTGGTG	**Gill:** 99	130	**Gill:** 0.5032	60	NR_145818
	R: GAACGCCACTTGTCCCTCTA	**Brain:**106.6		**Brain:** 0.7794		
RPS18	**F:** GAGGTTGAGAGGGTGGTGAC	**Gill:** 94.2	111	**Gill:** 0.3621	60	NM_173234
	R: AAGGACCTGGCTGTATTTCCC	**Brain:**102.3		**Brain:** 0.5350		
EF1α	**F:** CAAGGAAGTCAGCGCATACA	**Gill:** 97.4	107	**Gill:** 0.3621	60	NM_131263
	R: TCTTCCATCCCTTGAACCAG	**Brain:** N/A		**Brain:** N/A		
β -actin	**F:** TCCATTGTTGGACGACCCAG	**Gill:** N/A	80	**Gill:** N/A	60	NM_131031.2
	R: TGGGCCTCATCTCCCACATA	**Brain:**107.5		**Brain:** 0.5350		
IL-1β	**F:** TGGACTTCGCAGCACAAAATG	**Gill:** N/A	150	N/A	57	NM_212844.2
	R: GTTCACTTCACGCTCTTGGATG	**Brain:**106.1				
IL-8 (CXCL8)	**F:** GCTGTCGCTGCATTGAAACA	**Gill:** 107.7	118	N/A	57	XM_009306855.3
	R: GTTGTCATCAAGGTGGCAATGA	**Brain:** N/A				
TNFα	**F:** CCATGCAGTGATGCGCTTTT	**Gill:** 98.3	76	N/A	**Gill:** 63.3	NM_212859.2
	R: CGTGCAGATTGAGCGGATTG	**Brain:** 93.9			**Brain:** 65	
HSP47	**F:** AGTGAGCATGGAAGTCAGCC	**Gill:** 108.8	146	N/A	**Gill:** 65	NM_131204.2
	R: AGGAGGAGGCATGGAAGACA	**Brain:** N/A			Brain: N/A	
HSP70	**F:** AAAGCACTGAGGGACGCTAA	**Gill:** 102.7	127	N/A	60	AF_210640.1
	R: TGTTCAGTTCTCTGCCGTTG	**Brain:** 93.8				
HSP90	**F:** CACGATCATGGCGATAAGTG	**Gill:** 104.7	100	N/A	60	BC134081
	R: ACAGCGGTTTGGTTTTTGTTC	**Brain:**100.3				
HIFαα	**F:** CGTGCAGCAGAAAAACAAAA	**Gill:** N/A	103	N/A	60	NM_001308559.1
	R: CTTGAGTAGCTTCGGGTGG	**Brain:**106.6				

1.11.4 Enzymatic Analysis

For enzymatic analysis, frozen brain and gill tissue were homogenized in 20 mL of homogenization buffer per 1 mg of tissue (20 mM Hepes, 1 mM EDTA, 0.1% Triton X-100, and 1 anti-protease pill (1 pill per 10 mL of homogenization buffer); pH 7.4) using the OMNI Tissue Homogenizer TH (OMNI International, Kenneaw, USA) on ice. The homogenate was spun down at 12000xg for 10 min at 4°C using the Eppendorf® Centrifuge 5810/5810R (Eppendorf Canada Ltd., Mississauga, ON) and the supernatant was used for analysis. Enzyme assays were carried out in 96-well plates using the Molecular Device SpectraMax 190 spectrophotometer (San Jose, California, USA). Cytosolic enzymes were measured first—pyruvate kinase (PK; E.C. 2.7.1.40) and lactate dehydrogenase (LDH; E.C. 1.1.1.27), which were analyzed using fresh homogenate at 340 nm and 25°C for 10 min. Following one freeze-thaw cycle at -80°C, mitochondrial enzyme—citrate synthase (CS; E.C. 2.3.3.1), was analyzed at 412 nm and 25°C for 10 min. Enzymatic soups for PK, LDH, and CS were made as outlined in Medhi et al. (2018) and all chemicals were purchased from Sigma-Aldrich. Bicinchoninic acid (BCA) assays were used to determine protein concentration of each homogenate, and bovine serum was used as a relative standard at 340 nm. Enzymatic activity was normalized to individual protein concentrations via BCA.

1.11.5 Statistics

Data was analyzed using GraphPad Prism 9.5.1 Software for statistical testing. Two-way ANOVAs were performed, and results were analyzed via Tukey post-hoc test to determine statistical significance between temperature and VFX treatments. Assumptions of ANOVA were met by assessing normality using the Shapiro-Wilk's or the D'Agostino-Pearson omnibus (K2) normality test, variation of parameters was assessed using an F-test, and the Spearman test for heteroskedasticity. The p-values for the ANOVA were compared to the 5% alpha value (α=0.05), and p was deemed significant if $p<\alpha$. Data that failed to meet ANOVA assumptions were log or square root transformed and reassessed using two-way ANOVA. Data that failed ANOVA assumptions following transformation were assessed using the Mann-Whitney non-parametric test. The Holm-Sidak correction was applied to non-parametric assumptions to control for

pairwise error, which reports the multiplicity adjusted p-value (Wright, 1992). Outliers in data were identified using ROUT outlier test (Q=0.01= 1%) following Motulsky & Brown (2006) and if detected, were removed from analysis. Figures are presented as untransformed for visual clarity; figures and data are presented as the mean± standard error of the mean (SEM).

1.12 Results

1.12.1 Body Length and Weight

No significant differences were seen across treatments in fish body length (Tukey HSD, $p>0.05$), and weights (Tukey HSD, $p>0.05$). A significant difference in length was seen across Trial groups in the gills (Two-way ANOVA, $F_{2,12}=4.383$, $p=0.0372$) and the brain (Two-way ANOVA, $F_{2,12}=11.00$, $p=0.0019$), but not treatment groups (Gills: Two-way ANOVA, $F_{6,12}=1.235$, $p>0.05$; Brain: Two-way ANOVA, $F_{6,12}=0.55373$, $p>0.05$). Similarly, a difference in body weight was seen across trial groups in the gills (Two-way ANOVA, $F_{2,12}=9.152$, $p=0.0039$), and the brain (Two-way ANOVA, $F_{2,12}=8.645$, $p=0.0047$), but not across treatments (gill: Two-way ANOVA, $F_{6,12}=0.4565$, $p>0.05$; brain: $F_{6,12}=0.2418$, $p>0.05$).

1.12.2 Water Chemistry

VFX concentrations were below detection levels in and across all *Control* and *Temp.* replicate tanks (<5 ng/L). No significant differences in VFX concentrations were observed between *Temp.+VFX* treatments (Tukey HSD, $p>0.05$; Two-way ANOVA, $F_{2,2}=0.8798$, $p>0.05$). Additionally, no significant differences were observed between time points (1-hour vs. 24 hours) between groups (Two-way ANOVA, $F_{1,2}=2.373$, $p>0.05$; Fig 3.5). Graph data is reported in Appendix Table 6.2.

