INTERACTIVE EFFECTS OF WASTEWATER EFFLUENT AND HYPOXIA ON THE METABOLIC PHYSIOLOGY AND HEALTH OF MUMMICHOG KILLIFISH (*FUNDULUS HETEROCLITUS*)

INTERACTIVE EFFECTS OF WASTEWATER EFFLUENT AND HYPOXIA ON THE METABOLIC PHYSIOLOGY AND HEALTH OF MUMMICHOG KILLIFISH (*FUNDULUS HETEROCLITUS*)

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TITLE: Interactive effects of wastewater effluent and hypoxia on the metabolic physiology and health of mummichog killifish (*Fundulus heteroclitus*)

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LAY ABSTRACT

Low oxygen conditions, known as 'hypoxia', frequently occur in aquatic ecosystems that receive municipal wastewater treatment plant (WWTP) effluent. WWTP effluent is a continuous and complex source of pollution, including contaminants that can disrupt fish physiology, affecting their ability to cope with stressors, like hypoxia. The effects of WWTP effluent on the responses of fish to chronic hypoxia are poorly understood. To address this research gap, I examined the effects of hypoxia and WWTP effluent on chronically exposed mummichog killifish. I provide evidence that combined exposure to hypoxia and wastewater affected hypoxia tolerance, gill structure, and depleted energy stores in the brain. My thesis demonstrates that WWTP effluent can disrupt mechanisms that fish use to cope with chronic hypoxia and impair hypoxia tolerance. These findings contribute to the existing body of work that documents the interactive effects of combined stressors in effluent-dominated ecosystems on the physiology and health of fish.

ABSTRACT

Hypoxia often occurs in aquatic ecosystems that receive effluent from municipal wastewater treatment plants (WWTP). WWTP effluent contains contaminants that could disrupt the complex physiological pathways fish use to cope with hypoxia (*e.g.,* pharmaceuticals, polychlorinated biphenyls, and polycyclic aromatic hydrocarbons), but the effects of WWTP effluent on the physiological responses of fish to chronic hypoxia is poorly understood. We exposed mummichog killifish (*Fundulus heteroclitus*) to hypoxia (5 and 2 kPa O_2) and/or WWTP effluent for 21 days in a full factorial design. We then measured hypoxia tolerance, whole-animal metabolism, gill morphology, haematology, and tissue metabolites. In clean water, killifish responded to chronic hypoxia with improvements in hypoxia tolerance – increases in time to loss of equilibrium at 0.5 kPa (t_{LOE}) and decreases in critical O_2 tension (P_{crit}) – in association with increased gill surface area as a result of regression of the interlamellar cell mass (ILCM). Concurrent exposure to wastewater attenuated the increases in t_{LOE} and gill remodeling in chronic hypoxia, and nearly depleted brain glycogen stores. Therefore, exposure to WWTP effluent can disrupt the physiological mechanisms fish use to cope with chronic hypoxia and impair hypoxia tolerance. My research suggests that the combination of stressors near WWTPs can have interactive effects on the physiology and health of fish.

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LIST OF ABBREVIATIONS AND SYMBOLS

5-HT: Serotonin

AHR: Aryl hydrocarbon receptor

AMPK: Adenosine monophosphate protein kinase

ANOVA: Analysis of variance

ARNT: Aryl hydrocarbon receptor nuclear translocator

AS: Aerobic scope

ASR: Aquatic surface respiration

ATP: Adenosine triphosphate

BPA: Bis-phenol A

CEC: Contaminants of emerging concern

CYP1A: Cytochrome P450-1A

EDC: Endocrine disrupting chemical

EPO: Erythropoietin

Hb: Haemoglobin

HIF: Hypoxia-inducible factor

HPLC: High performance liquid chromatography

ILCM: Interlamellar cell mass

LC-MS/MS: Liquid chromatography tandem mass spectrometry

LOE: Loss of equilibrium

MCHC: Mean corpuscular haemoglobin concentration

MMR: Maximum metabolic rate

MO_2: Metabolic rate; O_2 consumption rate

MOD: Moderate hypoxia

N_2: Nitrogen

NEC: Neuroepithelial cell

NH_3: Ammonia

NO_2: Nitrite

NO_3: Nitrate

NOR: Normoxia

NSAID: Non-steroidal anti-inflammatory agent

O_2: Oxygen

PAH: Polyaromatic hydrocarbon

PCB: Polycyclic biphenyl

P_{crit}: Critical oxygen tension

P_{LOE}: Partial pressure of oxygen at loss of equilibrium

PO_2: Partial pressure of oxygen

PPCP: Pharmaceuticals and personal care products

RMR: Resting metabolic rate

SEM: Standard error of the mean

SEV: Severe hypoxia

SMR: Standard metabolic rate

SPE: Solid phase extraction

SSRI: Selective serotonin reuptake inhibitor

t_{LOE}: Time to loss of equilibrium

VEGF: Vascular endothelial growth factor

WW: Wastewater

WWTP: Wastewater treatment plant

THESIS ORGANIZATION AND FORMAT

This thesis is organized in "sandwich" format, as recommended by my supervisory committee. It consists of three main chapters. Chapter one is a general introduction and outlines the background information leading to the objectives and hypotheses of my thesis research. Chapter two is a manuscript prepared for submission to a peer-reviewed scientific journal. Chapter three is an overview of the major findings of this thesis, their implications in fish physiology and ecotoxicology, including suggestions of future directions of research. Appendix A contains data from an additional series of experiments that were conducted during my thesis but are not included as a full data chapter. It will be prepared for publication after my defence.

CHAPTER ONE: **GENERAL INTRODUCTION**

CHAPTER TWO: **EXPOSURE TO WASTEWATER EFFLUENT DISRUPTS HYPOXIA RESPONSES IN KILLIFISH (*Fundulus heteroclitus*)**

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CHAPTER THREE: **GENERAL DISCUSSION**

APPENDIX A

REFERENCES

CONTRIBUTIONS NOT APPEARING IN THESIS

Mehdi, H., Lau, S.C., Synyshyn, C., Salena, M.G., McCallum, E.S., Muzzatti, M., Bowman, J.E., Mataya, K., , Bragg, L.M., Servos, M.R., Kidd, K.A., Scott, G.R., and Balshine, S. (2020). Municipal wastewater as an ecological trap: Effects on fish communities across seasons. Manuscript submitted for publication on August 19, 2020 to Science of the Total Environment.

CHAPTER ONE: GENERAL INTRODUCTION

1.1 Anthropogenic stress in aquatic environments

Global climate change has drastically altered physico-chemical variables (e.g., temperature, precipitation, salinity, pH, and oxygen availability) in aquatic environments. The worsening of environmental abiotic stressors can interact with the sensitivity of aquatic organisms to chemical stressors, like the exposure to anthropogenic pollutants (Hooper et al., 2013). Hypoxia (*i.e.,* low or depleted oxygen) is a prominent abiotic stressor in aquatic systems, which can be attributed to seasonal variations as well as the inputs of anthropogenic pollution (Diaz, 2001; Friedrich et al., 2014). Human impacts have largely accelerated the occurrence of aquatic hypoxia, often in habitats that are not historically hypoxic, which can lead to rapid loss of biodiversity, alteration of food webs, and ecosystem collapse (Diaz, 2001; Diaz & Rosenberg, 2008). Oxygen is essential for aerobic metabolism and generates nearly all of the cellular energy (ATP) required for animal life, so declines in the partial pressure of O_2 (PO_2) in the environment have the potential to be catastrophic to normal physiological function. Environmental hypoxia in freshwater and marine systems is predicted to increase with the progression of climate change (Friedrich et al., 2014; Rabalais et al., 2010). Although some fish have evolved a range of adaptive physiological and behavioural responses to hypoxia, it is unknown how these species and others will respond to the increased occurrence and frequency of hypoxic events.

Aquatic systems that receive excessive nutrient loading from point sources, like wastewater effluent discharges often have an exhausted oxygen regime (Holeton et al., 2011). The sustained input of oxygen-demanding wastes and nutrients, like nitrogen and phosphorus from wastewater treatment plants (WWTP) can lead to increased rates of eutrophication (Brooks et al., 2006; Carey & Migliaccio, 2009).

Fluctuations in abiotic variables altered by global climate change can increase sensitivity to chemical stressors, and simultaneously, exposure to pollutants can make organisms more sensitive to abiotic stress (Hooper et al., 2013). Pollution-dominated ecosystems are often hypoxic, but the interactive effects of combined exposure to wastewater effluent and hypoxia have not been researched. Anthropogenic stressors can

disrupt the natural adaptive responses to hypoxia, but it is largely unknown if the concentrations of contaminants in wastewater effluent are sufficient to disrupt the ability of fish to respond and cope with hypoxia (Ackerly & Esbaugh, 2020; Blewett et al., 2017; Fitzgerald et al., 2020, 2019; Kraemer & Schulte, 2004; Landman et al., 2006; Mu et al., 2017). My thesis aims to examine the interactive effects of wastewater effluent and hypoxia on the health and metabolic physiology in an estuarine fish model.

1.2 Hypoxia in aquatic environments

Many tide pools, estuarine habitats, stratified or ice-covered lakes and ponds are characterized by natural fluctuations in oxygen availability (Diaz & Rosenberg, 2008), and many fish have evolved the means to live and sometimes thrive in such conditions. The depletion of available oxygen can also be linked to eutrophication events in formerly pristine aerated waters due to nutrient loading from fisheries, agricultural run-off, and WWTP discharges (Breitburg et al., 2009; Brooks et al., 2006; Carey & Migliaccio, 2009; Diaz, 2001; Friedrich et al., 2014). Eutrophic events can be complex, leading to increased algal biomass, decreased water clarity, and proliferation of toxic phytoplankton (Breitburg et al., 2009; Carey & Migliaccio, 2009; Diaz & Rosenberg, 2008). The resulting hypoxic (low oxygen) and anoxic (without oxygen) events are especially detrimental to hypoxia-sensitive species and have been linked to major fish kills (Breitburg et al., 2009; Diaz & Rosenberg, 2008; Friedrich et al., 2014; Wannamaker & Rice, 2000). The occurrence of hypoxic events is predicted to increase along with global climate change, urbanization, and pollution (Friedrich et al., 2014).