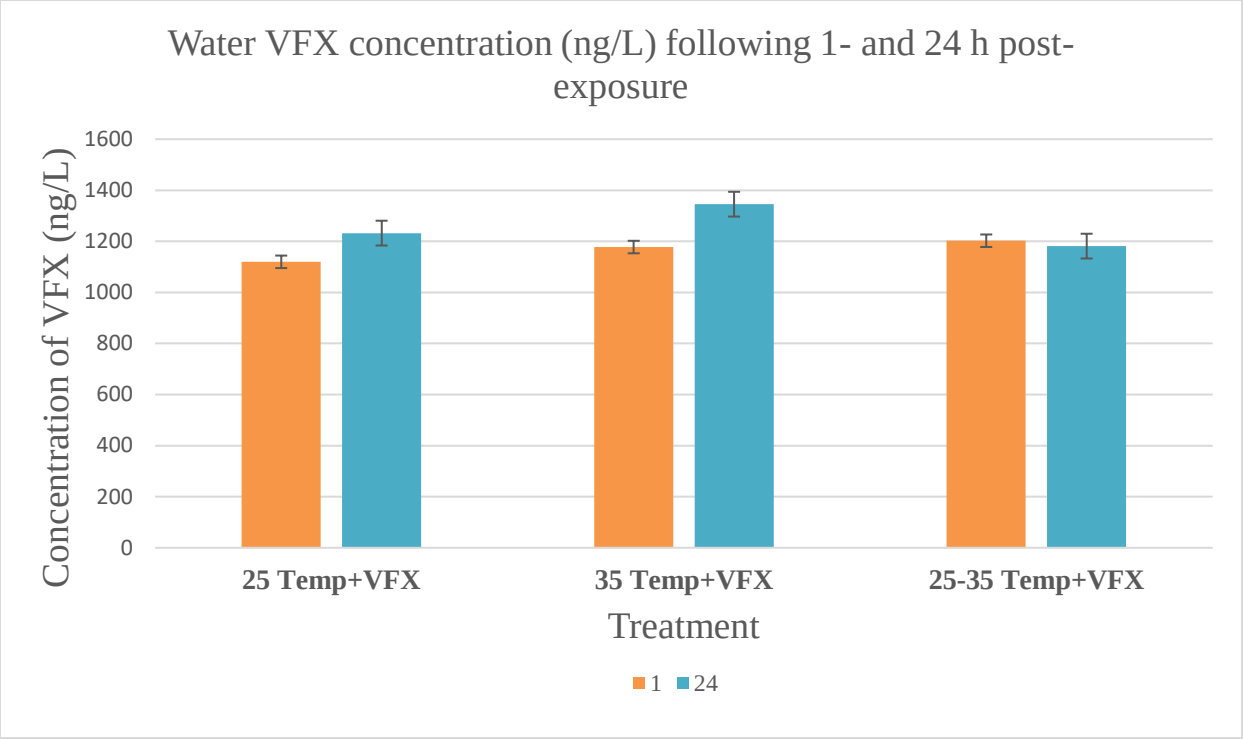

Figure 0.5 Average concentration of VFX (ng/L).

Average concentration of VFX across tank replicates at 1- and 24 h post-addition. For visual clarity, *Control* and *Temp.* tanks were removed from graph as their concentrations were 0.0 ng/L (detection limit <5 ng/L). The average concentration of VFX was taken across *Temp.+VFX* replicates (n=3 per treatment group) at 1- and 24 h, respectively.

1.12.3 Temperature Analysis

The average temperature across replicate groups is shown in Table 3.3. No significant temperature differences were seen between 25°C group replicates (Tukey HSD, $p>0.05$), 35°C group replicates (Tukey HSD, $p>0.05$), and 25-35°C replicate groups (Tukey HSD, $p>0.05$; Table 3.3). No differences were seen between 25°C treatment groups (*25°C control*, *Temp*., and *Temp.+VFX*; Two-way ANOVA, $F_{2,4}= 0.7870$, $p>0.05$), 35°C treatment groups (35°C *Temp.* and *Temp. +VFX*; Two-way ANOVA, $F_{1,2}=3.131$, $p>0.05$), and 25-35°C treatment groups (25-35°C *Temp*. and *Temp.+VFX*; Two-way ANOVA, $F_{1,2}= 0.8795$, $p>0.05$). The diurnal temperature cycle is shown in fig 3.6, demonstrating the two-week acclimation. DO and heat stress temperature data is reported in Appendix Table 6.6 and 6.7, respectively.

Table 0.1 Average temperature (°C) across treatment groups

The average temperature (°C) and standard error of the mean (SEM) of each tank replicate over the course of the two-week acclimation.

	Temperature (°C)				
Treatment	**Replicate 1**	**Replicate 2**	**Replicate 3**	**Avg. Temp.**	**SEM**
25 Control	25.08	25.27	25.75	25.37	0.2809
25 Temp	25.45	25.38	24.71	25.18	0.3352
25 Temp+VFX	24.64	25.42	24.72	24.92	0.3532
35 Temp.	34.12	33.79	34.31	34.07	0.2133
35 Temp+VFX	34.19	34.34	34.47	34.33	0.1111
25-35 Temp.	29.94	29.99	30.09	30.01	0.0616
25-35 Temp.+VFX	29.96	29.99	29.90	29.95	0.0358

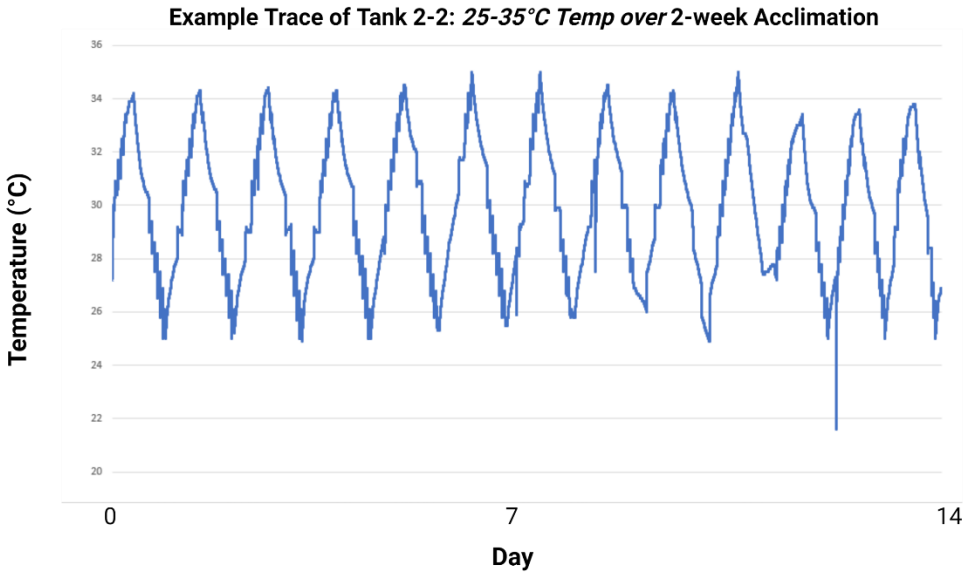

Figure 0.6 Representative trace of tank 2-2 (25-35 Temp.).

Example of fluctuating temperature regime over two-week acclimation. Random drops in temperatures are attributed to system failure.

1.12.4 Brain and gill hsp47 and hsp90 are upregulated in the presence of multiple stressors

Hsp47 was significantly different across all *Temp.* treatments when compared to the *Control* in the gills*;* ~5.4-fold at 27°C (Tukey HSD, p<0.0001), ~9.8-fold at 37°C (Tukey HSD, p=0.0003), and ~16.7-fold at 27-37°C (Tukey HSD, p<0.0001; Fig 3.7Ai). Likewise, in the presence of VFX, *hsp47* was increased ~6.9-fold in the *27°C Temp.+VFX* group (Tukey HSD, p<0.0001), ~2.3-fold in the *37°C Temp.+VFX* (Tukey HSD, p=0.0251), and ~6.1-fold in the *27-37°C Temp.+VFX* (Tukey HSD, p=0.0007) compared to the *Control* (Fig 3.7Ai). *Hsp47* was significantly different across treatment groups (Two-way ANOVA, $F_{2,62}$=54.28, p<0.0001), but not across temperatures (Two-way ANOVA, $F_{2,62}$=1.885, p>0.05) and no interaction was observed (Two-way ANOVA, $F_{4,62}$=1.136, p>0.05; Fig 3.7Ai).

Hsp70 expression in the gills increased ~3.0-fold in the *25-35°C Temp.* group (Mann Whitney U=7.00, p=0.020833), but no significant changes were seen in the *25°C* or *35°C Temp.* groups when compared to the control (Mann Whitney U=11.00, p>0.05; Mann-Whitney U=12.00, p>0.05; Fig 3.7Aii). No significant changes were seen across VFX groups when compared to the *Control* nor between *Temp.-Temp-VFX* groups (Mann Whitney U>10.00, p>0.05; Fig 3.7Aii).