1.2.1 Hypoxia tolerance

The ability to cope with and survive reductions in oxygen availability is described using several common indices of hypoxia tolerance, including critical oxygen tension (P_{crit}) and the capacity to resist loss of equilibrium (LOE) in deep, severe hypoxia. P_{crit} is the PO_2 at which the organism's oxygen consumption rate transitions from being relatively constant and independent of external oxygen levels (oxyregulation) to being dependent and

declining with external oxygen levels (oxyconformation; Mandic et al., 2009). Therefore, species with a lower P_{crit} can maintain resting rates of aerobic metabolism in deeper levels of hypoxia and are often considered to be more hypoxia tolerant (Mandic et al., 2009; Richards, 2011). LOE is denoted as the disruption of dorsal-ventral equilibrium, the inability to maintain an upright position in the water column. The ability to resist LOE in hypoxia can be quantified by both 1) the time to LOE at a constant level of severe hypoxia (t_{LOE}), and 2) the PO_2 at LOE during progressive hypoxia (P_{LOE}), are also common experimental and ecologically-relevant indices used to assess hypoxia tolerance (Borowiec et al., 2015, 2020; Regan & Richards, 2017). These indices of tolerance are at PO_2 well below P_{crit}, and P_{LOE} may approach complete anoxia in very tolerant species. It is thought that LOE is linked with the loss of ATP homeostasis in hypoxia-sensitive tissues like the brain (Speers-Roesch et al., 2013; van Raaij et al., 1994).

1.2.2 Physiological responses to hypoxia

Many fish species make physiological adjustments when exposed to fluctuations in external oxygen levels that help support hypoxia tolerance (Mandic et al., 2009; Pollock et al., 2007). Initial detection of low environmental oxygen may lead to behavioural adjustments, including avoidance of hypoxic regions, actively seeking out oxygenated areas, and/or relocating near the air-water interface which is comparatively more oxygenated (aquatic surface respiration, ASR; Kramer & McClure, 1982; Pollock et al., 2007; Wu, 2002). However, when hypoxia cannot be avoided, fish must make appropriate physiological adjustments in order to maintain the balance between tissue oxygen supply and demand. Rapid physiological adjustments help offset declines in O_2 supply to tissues, including increases in gill ventilation to augment water flow across the gas-exchange surface (Tzaneva et al., 2011; Wu, 2002). Blood flow is also redistributed towards the hypoxia-sensitive areas, such as brain tissue, which needs oxygen the most (Axelsson & Fritsche, 1991; Randall, 1982; Sundin et al., 1995). Fish can also make rapid metabolic adjustments to shift energy production towards oxygen-independent pathways, like

anaerobic glycolysis, which is particularly prevalent at PO_2 below P_{crit} (Borowiec et al., 2018; Omlin & Weber, 2010; Regan et al., 2017; Richards, 2011; Scott et al., 2008).

With chronic exposure to hypoxia, plastic changes are made along the oxygen transport cascade to further improve the capacity for O_2 extraction from the environment, lower activity, and depress O_2 and energy demands. For example, crucian carp (*Carassius carassius*), goldfish (*Carassius auratus*), mangrove killifish (*Kryptolebias marmoratus*) and mummichog killifish (*Fundulus heteroclitus*) have demonstrated the ability to remodel their gill morphology, reducing the interlamellar cell mass to reveal more surface area for O_2 uptake (McBryan et al., 2016; Nilsson, 2007; Sollid et al., 2003; Sollid & Nilsson, 2006; Turko et al., 2012). Fish can also augment blood O_2 content by increasing hemoglobin (Hb) content via erythropoiesis and/or by lowering intraerythrocytic concentrations of allosteric modulators to increase Hb-O_2 affinity (Borowiec et al., 2015; Mandic et al., 2009; Val et al., 2015). Hypoxia-tolerant fishes, like carp, goldfish, and killifish can decrease metabolic rate in hypoxia, resulting in low routine O_2 demands (Borowiec et al., 2015, 2018; Regan et al., 2017; Richards, 2011). Plastic changes in response to chronic hypoxia vary between species, and the magnitude of these differences contribute to differences in hypoxia tolerance and the ability to cope with prolonged hypoxia exposure (Borowiec et al., 2020; Regan et al., 2017).

1.2.3 Control systems and signalling pathways involved in hypoxia responses

Hypoxia responses are controlled by intricate pathways that involve oxygen sensing, neural reflexes, gene transcription, and cellular metabolism (Perry & Tzaneva, 2016). The hypoxic chemoreflex, which controls the ventilatory and circulatory responses to hypoxia, is initiated by neuroepithelial cells (NECs) that act as O_2 chemoreceptors in fish gills, which secrete serotonin in response to hypoxic conditions (Perry & Tzaneva, 2016). This reflex then increases the activity of the efferent neurons that drive gill ventilation, and also drives the activity of the sympathetic nervous system, stimulating catecholamine release by chromaffin cells into the bloodstream to elicit many of the cardiovascular responses to hypoxia (Reid & Perry, 2003). Changes in serotonin levels

could disrupt signalling by NECs in hypoxia and could therefore impair the cardiorespiratory adjustments that are involved in the hypoxic response.

Cells respond to hypoxia with changes in gene expression and other molecular responses. Many of these responses are governed by hypoxia-signaling pathways involving hypoxia inducible factors (HIF; Gorr et al., 2010; Wu, 2002). There are multiple isoforms of the HIF-α subunit, of which HIF-1α and HIF-2α are best understood. HIF-α is normally degraded in normoxia but is stabilized in hypoxia and dimerizes with HIF-1β, and this functional heterodimer moves to the nucleus where it binds specifically to hypoxia-response elements in target genes (Gorr et al., 2010). These hypoxia-inducible genes direct many cell and tissue responses to hypoxia, including angiogenesis (by inducing VEGF, vascular endothelial growth factors), enhanced anaerobic metabolism (by regulating glucose transporters and enzymes in anaerobic pathways), and red blood cell production (by inducing EPO, erythropoietin; Pollock et al., 2007; Wu, 2002). Response to hypoxia may also involve cellular pathways involved in energy sensing and metabolic control, such as AMP-activated protein kinase (AMPK; Jibb & Richards, 2008; Stensløkken et al., 2008). As a result, exposure to pollutants that disrupt HIF or AMPK signalling pathways could have a range of detrimental effects on the cellular mechanisms fish use to respond to chronic hypoxia.

1.3 Wastewater effluent

Wastewater is defined as any water that is affected by human usage. It is collected from residential, commercial, and industrial sources and brought to a wastewater treatment plant (WWTP) to be processed for discharge into a nearby waterbody. In Canada, wastewater effluent is the greatest point source of aquatic pollution by volume (Holeton et al., 2011). Wastewater is characterized by excess nutrients, like nitrogen and phosphorus, and is known to contain chemicals like pharmaceuticals and personal care products (PPCPs), polyaromatic hydrocarbons (PAHs), and polycyclic biphenyls (PCBs; Carey & Migliaccio, 2009; Pal et al., 2010; Zenker et al., 2014).

Exposure to pollutants found in wastewater can lead to adverse effects in fish and fish populations, with effects observed in developmental and reproductive biology, metabolic physiology, and behaviour in caging studies (Almroth et al., 2008; Cazenave et al., 2014; Du et al., 2018; McCallum et al., 2017), field collection studies (Bahamonde et al., 2015; Bugel et al., 2010; Fuzzen et al., 2016; Mehdi et al., 2018), (Fontaínhas-Fernandes et al., 2008; Galus et al., 2013; Kakuta & Murachi, 1997; McCallum et al., 2017b).. The potential interactions between contaminants and abiotic stressors are poorly understood, and it is unknown whether the concentrations of these contaminants in wastewater effluent may impact the natural adaptive responses of fish coping with other abiotic stressors.

1.3.1 Biological effects of wastewater

The effects of contaminants of emerging concern (CECs), like those identified in wastewater effluent, are an ever-growing area of research. CECs include endocrine disruptors, disinfection by-products, industrial chemicals, persistent organic pollutants, pesticides, and PPCPs. These chemical compounds are designed specifically for use in medical, veterinary, agricultural, and industrial settings, but it is unknown if these modes of action are conserved across animal phyla (Corcoran et al., 2010; Holeton et al., 2011).

The impacts of wastewater effluent and pollutant exposure have been studied at various levels of biological organization. Exposure to contaminants found in wastewater can be detrimental to developmental and reproductive physiology. One of the most notable effects is the feminization of male fish living downstream of WWTPs (Hicks et al., 2017). A greater incidence of intersex is reported near wastewater outfalls, with males showing reduced levels of testosterone and 11-ketotestosterone, induction of vitellogenin production, and in severe cases the development of testicular oocytes (Bahamonde et al., 2015; Fuzzen et al., 2016). Furthermore, the exposure to relevant concentrations of pharmaceutical compounds found in wastewater increased embryonic mortalities and developmental abnormalities, like spinal deformation, yolk, and pericardial edema in zebrafish (*Danio rerio*; Galus et al., 2013). Environmental pollutants are known to disrupt

complex fish behaviours (Jacquin et al., 2020; Scott & Sloman, 2004). In another study, exposure to 100% wastewater effluent reduced aggression in adult round goby (*Neogobius melanostomus*), a behaviour which is crucial in acquiring and defending nest territories for reproduction (McCallum et al., 2017b). These disruptions in reproductive and developmental physiology can have deleterious impacts on fish populations in effluent-dominated ecosystems.

Sewage exposure led to edema, lifting of lamellar and filamentary epithelium, and hematoma formation in the gills, and thickening of glomeruli and altered proximal tubule morphology in the kidney (Fontaínhas-Fernandes et al., 2008; Galus et al., 2013; Kakuta & Murachi, 1997). Pathological changes in gill morphology may impact the capacity for oxygen uptake. It is noted that the severity of histopathological damage was shown to increase with the time of exposure (Fontaínhas-Fernandes et al., 2008).

Wastewater can also interfere with bioenergetics and metabolism, as exhibited in increased metabolic rates in bluegill sunfish (*Lepomis macrochirus*) and rainbow darter (*Etheostoma caeruleum*; Du et al., 2018; Mehdi et al., 2018). Prolonged exposure impacted energy reserves, where caging near wastewater outfalls depleted liver glycogen in curimbata (*Prochilodus lineatus*; Cazenave et al., 2014). Mitochondrial respiratory capacity for oxidative phosphorylation was increased in livers of bluegill sunfish caged near a wastewater outfall, which is postulated to offset reductions in glycogen content and support energetic demands of detoxification (Du et al., 2019). Prooxidants found in wastewater effluent can also lead to oxidative damage, which is observed as elevated levels of protein carbonyls, lipid peroxides, and antioxidants (GSH, glutathione) in rainbow trout caged outside a sewage treatment plant (Almroth et al., 2008)

1.3.2 Underlying molecular mechanisms

Endocrine disrupting chemicals (EDCs) can affect hormone and neural signalling in fish, and include hormone receptor agonists or antagonists, like estrogens, androgens, and industrial compounds. Recent findings show that endocrine disruption in fish can be attributed to mechanisms outside of classical hormone receptor pathways, including the

neuroendocrine system and insulin signalling axis (Niemuth & Klaper, 2015). Non-hormone pharmaceuticals, like metformin (antidiabetic) and fluoxetine (antidepressant) are also known to cause endocrine disruption, resulting in intersex and reduced fecundity (Niemuth & Klaper, 2015, 2018). Other EDCs, like the pesticide linuron, which has anti-androgenic activity, can also activate the aryl hydrocarbon receptor (AHR) pathway (Fitzgerald et al., 2020).