Gill *hsp90* expression was upregulated across all *Temp.* groups; ~5.8-fold at 25°C (Mann-Whitney U=5.00, p=0.0088), ~10.5-fold at 35°C (Mann-Whitney U=10.00, p=0.029), and ~5.3-fold at 27-37°C (Mann-Whitney U=9.00, p=0.029; Fig 3.7Aiii) compared to the *Control*. Similarly, *hsp90* expression significantly increased in all *Temp.+VFX* groups; ~5.9-fold at 27°C (Mann-Whitney U=4.00, p=0.0056), ~0.96-fold at 35°C (Mann-Whitney U=12.00, p=0.038), and ~2.8-fold at 25-35°C (Mann-Whitney U=6.00, p=0.019; Fig 3.6Aiii) relative to the *Control*. No significant differences were seen between *Temp-Temp.+VFX* groups (Mann Whitney U>19.00, p>0.05; Fig 3.7Aiii).

Brain *hsp70* was significantly increased ~5.9-fold in the *25°C Temp.* group (Tukey HSD, p<0.0001), but no changes were seen in the *35°C Temp.* and *25-35°C Temp.* groups relative to the *Control* (Tukey HSD, p>0.05; Fig 3.7Biv). Likewise, brain *hsp70* was significantly upregulated ~5.2-fold in the *25°C Temp.+VFX* group (Tukey HSD, p<0.0001), but no differences were seen at high or diurnal

temperatures compared to the *Control* (Tukey HSD, p>0.05; Fig 3.7Biv). No significant changes were seen between *Temp-Temp.+VFX* groups across all temperature treatments (Tukey HSD, p>0.05; Fig 3.7Biv). *Hsp70* was significant across temperature (Two-way ANOVA, $F_{3,68}$=46.14, p<0.0001), and treatment (Two-way ANOVA, $F_{1,68}$=6.897, p=0.011), but no interaction was seen between temperature and treatment (Two-way ANOVA, $F_{3,68}$=1.313, p>0.05).

Hsp90 expression in the brain was significantly upregulated across all *Temp* groups; ~7.7-fold at 25°C (Mann-Whitney U=0.000, p=0.00076), ~1.1-fold at 35°C (Mann-Whitney U=12.00, p=0.0058), and ~0.87-fold at 25-35°C when compared to the *Control* (Mann-Whitney U=19.00, p=0.019; Fig 3.7Bv). Similarly, brain *hsp90* increased ~1.2-fold in the *25°C Temp.+VFX* (Mann-Whitney U= 0.00, p=0.000032), and ~0.36-fold in the *35°C Temp.+VFX* (Mann-Whitney U=20.00, p=0.046). No differences were observed in the *25-35°C Temp.+VFX* group (Mann-Whitney U=44.00, p>0.05). A significant difference was observed in the *25°C Temp.- 25°C Temp.+VFX* group (Mann-Whitney U=4.00, p=0.0090), but no changes were seen at 35°C or 25-35°C between treatments (Mann-Whitney U>28.00, p>0.05; Fig 3.7Bv). ANOVA statistics are reported in Appendix Table 6.4.

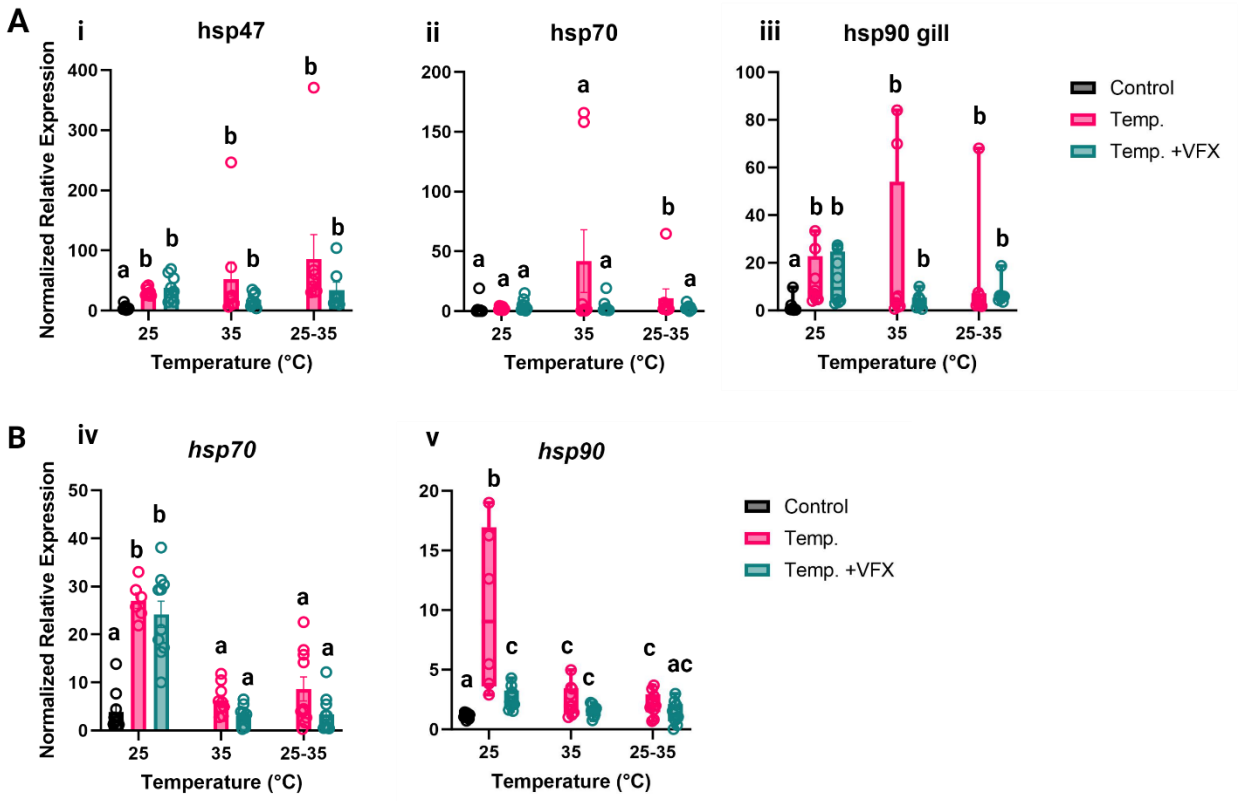

Figure 0.7 Normalized relative expression of HSPs.

Adult mixed-sex zebrafish A) gill i) *hsp47*, ii) *hsp70*, and iii) *hsp90*, and B) brain iv) *hsp70*, and v) *hsp90* was assessed following 24 h acute heat stress with or without VFX (n=10 fish/group; n=6 fish/group in the *25°C Temp.* group). Data was log or sqrt transformed followed by Two-way ANOVA and Tukey post hoc. Alternatively, a Mann-Whitney U test was performed with the Holm-Sidak correction ($p<0.05$) and presented as a boxplot with the median. Different lettering indicates significance.

1.12.5 VFX does not impact cytokine expression in the gills and brain of zebrafish following acclimation.

Il-8 expression in the gills was not significantly impacted across *Temp.* groups (Tukey HSD, p>0.05) nor *Temp.+VFX* groups (Tukey, HSD, p>0.05) when compared to the *Control* (Fig 3.8Ai). No impacts were seen in temperature treatment between *Temp.-Temp.+VFX* groups (Tukey HSD, p>0.05). A significant temperature impact was seen (Two-way ANOVA, $F_{2,61}$=4.440, p=0.016), but no changes were seen between treatment groups (Two-way ANOVA, $F_{2,61}$=0.4523, p>0.05) and no interaction was observed (Two-way ANOVA, $F_{4,61}$=2.462, p>0.05; Table A1).

Gill *tnfα* expression was not significantly changed across all groups relative to the *Control* (Tukey HSD, p>0.05; Fig 3.8Aii). Additionally, no changes were seen across temperature groups (Tukey HSD, p>0.05), treatment groups (Tukey HSD, p>0.05), and no interactive impacts were demonstrated (Tukey HSD, p>0.05; Fig 3.8Aii).