Metformin, an anti-diabetic drug, is one of the most abundant PPCPs found in aquatic ecosystems (Oosterhuis et al., 2013). Metformin (METF) inhibits complex I of the electron transport chain, thereby activating AMP-activated protein kinase (AMPK), which is a protein that maintains energy homeostasis. AMPK has been identified as a useful energetic status biomarker for wastewater effluent exposure in freshwater mussels (Goodchild et al., 2015). AMPK-activation phosphorylates a variety of metabolic enzymes and transcription factors, leading to downregulation of gluconeogenesis, activation of glycolysis, and promotion of fatty acid oxidation (Hertz et al., 1989; Ussery et al., 2018). These changes in energy turnover can lead to metabolic dysfunction in fish that are exposed to these PPCPs.

Antidepressants, like selective serotonin reuptake inhibitors (SSRIs), provide therapeutic effects by increasing free serotonin (5-HT) levels via the inhibition of monoamine transporters at presynaptic membranes (Corcoran et al., 2010; Kreke & Dietrich, 2008). 5-HT is considered a ubiquitous neuromodulator, and its role is considered well-conserved in vertebrates (Prasad et al., 2015). In fish, elevated 5-HT levels can influence social, courting, and feeding behaviours, and has been implicated in physiological functions within endocrine and reproductive systems, where 5-HT plays a stimulatory role in steroidogenesis (Corcoran et al., 2010).

β-blockers, or β-adrenergic receptor antagonists are a group of drugs prescribed to treat a variety of cardiovascular diseases. Catecholamine signalling via β-adrenergic receptors is relatively conserved across vertebrates, and is responsible for regulating cardiac output, ventilation rates, metabolism, and oxygen chemoreception (Corcoran et al., 2010; Owen et al., 2007). The effects of pollutants in wastewater on aquatic biota are

difficult to predict, as these complex mixtures may produce varied responses and are affected by abiotic changes.

1.4 Implications of combined exposure to wastewater and hypoxia

Contaminants found in WWTP effluents, like endocrine disrupting chemicals, diabetes medications, antidepressants, beta-blockers, PAHs, PCBs, etc. could disrupt many of the physiological pathways that fish use to cope with hypoxia.

In addition to many CECs found in wastewater, hypoxia is also an endocrine disruptor and has been noted to significantly affect sex steroid levels in common carp (*Cyprinus carpio*), resulting in adverse effects on key reproductive processes, including spawning, fertilization, and hatching (Wu et al., 2003). Co-exposure to both stressors may exacerbate hormonal and neural signalling. AMPK signalling pathways are responsible for mediating cellular mechanisms fish use to respond to chronic hypoxia (Jibb & Richards, 2008; Stensløkken et al., 2008). Metformin, which disrupts AMPK, has been shown to affect gene expression, metabolism, glucose metabolism, and protein synthesis (Hertz et al., 1989; Renquist et al., 2013; Ussery et al., 2018). The blockage of AMPK sensitivity in *C. carassius* resulted in elevated metabolic rate and increased ethanol production in anoxia (Stensløkken et al., 2008). Therefore any interference of cellular pathways in chronic hypoxia may be harmful.

Serotonin plays a critical role in oxygen sensing, such that the inhibition of serotonergic neuroepithelial cells may lead to disruption in the hypoxia response across multiple levels of organization (McDonald, 2017). The exposure to SSRI antidepressants, like fluoxetine, was shown to attenuate cardiovascular and ventilatory responses to hypoxia in Gulf toadfish (*Opsanus beta*; Amador et al., 2018; Panlilio et al., 2016). Beta blockers (such as propranolol) could also disrupt signaling by catecholamines like epinephrine and norepinephrine. Indeed, beta blockers can disrupt chemoreceptor discharge in rainbow trout (*Oncorhynchus mykiss*) gills, which can involve catecholamines as well as serotonin (Burleson & Milsom, 1990; Porteus et al., 2012). Exposure to these contaminants could

foreseeably disrupt the cardiovascular responses to hypoxia that result from activation of the sympathetic nervous system in any tissues that express beta-adrenergic receptors.

Exposure to persistent organic pollutants (*e.g.*, PCBs, PAHs) can also disrupt oxygen signaling pathways that underlie the physiological adjustments to chronic hypoxia (*e.g.*, hypoxia inducible factor [HIF] pathway; Chan et al., 1999; da Silva et al., 2017; Kraemer & Schulte, 2004; Vorrink & Domann, 2014). The oxygen signalling pathway (HIF-α) and dioxin signalling pathway (AHR) share an identical dimerization partner (HIF-β or ARNT; Chan et al., 1999; Vorrink & Domann, 2014). Responses attributed to this antagonistic interaction include the suppressed upregulation of hypoxia-inducible enzymes in killifish exposed to PCBs, and the overexpression of HIF-1α in tambaqui (*Colossoma macropomum*) injected with benzo[a]pyrene (PAH; da Silva et al., 2017; Kraemer & Schulte, 2004). Early life co-exposures to real-world PAH mixtures in hypoxia are also linked with altered growth rates, impaired reproductive capacity, and reduced the quality of offspring (Mu et al., 2017). Due to the reciprocal crosstalk and competition between HIF-α and AHR for ARNT (HIF-β), hypoxia exposure may enhance the susceptibility to xenobiotics by limiting detoxification processes. It is largely unknown whether the concentrations of these contaminants in wastewater effluent are sufficient to disrupt the ability of fish to cope with hypoxia, and conversely, how species-specific hypoxia tolerance limits detoxification in fish.