Brain *il-1β* expression was significantly downregulated ~0.59-fold in the *25-35°C Temp.* group relative to the *Control* (Tukey HSD, p=0.0027), but no changes were observed in the *Temp.* groups at constant temperatures (Tukey HSD, p>0.05). No significant changes in expression were seen across *Temp.+VFX* groups (Tukey HSD, p>0.05; Fig 3.8Biii). *il-1β* was significant across temperature groups (Two-way ANOVA, $F_{2,73}$=3.760, p=0.028), and treatment groups (Two-way ANOVA, $F_{2,73}$=7.626, p=0.0010), but no interactive impacts were observed (Two-way ANOVA, $F_{4,73}$=1.872, p>0.05; Fig 3.8Biii).

Tnfα expression in the brain was not significantly different across *Temp.* groups (Mann-Whitney U>24, p>0.05; Fig 3.8Biv). Interestingly, *tnfα* expression was downregulated ~0.68-fold in the *25°C Temp.+VFX* (Mann-Whitney U=19.00, p=0.036743), and in the *35°C Temp.+VFX* group (Mann-Whitney U=9.00, p=0.003147), but not in the *25-35°C Temp.+VFX* group (Mann-Whitney U=41.00, p>0.05). No changes were observed between *Temp.-Temp.+VFX* groups (Mann-Whitney U>13.00, p>0.05; Fig 3.8Biv). ANOVA statistics are reported in Appendix Table 6.4.

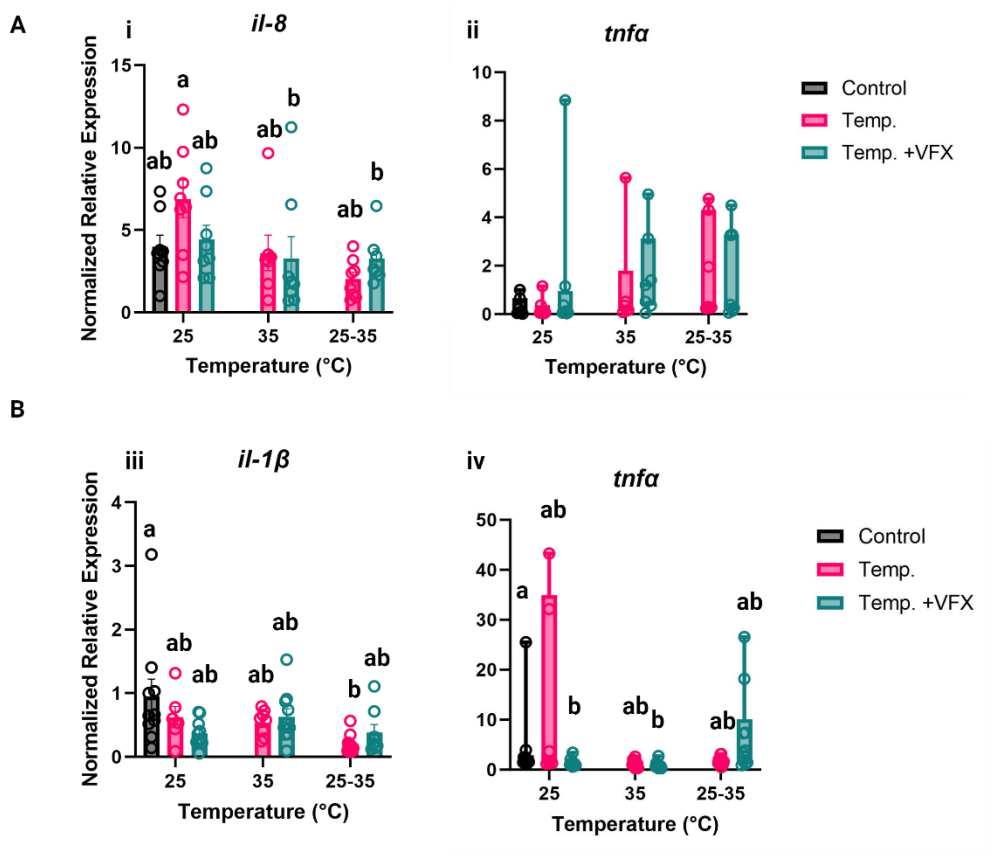

Figure 0.8 Normalized relative expression of immune cytokines.

The expression of zebrafish A) gill i) *il-8*, and ii) *tnfα*, and B) brain iii) *il-1β*, and iv) *tnfα* were assessed with or without 1µg/L of VFX (gills: n=8; brain: n=10, excluding *25°C Temp* n=6). Two-way ANOVA or Mann-Whitney non-parametric tests were performed, and Tukey post hoc test was used for pairwise analysis. The Holm-Sidak correction was applied to non-parametric data to correct for pairwise error and data is presented in a boxplot with the median. Different lettering indicates significance.

1.12.6 VFX does not impact enzymatic activity in the brain and the gills of zebrafish

Gill PK was not statistically different across treatment groups when compared to the *Control* (Tukey HSD, p>0.05; Fig 3.9Ai). PK was significant across treatment groups (Two-way ANOVA, $F_{2,61}$=4.039, p=0.023), but not across temperature groups (Two-way ANOVA, $F_{2,61}$=1.248. p=0.29), and no interaction was noted (Two-way ANOVA, $F_{4,61}$=0.6095, p=0.66; Fig 3.9Ai; Table A1).

LDH activity in the gill was significantly upregulated ~1.6-fold in the *35°C Temp.* (Tukey HSD, p=0.0030) and ~1.8-fold in the *25-35°C Temp.* (Tukey HSD, p=0.0191), but no changes were observed in the *25°C Temp.* group relative to the *Control* (Tukey HSD, p>0.05; Fig 3.9Aii). Additionally, LDH expression was significantly increased in all *Temp.+VFX* groups; ~1.8-fold at 25°C (Tukey HSD, p=0.0042), ~1.5-fold at 35°C (Tukey HSD, p=0.0086), and ~2.5-fold at 25-35°C when compared to the *Control* (Tukey HSD, p<0.0001). No differences were seen between *Temp.-Temp.+VFX* groups (Tukey HSD, p>0.05). LDH activity was significant across treatment groups (Two-way ANOVA, $F_{2,63}$=33.16, p<0.0001), but not across temperature groups (Two-way ANOVA, $F_{2,63}$=0.5688, p=0.57), and no interactive impacts were seen (Two-way ANOVA, $F_{4,63}$=0.7400, p=0.57; Fig 3.9Aii; Table A1).

Brain PK expression was not significantly different across *Temp.* groups when compared to the *Control* (Tukey HSD, p>0.05; Fig 3.9Biii). Interestingly, compared to the *Control*, brain PK was not significantly upregulated at constant temperatures in *Temp.+VFX* groups (Tukey HSD, p>0.05), however, under diurnal temperature fluctuations, a ~1.3-fold increase was seen in the *25-35°C Temp.+VFX* group (Tukey HSD, p=0.018; Fig 3.9Biii). Brain PK was significant across treatment groups (Two-way ANOVA, $F_{2,63}$=4.516, p=0.015), and an interactive impact was noted (Two-way ANOVA, $F_{4,63}$=3.131, p=0.021), but no changes were seen across temperature groups (Two-way ANOVA, $F_{2,63}$, p=0.156; Fig 3.9Aiii; Table A1).

Brain LDH activity was not statistically significant across temperature and treatment groups when compared to the *Control* (Tukey HSD, p>0.05; Fig 3.9Biv). LDH was significant between temperature groups (Two-way ANOVA, $F_{2,63}$=3.917, p=0.025). No significant changes were seen between treatment

groups (Two-way ANOVA, $F_{2,63}$=1.802, p=0.173), and no interaction between temperature and treatment was seen (Two-way ANOVA, $F_{4,63}$=1.531, p=0.204; Fig 3.9Biv).