1.5 Model organism

Mummichog killifish (*Fundulus heteroclitus*) are an ideal model organism for this study as they are a robust species that experiences complex stressors in their estuarine habitat. Living in tide pools along the east coast of North America, *F. heteroclitus* encounter dynamic fluctuations in physicochemical factors, including salinity, temperature, oxygen availability, carbon dioxide, and pH (Burnett et al., 2007). In their natural habitat, this species experiences fluctuations between oxygen supersaturation and complete anoxia, and are able to survive dissolved oxygen levels as low as 1.5-2.0 ppm (Schulte, 2007, 2014). Even when denied access to aquatic surface respiration (ASR), *F. heteroclitus* is able to

maintain moderate growth rates and resist hypoxia-related mortality seen in other species, at levels of oxygen <1 mg O_2/L (Stierhoff et al., 2003). To survive in chronic hypoxia, mummichog killifish depress metabolism, lowering oxygen consumption rates by ~50% (Borowiec et al., 2015, 2018). Increased haemoglobin content, gill filament length, and other physiological adjustments contribute to improvements in oxygen uptake and transport to cope with hypoxic conditions (Borowiec et al., 2015).

Previous work on mummichog killifish have investigated the integrated physiological, developmental, and genomic responses of *F. heteroclitus* which enable survival and fitness in the wild. *F. heteroclitus* is a unique teleost model to study individual and population responses to environmental stress, noted for their adaptive resistance to pollutants (Schulte, 2007). There are wild populations observed in contaminated habitats with high levels of persistent organic pollutants, including dioxins, PAHs, PCBs, and heavy metals (Bugel et al., 2010; Burnett et al., 2007; Crawford et al., 2020; Schulte, 2007; Weis, 2002). Evolutionary physiologists attribute the rapid adaptation of *F. heteroclitus* to toxic pollution to their large population sizes, impressive genetic diversity, and polygynous mating system, which provides a potential for rapid gene frequency changes (Crawford et al., 2020; Weis, 2002). The genes in the aromatic hydrocarbon receptor (AHR) pathway have been identified as key, repeated targets of selection in tolerant *F. heteroclitus* populations (Reid et al., 2016). The lack of inducibility of CYP1A seen in resistant populations demonstrates that an altered aromatic hydrocarbon receptor (AHR) pathway may be involved in the adaptive resistance to dioxin-like compounds (Burnett et al., 2007; Reid et al., 2016; Weis, 2002). This adaptive suppression of the AHR pathway in polluted settings may raise physiological trade-offs due to the crosstalk with other signalling pathways involving estrogens, oxidants, and hypoxia (Whitehead et al., 2017). It is vital to understand how adaptive phenotypes in urban settings may be altered in response to interactive stressors, like elevated hypoxia and contaminant exposure.

1.6 Objectives & hypotheses

For my thesis, the **aim** of my research was to examine the effects of wastewater exposure on the adaptive responses to chronic hypoxia in mummichog killifish. Using a full factorial design, my work had several **objectives**:

1) Determine whether wastewater exposure disrupts the changes in metabolism and hypoxia tolerance in response to hypoxia acclimation.
2) Assess the underlying mechanisms along the oxygen transport cascade to explain these changes in metabolism and hypoxia tolerance.

I **hypothesized** that strategies fish use to cope with hypoxia in clean water may be disturbed with exposure to contaminants found in wastewater, such that exposed killifish would have impaired hypoxia tolerance and disruptions within the natural respiratory and metabolic adjustments to hypoxia.

Acknowledging the ability of *F. heteroclitus* to thrive in challenging environments, it is a unique model to study the interactive effects of two prevalent co-occurring stressors, a combination that can be lethal to much more sensitive species. Previous studies have focussed on the effects of individual contaminants on aquatic organisms, and it is vital that we understand the complex interactive impacts of multiple environmental stressors on aquatic life. The outcomes of my MSc project will provide valuable groundwork to fish population management in the future, as anthropogenic stressors in the aquatic environment are anticipated to worsen with climate change.