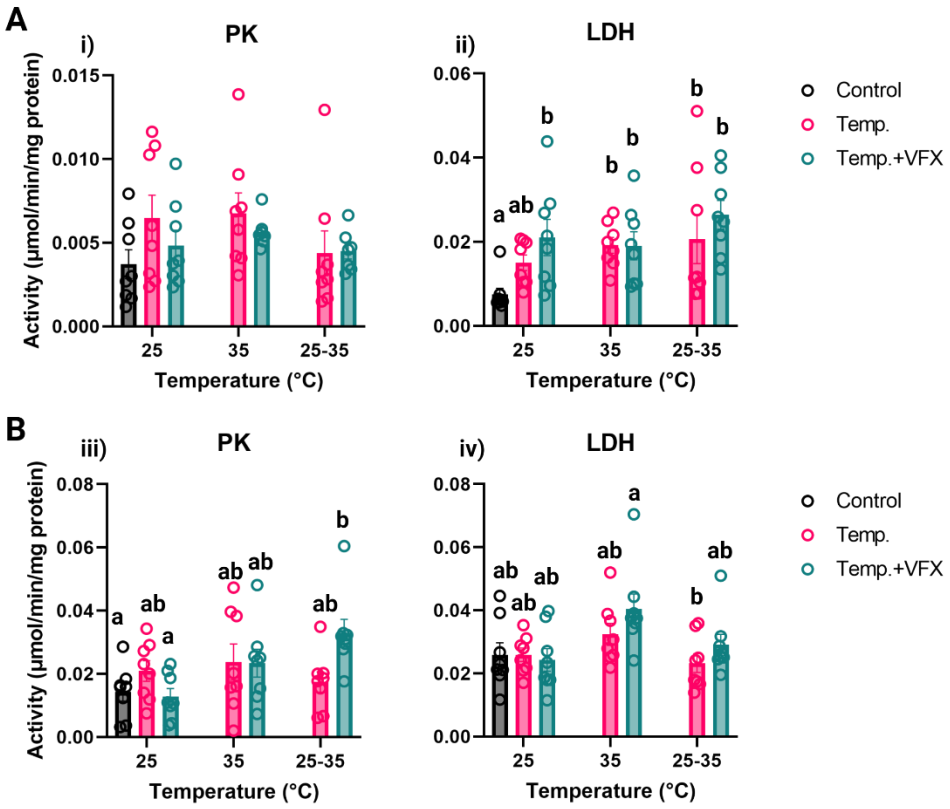

Figure 0.9 Enzymatic activity of cytosolic PK and LDH.

Zebrafish (A) gill i) PK, ii) LDH, and (B) brain iii) PK, and iv) LDH activity was determined following 24 h heat stress with or without 1 µg/L of VFX. Data was log or sqrt transformed and analyzed via two-way ANOVA, followed by Tukey post hoc test. (gills: n=8 fish/group). Different lettering indicates significance.

Gill CS was significantly increased across all *Temp.* groups; ~0.71-fold at 25°C (Mann-Whitney U=5.00, p=0.012), ~2.2-fold at 35°C (Mann-Whitney U=0.00, p=0.0009), and ~2.1-fold at 25-35°C relative to the *Control* (Mann-Whitney U=6.00, p=0.012; Fig 3.10Ai). Additionally, compared to the *Control*, CS expression was upregulated ~2.2-fold in the *25°C Temp.+VFX* group (Mann-Whitney U=0.00, p=0.0009), ~3.0-fold in the *35°C Temp.+VFX* group (Mann-Whitney U=0.00, p=0.0009), and ~3.2-fold in the *25-35°C Temp.+VFX* group (Mann-Whitney U=0.00, p=0.0009; Fig 3.10Ai). No significant differences were seen between *Temp-Temp.+VFX* groups (Mann-Whitney U>13.00, p>0.05; Fig 3.10Ai).

Expression of brain CS was upregulated ~1.7-fold in the *35°C Temp.* group (Mann-Whitney U=0.00, p=0.0004), and ~1.8-fold in the *25-35°C Temp.* group (Mann-Whitney U=0.00, p=0.0004) compared to the *Control* (Fig 3.10Bii). No significant changes were seen in the *25°C Temp.* group relative to the *Control* (Mann-Whitney U= 16.00, p>0.05; Fig 3.10Bii). Across all *Temp.+VFX* groups, brain CS expression was increased ~1.5-fold at 25°C (Mann-Whitney U=2.00, p=0.0006), ~2.0-fold at 35°C (Mann-Whitney U=0.00, p=0.0004), and ~2.3-fold at the 25-35°C (Mann-Whitney U=0.00, p=0.0006) relative to the *Control* (Fig 3.10Bii). Interestingly, a significant difference was seen between the *25°C Temp.* and *25°C Temp.+VFX* group (Mann-Whitney U=8.00, p=0.031), but no changes were seen between *35°C* or *25-35°C Temp-Temp+VFX* groups (Mann-Whitney U>25.00, p>0.05; Fig 3.10Bii).

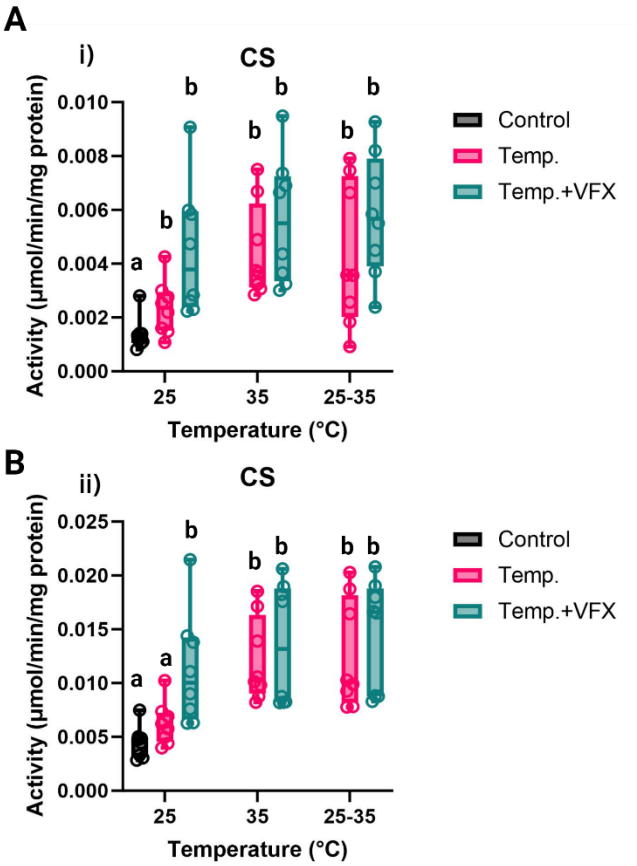

Figure 0.10 Enzymatic activity of mitochondrial CS.

Zebrafish CS was assessed in the (A) gills, and (B) brain following 24 h heat stress with or without 1 µg/L of VFX (n=8 fish/treatment group). Data was assessed via Mann-Whitney U test and the Holm-Sidak correction was applied ($\alpha<0.05$) and data is presented as a boxplot with the median. Different lettering indicates significance.

1.12.7 Diurnal temperature impacts Hif1α expression in the zebrafish brain

The activity of one Hif were measured following 96 h exposure to determine if temperature and VFX modulate hif activity in zebrafish brain (Fig 3.11). *Hif1α* activity showed no differences across *25°C* and *35°C Temp*. groups when compared to the *Control* (Mann-Whitney U>16.00, p>0.05; Fig 3.11). However, in the *25-35°C Temp.* group *hif1a* was downregulated 0.67-fold compared to the *Control* (Mann-Whitney U= 10.00, p=0.0089). Similar results were seen for VFX groups as no changes were seen at constant temperatures (Mann-Whitney U>22.00, p>0.05), and *25-35°C Temp.+VFX* was downregulated 0.49-fold relative to the *Control* (Mann-Whitney U=11.00, p=0.045). No differences were seen between *Temp.-Temp+VFX* groups (Mann-Whitney U>17.00, p>0.05; Fig 3.11).

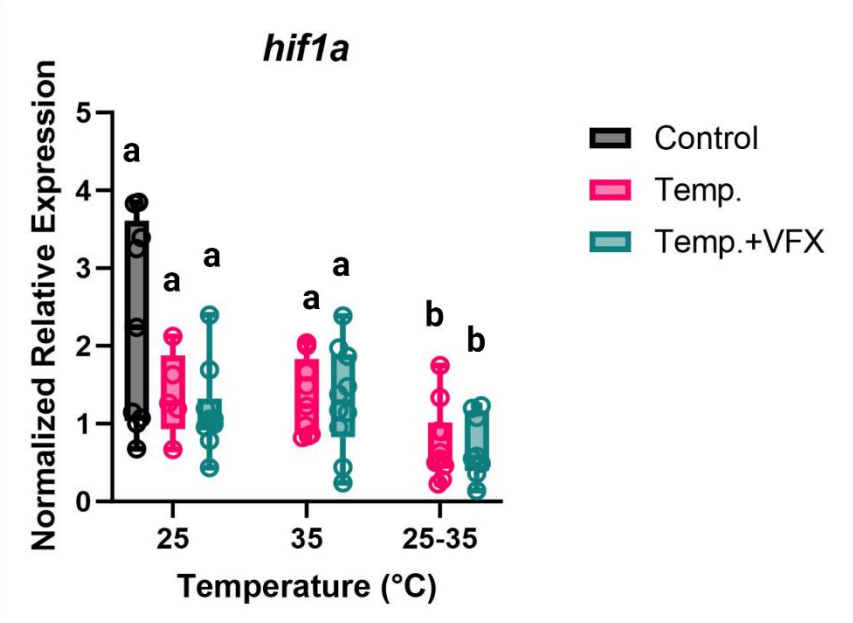

Figure 0.11 Normalized relative expression of Hif1-alpha.

Zebrafish *hif1α* expression was assessed following 15 d exposure. Data were log transformed and analyzed via a Mann-Whitney U test was performed with Holm-Sidak correction ($p<0.05$) and data is presented as a boxplot with the median. Different letters indicate significance (n=10 fish/ treatment group, excluding *25°C Temp.* n=6).

Chapter 4 Discussion

Several studies have addressed the impacts of contaminant exposure on various physiological parameters, but little to date is known about their impact on the immune system of fish. Further, investigations into multiple stressor environments, particularly pertaining to the impacts of diurnal temperature is a newer avenue of study in relation to fish physiology. To our knowledge, this is the first study aimed at understanding the inflammatory implications of diurnal temperature and contaminant exposure in fish.

1.12.8 Cytokine responses

Il-1β gill expression was significantly downregulated in the presence of VFX and diurnal temperature exposure (Ch. 2, Fig. 2.4i). This finding is similar to Dawe et al., (2024), who noted wild-caught female johnny darters (*Etheostoma nigrum*) exposed to WWTP effluent had decreased *il-1β* expression in their gills. Alternatively, unpublished data from our lab suggests that *il-1β* increases in expression following 96 h of exposure to VFX at 27ºC (Dawe and Craig, unpublished data). These findings prompted us to question whether *il-1β* expression in constant laboratory settings are capturing the dynamic range of the immune response that would be occurring in the wild. *Il-1β* is a complex and diverse immunological molecule, and it's well-established that the cell type, animal species, microenvironment, and concentration of PAMPs/DAMPs impact its stimulation and secretion (Lopez-Castejon & Brough, 2011). Additionally, due to its rapid onset and potency as a pro-inflammatory cytokine (Lopez-Castejon & Brough, 2011), it is plausible that following our 96 h exposure, we missed its initial stimulation. Nonetheless, this does not explain the differences we see at constant temperatures versus diurnal temperature exposure, suggesting some interaction between temperature and VFX is impacting *il-1β* expression. It is plausible the discrepancies seen in the data could be due to the high variability across treatment fish. Interestingly, these findings are consistent with mammalian models, such as mice, that typically show a depressed cellular and humoral immune response, including suppression of *il-1β* and *tnfα* in the presence of several

antidepressants, including VFX (Hajhashemi et al., 2015; Nazimek et al., 2016; Ohgi et al., 2013). Unfortunately, *il-1β* expression was not able to be measured in the gills during our acclimation exposure (Ch. 3), and thus further investigation is required to determine the nature of *il-1β* expression in the gills.

Gill *il-8 (CXCL8)* and *tnfα* exposure in our pilot study (Ch.2, Fig 2.4ii, iii) was significantly upregulated across all temperature groups in the presence of VFX. I*l-8* is considered a key biomarker for immune activation due to its role in neutrophil recruitment (Semple & Dixon, 2020). This may suggest that it promotes inflammation in the gills of zebrafish. However, our acclimation study (Ch. 3) revealed no significant changes in *il-8* or *tnfα* expression in the gills (Fig 3.8A). Weber et al. (2023) saw similar results as the acclimation study (Ch. 3), as *il-8* expression remained unchanged following VFX and CT_{max} exposure. Another study on zebrafish eggs revealed that exposure to paroxetine, another common antidepressant, did not stimulate *il-8* or *tnfα* expression at 1 or 5 mg/L after 5 d (Y. Zhu et al., 2023). The lowest dose from this study is 1000x larger than the concentration used in our studies but demonstrates similar results to our acclimation study, as *il-8* and *tnfα* expression remained unchanged (Ch. 3. Fig 3.8A). It is possible the cytokine responses between the two studies were dissimilar if the fish in the pilot study (Ch. 2) were ill prior to experimentation. Unfortunately, there is no way to confirm these results due to a lack of tank replicates used in our initial experiment. Alternatively, the time of exposure to VFX (96 h vs. 24 h) could account for these differences. Therefore, it is difficult to draw conclusions regarding the impacts of VFX exposure on the inflammatory response in zebrafish gills without further experimentation. Future studies could look at earlier timepoints, as cytokine mRNA tend to have a rapid onset, with peak concentrations occurring between 4-8 h (Sullivan et al., 2000).

1.12.9 HSPs

To determine if temperatures and VFX exposure impacted the HSP response, we measured three key HSPs: *hsp47*, *hsp70,* and *hsp90*. *Hsp47, hsp70,* and *hsp90* are important molecular chaperones and are essential for protein folding and protein assembly and it is well established these proteins play an integral role in the heat shock response (Roberts et al., 2010). Following 96 h of exposure to temperature (Ch. 2),

gill *hsp70* expression returned to baseline, while *hsp47* and *hsp90* were only comparable to the control at diurnal temperatures and remained upregulated at 37°C (Fig. 2.5). Elevations of *hsp47,* and *hsp90* at 37°C could be explained by the chosen temperature in this exposure, which is close to the thermal maximum of zebrafish reported in previous studies (~40°C; Morgan et al., 2019; A. V. Weber et al., 2023). Nonetheless, it is interesting that *hsp70* was downregulated, as previous studies in *Channa striatus* liver showed *hsp70,* and *hsp90* remained significantly upregulated even after 4 d of heat stress at 36°C (Purohit et al., 2014). This may indicate that *hsp47* and *hsp90* could play a more significant thermal protective role in zebrafish compared to *hsp70* following acute heat stress. These considerations are odd however, as *hsp70* and *hsp90* are both cytoplastic HSPs with similar biological functions (Mohanty et al., 2018). Our acclimation study (Ch. 3) seems to further confirm these results, as *hsp47* and *hsp90* remained upregulated following acute thermal stress, but *hsp70* did not (Fig 3.7A). Manzon et al., (2022) saw similar yet conflicting results; following thermal acclimation to 18°C over several months, *hsp47* and *hsp70* were significantly upregulated following an acute heat stress in the liver, gill, and white muscle of lake whitefish, while no changes were seen in *hsp90.* Differences in regulatory mechanisms between *hsp47* and *hsp70* have been demonstrated in zebrafish, which could explain their differences in expression (Lele et al., 1997). However, this fails to explain the differences observed between *hsp70,* and hsp*90*.