CHAPTER TWO: EXPOSURE TO WASTEWATER EFFLUENT DISRUPTS HYPOXIA RESPONSES IN KILLIFISH (*Fundulus heteroclitus*)

2.1 Abstract

Hypoxia often occurs in aquatic ecosystems that receive effluent from municipal wastewater treatment plants (WWTP). WWTP effluent contains contaminants (*e.g.*, pharmaceuticals, polychlorinated biphenyls, and polycyclic aromatic hydrocarbons) that could disrupt the complex physiological pathways fish use to cope with hypoxia, but the effects of WWTP effluent on the physiological responses of fish to chronic hypoxia is poorly understood. In this study, we exposed mummichog killifish (*Fundulus heteroclitus*) to hypoxia (5 and 2 kPa O_2) and/or 100% WWTP effluent for 21 days in a full factorial design. We then measured hypoxia tolerance, whole-animal metabolism, gill morphology, haematology, and tissue metabolites. In clean water, killifish responded to chronic hypoxia with improvements in hypoxia tolerance – increases in time to loss of equilibrium at 0.5 kPa (t_{LOE}) and decreases in critical O_2 tension (P_{crit}) – in association with increases in the exposed surface of gill lamellae as a result of regression of the interlamellar cell mass (ILCM). Concurrent exposure to wastewater attenuated the increases in t_{LOE} and gill remodeling in chronic hypoxia, and nearly depleted brain glycogen stores. Therefore, exposure to WWTP effluent can disrupt the physiological mechanisms fish use to cope with chronic hypoxia and impair hypoxia tolerance. Our research suggests that the combination of stressors near WWTPs can have interactive effects on the physiology and health of fish.

2.2 Introduction

Environmental hypoxia, the depletion of available oxygen, is a common feature of many aquatic ecosystems, and it can occur as a result of natural causes as well as from anthropogenic pollution (Diaz, 2001; Friedrich et al., 2014). Many tide pools, estuarine habitats, stratified or ice-covered lakes and ponds are characterized by naturally low or fluctuating oxygen levels (Diaz & Rosenberg, 2008). In some other cases, the depletion of dissolved oxygen can be linked to eutrophication events due to nutrient loading from fisheries, agricultural run-off, or discharge from wastewater treatment plants that leads to

the proliferation of primary producers (Breitburg et al., 2009; Brooks et al., 2006; Carey & Migliaccio, 2009; Diaz, 2001; Diaz & Rosenberg, 2008; Friedrich et al., 2014). The occurrence of such hypoxic events is predicted to increase with further progression of climate change, urbanization, and pollution (Friedrich et al., 2014).

Fish exhibit a suite of behavioural, physiological, and biochemical responses to hypoxia that contribute to maintaining the balance between oxygen supply and demand and to thus cope with low oxygen conditions (Bickler & Buck, 2007; Perry et al., 2009; Pollock et al., 2007; Richards, 2011). Such responses have been well described in the literature and often act to improve O_2 extraction from the environment (*e.g.,* by increasing gill ventilation and surface area), reduce activity and metabolism, and/or increase anaerobic energy production (Borowiec et al., 2015; Kramer & McClure, 1982; Perry & Tzaneva, 2016; Regan et al., 2017; Sollid et al., 2003). However, hypoxia often occurs concurrent with other environmental stressors, including fluctuations in temperature and/or salinity or pollution (Friedrich et al., 2014). The potential interactions between hypoxia and many other types of stressors are poorly understood, and whether anthropogenic stressors can disrupt the natural adaptive responses to hypoxia remains unresolved.

Effluents discharged from municipal wastewater treatment plants (WWTP) are complex contaminant mixtures that can contain excess nutrients, pharmaceuticals and personal care products (PPCPs), and industrial chemicals (Brooks et al., 2006). The investigation of these contaminants of emerging concern (CECs) are necessary due to their increasing usage and potential for having significant effects on fish and other aquatic life (Corcoran et al., 2010; Kolpin et al., 2002; Metcalfe et al., 2003; Zenker et al., 2014). Exposure to WWTP effluent can have a number of physiological effects on fish, having been shown to disrupt reproductive function (Bahamonde et al., 2015) and metabolic rate (Du et al., 2019, 2018; Mehdi et al., 2018). Hypoxia exposure too is known to affect metabolic rate and its underlying physiological determinants (Hochachka et al., 1996; Mandic et al., 2009). The eutrophication of aquatic environments, including wastewater-dominated ecosystems can lead to reduced oxygen or hypoxic conditions, but the effects of combined exposure to wastewater effluent and hypoxia are largely unknown.

Given the molecular targets of many pharmaceuticals and other contaminants found in WWTP effluents, exposure to these effluents could disrupt the physiological pathways fish use to cope with hypoxia. For example, the pharmaceuticals in wastewater have molecular targets involved in oxygen sensing (*e.g.*, serotonin reuptake inhibitors), homeostatic regulation by the sympathetic nervous system (*e.g.*, beta blockers), and control of metabolic pathways (*e.g.*, anti-diabetics, lipid lowering drugs; Arlos et al., 2015; Corcoran et al., 2010; Kolpin et al., 2002; Metcalfe, 2013; Metcalfe et al., 2003). Indeed, exposure to the serotonin reuptake inhibitor, fluoxetine has been shown to attenuate the cardiovascular and ventilatory responses to hypoxia in Gulf toadfish (*Opsanus beta*; Panlilio et al., 2016). Industrial contaminants (*e.g.*, polychlorinated biphenyls, polycyclic aromatic hydrocarbons) can also disrupt hypoxia signaling pathways that underlie the physiological adjustments to chronic hypoxia (*e.g.*, hypoxia inducible factor [HIF] pathway; Chan et al., 1999; da Silva et al., 2017; Kraemer & Schulte, 2004; Vorrink & Domann, 2014). However, it is largely unknown whether the concentrations of these contaminants in wastewater effluent is sufficient to disrupt the ability of fish to respond to and cope with hypoxia.