Interestingly, in the presence of VFX, transcript expression remained elevated for most HSPs and temperature groups, apart from *hsp47* at 27°C in our pilot study (Ch.2, Fig. 2.5). These results corroborate findings from previous studies, which demonstrate that HSPs remain upregulated in the presence of VFX (Luu et al., 2021; A. V. Weber et al., 2023). Elevation of these transcripts is likely attributed to cytoprotective function as a means of mitigating and preventing protein damage during contaminant exposure (B. P. Mohanty et al., 2018). Gill HSP transcripts from our acclimation exposure (Ch. 3) yielded somewhat similar, but slightly dampened results, likely due to the shorter (24 h) VFX exposure (Fig. 3.7A). Interestingly, VFX did not elicit any changes in *hsp70,* while *hsp47* and *hsp90* were both significantly elevated across VFX groups (Fig 3.7A). It is plausible the lack of response seen in *hsp70* through the course

of both exposures may be explained by the tissue chosen, as HSP responses are tissue-dependent (Guo et al., 2023). However, in rainbow trout (*Oncorhynchus mykiss*), *hsp70* expression in the spleen and gills was not different between upper tolerance non-preconditioned and preconditioned fish, but expression was still significant when compared to the control (Guo et al., 2023). Why zebrafish *hsp70* was not upregulated despite being an inducible HSP is unknown. This prompts further investigation of the heat shock response in zebrafish gills, especially when considering isolated contaminants like VFX.

Brain HSPs showed relatively interesting results, as *hsp70* was only upregulated at 25°C following acute heat stress (Fig 3.7B). These results suggest that acclimation to elevated and diurnal temperatures may mitigate brain *hsp70* activation following a thermal stressor. These findings are positive, as there are survival trade-offs associated with the energetic demands of the heat shock response following chronic heat exposure (Narum et al., 2013) However, this does not explain why we saw no changes in *hsp70* following contaminant exposure at elevated and diurnal temperatures (Fig 3.7B). *Hsp70* has been well documented to have a response to environmental contaminants and has been implicated as useful biomarker of anthropogenic exposure (Mitra et al., 2018; Mohanty et al., 2010; Mohanty et al., 2018), thus it is interesting that no induction of *hsp70* was seen in the brain tissue. Additionally, toxicity of pollutants has been positively associated with elevated temperature exposure (Little & Seebacher, 2015). As previously mentioned, it is plausible that exposure time to VFX in our acclimation study (Ch. 3) was not long enough to induce *hsp70* expression. However, *hsp90* was downregulated in the presence of VFX when compared to the temperature group at 25°C (Fig 3.7B). These alterations in expression of *hsp90* would suggest that to some degree, the zebrafish brain must have been impacted by VFX within 24 h.

A future step would be to assess the protein concentrations of brain and gill hsp70. A recent study in adult zebrafish found that when their environment approaches lethal temperatures, translation of hsp70 mRNA is rapidly induced (Mottola et al., 2020). Importantly however, this study noted that both hsp70 mRNA transcription and translation were coupled, and translation did not occur without elevated mRNA transcript levels (Mottola et al., 2020). Therefore, it is hard to say for certain that protein expression would

be elevated in our situation as mRNA expression was obsolete, but this could be a starting point for future studies. Nonetheless, it is difficult to pinpoint exactly why *hsp70* was not impacted by VFX, and further investigation should be done on zebrafish to understand the underlying mechanisms of *hsp70* expression following temperature and contaminant exposure.

Brain *hsp90* showed elevated transcript expression across all treatment groups (Ch. 3). Interestingly an interactive suppressor impact between heat stress and VFX was seen in *hsp90* expression at 25°C (Fig 3.7B). Based on our understanding of temperature and environmental toxicity, we speculated that acute heat shock and VFX would have had an increasingly coupled impact on *hsp90* expression. This interaction could be problematic for brain function in zebrafish, especially for fish found in thermally constant environments. The suppression of *hsp90* suggests that the heat shock response may be attenuated in the presence of multiple stressors (Fig. 3.7Bii), which could have implications on the accumulation of misfolded proteins. HSPs have many neuroprotective roles, and when dysregulated can cause misfolded proteins to accumulate; this is the hallmark of several neurodegenerative diseases including Alzheimer's, Parkinson's, and Huntington's Disease (Dukay et al., 2019). Our study demonstrates that brain *hsp90* expression may be differentially impacted by constant temperatures, but thermal acclimation to wild-like temperatures may mitigate dysregulation caused by thermal stress and contaminant exposure.

1.12.10Hif1α

We assessed the impacts of elevated temperatures in the brain and gills of zebrafish, therefore we decided to look at Hif1α as an indicator of hypoxic stress. The gills revealed no changes in *hif1α* activity, which is unsurprising as dissolved oxygen content was >~6.5 mg/L (Ch. 2, Appendix Fig 6.5). There were no temperature impacts nor VFX impacts noted in the gills. Interestingly, brain *hif1α* in our acclimation study was significantly impacted, but only at diurnal temperatures (Ch. 3, Fig 3.11). This response did not seem to be driven by VFX contamination, suggesting that the thermal swings were responsible for alternations in *hif1α.* Hif1α is considered a primary regulator of the hypoxic response and increases in Hif1α are often associated with physiological impacts (Kopp et al., 2014). Interestingly, we saw a decrease in

hif1α expression in the brain, which may suggest that basal *hif1α* responses are different under diurnal temperature fluctuations (Ch. 3, Fig 3.11). It is also plausible that *hif1α* fluctuates as temperatures change. Previous studies have demonstrated that Hif1α increases in roughly 4-hours, then is dramatically reduced after 8-hours of exposure to hypoxia in primary endothelial cells (Jaśkiewicz et al., 2022). Since our zebrafish were sampled at 25°C, it is plausible that our results are reflective of the depression state of *hif1α*. To confirm this, it would be advisable to look at intermediate timepoints during the day and night to observe if these changes are seen on a whole-body level. Little is known regarding the impacts of diurnal temperature fluctuations and their implications on Hifs, thus it is hard to say with certainty what is causing *hif1α* downregulation in the brain.

1.12.11Enzymatic Responses

The glycolytic (PK, LDH) and mitochondrial (CS) enzymes assessed in this study were increased in both the gills and brain (Ch. 3, Fig 3.9). In the gills, increases were seen in LDH and CS across all treatment groups, but no changes were seen in PK (Ch. 3, Fig 3.9A). Furthermore, even though LDH activity was significantly increased in the presence of VFX, these changes were not different than our temperature stress groups, suggesting that VFX did not impact LDH and CS expression (Ch. 3, Fig 3.9A). The gills are important for several diverse physiological functions, including osmoregulation, ventilation, acid-base regulation and, elimination of waste (Foyle et al., 2020; Herrero et al., 2018; Perry & Walsh, 1989), thus the gills have a higher affinity for lactate and glucose as energy substrates (Mommsen, 1984; Perry & Walsh, 1989). Changes in ATP availability can induce lactate metabolism to maintain cellular ATP stores (Brooks, 2020). LDH activity has been shown to increase in response to increasing elevated acclimation temperatures (Cheng et al., 2018; Dalvi et al., 2017; Ekström et al., 2017), which suggests that LDH is necessary for anerobic metabolism at elevated temperatures. Furthermore, our results corroborate with past studies looking at contaminant exposure, where LDH activity did not change in the presence of anthropogenic stressors (Best et al., 2014; Ings et al., 2011; Mehdi et al., 2019). Like LDH, gill and brain CS were increased compared to the control, but these elevations in CS were not attributed to exposure to

VFX (Ch. 3, Fig 3.10). CS is implicated in oxidative metabolism and is a key enzyme in the citric acid cycle. Mehdi et al. (2019) saw similar results for CS expression in zebrafish muscle, as CS was upregulated due to an increase in temperature, but not VFX.

Although PK in the brain was upregulated under diurnal temperatures and VFX exposure, these results are likely inconclusive as the difference in expression was ultimately the result of one fish (Ch. 3, Fig. 3.9B). Other studies investigating the impacts of temperature and contaminant exposure have seen increased PK expression (Mehdi et al., 2018, 2019), but our results suggest neither temperature stress nor VFX impact brain PK activity (Ch. 3, Fig 3.9B). The brain is highly susceptible to lowered oxygen availability caused by hypoxia (Nilsson, 2001), thus temperature stressors would be expected to impact brain enzymatic activity. Studies on intertidal sculpins demonstrate that elevated PK and LDH activities in the brain are associated with a higher capacity for glycolysis and subsequent ATP production following hypoxic conditions (Mandic et al., 2013). Zebrafish are considered relatively hypoxia-tolerant species (Kopp et al., 2014), but substantial elevation in PK and LDH were not seen. It is possible that the elevation in temperature (35°C) was not enough to induce profound changes in brain enzymes. Zebrafish in the wild experience dynamic and sudden shifts in temperature, ranging from 6°C up to 40°C throughout the year, and upwards of 6°C diurnal fluctuations (Angiulli et al., 2020). Therefore, it is plausible that zebrafish metabolism is relatively robust during acute thermal stress, ultimately resulting in our study seeing little changes in anerobic enzyme activity in the brain.

1.12.12Conclusions and Future Directions

Climate change and increases in man-made alterations to ecosystems are predicted to have coupled impacts on thermal warming. Furthermore, effluent release continues to introduce various environmental contaminants into global watersheds, having several physiological impacts on fish species. Here we demonstrate some of the first insights into the impacts of diurnal temperature and xenobiotic exposure in the context of fish immunology. This study demonstrates that VFX exposure does not seem to impact the inflammatory response in zebrafish gills or brain. Interactive impacts of VFX at diurnal temperatures seems

to have comparable results to constant elevated temperatures, suggesting that constant temperatures often used in lab provide reliable comparisons to environmental temperature dynamics. Contrastingly, we show evidence that basal expression of HSPs may be different under fluctuating temperature regimes, thus further work should be done to assess how temperature fluctuations impact baseline expression of transcripts. Furthermore, we demonstrated that acclimation to elevated and diurnal temperatures may have cytoprotective impacts for organisms living in diverse environments, especially in the context of the heat shock response. Future studies should be done to address the discrepancies associated with earlier timepoints to gain a full understanding of inflammatory and heat shock responses in the presence of multiple stressors. It would also be advisable to look at infection with a live pathogen to better understand the threshold of the inflammatory response during diurnal temperature exposures. Taken together, this study represents a new direction in fish physiology aimed at addressing how laboratory studies can better integrate field-based dynamics to understand fish physiology in a controlled setting.

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Appendix

Table 0.1 Concentrations of VFX (ng/L).

Concentrations of VFX each day 1-hour post-addition over the course of 96-hour exposure.

Day	**0**	**1**	**2**	**3**	**4**
27 VFX Temp+VFX	0	1430.3	N/A	1832	1419.9
37 VFX Temp+ VFX	0	1626.9	N/A	2568.5	1661.6
27-37 Temp+VFX	0	1628.1	N/A	1301.4	1324

Note that amber bottles with Day 2 samples cracked and were excluded from analysis.

Table 0.2 Average concentration of VFX (ng/L) across tank replicates.

Concentrations of VFX 1- and 24 h post-addition for acclimation exposure.

	Hour	
Treatment	*1*	*24*
25 Temp+VFX	1119.8	1232.2
35 Temp+VFX	1177.7	1345.5
25-35 Temp+VFX	1202.4	1181.3

Averages are of tank replicate for each treatment (n=3)

Table 0.3 Two-way ANOVA F-statistics for gene targets.

Mann-Whitney U test were performed on N/A targets.

	Two-Way ANOVA [F (DFn, DFd) =F-ratio, p-value]		
Targets	Temperature	VFX	Temperature x VFX
Cytokines			
il-1β	**F (2,41) =19.11; p<0.0001**	**F (1,41) =11.02; p=0.0019**	**F (2,41) =10.91; p=0.0002**
tnfα	N/A	N/A	N/A
il-8	F (2,41) = 0.3723; p=0.6914	**F (1,41) =28.71; p<0.0001**	F (2,41) =1.696; p=0.1957
HSPs			
hsp70	**F (2,39) =16.22; p<0.0001**	**F (1,39) = 27.82; p<0.0001**	F (2,39) =1.722; p=0.1920
hsp90	N/A	N/A	N/A
hsp47	**F (2,42) =26.09; p<0.0001**	**F (1,42) =20.45; p<0.0001**	**F (2,42) =3.624; p=0.0353**
HIFs			
hif1α	F (2,41) = 0.3880; p=0.6809	F (1,41) = 0.07882; p=0.7803	F (2,41) =1.1250; p=0.2973

**Note: DFn= degrees of freedom numerator; DFd= degrees of freedom denominator (α=0.05), significant results are shown in bold.*

Table 0.4 Two-way ANOVA F-statistics for gene targets.

Mann-Whitney U test were performed on N/A targets.

		Two-Way ANOVA [F (DFn, DFd) =F-ratio, p-value]		
Targets		Temperature	VFX	Temperature x VFX
Cytokines				
il-1β	**Gill:**	N/A	N/A	N/A
	Brain:	**F(2,73)=3.760; p=0.0279**	**F(2,73)= 7.626; p=0.0010**	F(4,73)= 1.872; p=0.1245
tnfα	**Gill:**	F(2,62)= 2.938; p=0.0604	F92,62)= 2.669; p=0.0773	F(4,62)=0.8592; p=0.4935
	Brain:	N/A	N/A	N/A
il-8	**Gill:**	**F (2,61) =4.440 ; p=0.158**	F (2,61) =0.4523; p=0.6383	F (4,61) =2.462; p=0.0546
	Brain:	N/A	N/A	N/A
HSPs				
hsp70	**Gill:**	N/A	N/A	N/A
	Brain:	**F(3,68)= 46.14; p<0.0001**	**F(1,38)= 6.897; p=0.0107**	F(3,68)= 1.313; p=0.2773
hsp90	**Gill:**	N/A	N/A	N/A
	Brain:	N/A	N/A	N/A
hsp47	**Gill:**	F (2,62) =1.885; p=0.1605	**F (2,62) = 54.28; p<0.0001**	F (4,62) =1.136; p=0.3481
	Brain:	N/A	N/A	N/A
HIFs				
hif1α	**Gill:**	N/A	N/A	N/A
	Brain:	N/A	N/A	N/A

**Note: DFn= degrees of freedom numerator; DFd= degrees of freedom denominator (α=0.05), significant results are shown in bold.*

Table 0.5 DO content (mg/L).

The DO content over the course of 96-hour exposure. Data was obtained from LoggerPro and averages were calculated.

Experimental Group	Average Dissolved Oxygen (mg/L)	Standard error of the mean (SEM)
Control		
27°C	8.9	0.1
Temp.		
37°C	6.5	0.2
27-37°C	9.1	0.8
Temp.+VFX		
27°C	9.2	0.06
37°C	6.8	0.1
27-37°C	9.7	0.9

Table 0.6 Average DO Content (mg/L).

Average DO content in mg/L over the course of 15-day exposure.

Experimental Group	Average DO (mg/L)	Standard error of the mean (SEM)
Control		
25°C	6.9	0.1
Temp.		
25°C	6.8	0.5
35	5.3	0.6
25-35°C	6.2	0.6
Temp.+VFX		
25°C	7.0	0.2
35°C	6.0	0.2
25-35°C	6.5	0.3

Table 0.7 Average temperatures for heat exposure.

The average temperatures of experimental groups over 24 h heat exposure.

Experimental Group	Average Temperature (°C)	Standard error of the mean (SEM)
Control		
25°C	N/A	N/A
Temp.		
25°C	33.5	0.6
35	34.1	0.2
25-35°C	34.3	0.1
Temp.+VFX		
25°C	33.8	0.7
35°C	34.1	0.1
25-35°C	34.2	0.3

Note that the control was not subjected to heat stress.