The objective of this study was to determine whether exposure to WWTP effluent disrupts the adaptive physiological responses of mummichog killifish (*Fundulus heteroclitus*) to hypoxia. We examined the effects of chronic exposure to hypoxia and/or municipal wastewater effluent in a full factorial design on metabolism, hypoxia tolerance, and several underlying respiratory and metabolic traits. Killifish are an ideal model organism for this study as they naturally experience hypoxia and fluctuations in various other environmental conditions in their estuarine habitat (Burnett et al., 2007). This species can also be found in some heavily polluted estuarine environments containing high levels of persistent organic pollutants, including dioxins, polyaromatic hydrocarbons (PAHs), polycyclic biphenyls (PCBs), and heavy metals, and some wild populations from such environments have evolved exceptional tolerance of pollution exposure (Bugel et al., 2010; Reid et al., 2016; Weis & Weis, 1989). We hypothesized that the response of killifish to

chronic hypoxia would be disrupted by exposure to municipal wastewater, such that fish would be less tolerant of hypoxia.

2.3 Materials and methods

Study animals and housing

Adult mummichog killifish (*Fundulus heteroclitus*) were wild-caught by a commercial supplier (Aquatic Research Organisms, Hampton, New Hampshire, USA) and shipped to McMaster University in Hamilton, Ontario, Canada in the Fall of 2018. Killifish were kept in well-aerated brackish water (4 ppt), produced by mixing artificial sea salt (Reef Pro Mix Redline Complete Sea Salt, Fritz Aquatics, Mesquite, Texas) with dechlorinated City of Hamilton tap water. Holding tanks were kept at room temperature (~18°C), and killifish were fed 5 days a week to satiation with commercial pellets (AgloNorse Complete Fish Feed, 0.6-0.9 mm, Tromsø, Norway). The photoperiod was 12 h:12 h light:dark between 0700 and 1900 local time. All animal protocols were developed in accordance with guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board (AUP # 19-07-20).

Wastewater effluent collection

Wastewater effluent was collected from Woodward Avenue Wastewater Treatment Plant (43°15'15.6"N; 79°46'25.4"W) in Hamilton, Ontario from March-May of 2019. Woodward WWTP is a secondary conventional activated sludge treatment plant with sludge dewatering and digestion and has a maximum daily capacity of 409 million litres (City of Hamilton, 2019). Effluent was collected every Tuesday and Saturday during the exposure period between the hours of 0930 and 1130 local time. Using a submersible sump-pump, wastewater effluent from the final clarifier chimney was collected into 20 L carboys (Reliance Rectangular AquaPak Containers, BPA Free) for transportation to McMaster University. This effluent was representative of the final effluent discharged into Hamilton Harbour via the Windermere Basin. Effluent was stored in a dark and refrigerated cold

room at 4°C for at most three days before use in chronic exposures (see below). We obtained water quality data of 24 h composite samples from each day of effluent collection from the City of Hamilton Environmental Laboratory (collected as per Wastewater Systems Effluent Regulations), including total Kjeldahl nitrogen, ammonia + ammonium as N, NO_3 as N, NO_2 as N, chemical oxygen demand, cBiochemical oxygen demand, conductivity, total phosphorus, *Escherichia coli*, and total suspended solids (Figs. S1-3). For each effluent collection, we also preserved a sample of effluent (500 mL) in pre-cleaned amber glass bottles containing sodium azide (1 g/L) and ascorbic acid (50 mg/L), and stored these samples at 4°C until extractions for analytical chemistry.

Chronic exposures of fish

Killifish were exposed to wastewater and/or hypoxia in a full factorial design in 40 L aquaria for 21 days at room temperature (~18°C). Conditions of normoxia (normal oxygen conditions; well aerated to ~20 kPa O_2), moderate hypoxia (5.0 ± 0.1 kPa), or severe hypoxia (2.0 ± 0.1 kPa) were maintained in clean water (Hamilton dechlorinated tap water adjusted to 4 ppt with artificial sea salt) or in 100% wastewater effluent. Each of these 6 chronic exposure groups was replicated 2-3 times over the period of wastewater collection (n=24 fish per tank). Wastewater effluent was brought up to room temperature and artificial sea salt was added to match the 4 ppt salinity of control water immediately before use. Killifish were fed daily (0.2 g per tank) using the same diet mentioned above. For all hypoxic treatments, oxygen tension (PO_2) was reduced from 20 kPa to the treatment setpoint over the first three days of exposure, and was maintained by injecting N_2 gas using O_2 controllers that we have previously described (Borowiec et al., 2015). Bubble wrap was held on the water surface in the hypoxic treatments to reduce O_2 diffusion from the air and to prevent aquatic surface respiration. Fish were also provided a black PVC tube as a refuge. Water was gently mixed with a submersible aquarium pump and filtered through a sponge filter. Every 72 hours, faeces and other debris were removed, various physicochemical parameters were measured (pH, conductivity, total dissolved solids, salinity, temperature, and NH_3, NO_2, NO_3 levels), and the appropriate tank water was renewed (clean water or

wastewater). Because effluent was stored for ~3 days after collection before use, we anticipated the degradation of some contaminants found in the water during storage and over the 3 days between water changes. Paired water samples (125 mL) were performed once during a randomly selected water change for each tank replicate to provide an indication of the level of exposure - where one sample was taken immediately after a water change, and the second sample taken prior to the next water change (n=10-14). Tank samples were preserved using sodium azide and ascorbic acid as described above. After completing the 21-day exposure, one set of fish (n=4 per tank replicate) in each chronic exposure group was immediately sampled at rest, and a second and third set was used for respirometry and hypoxia tolerance measurements, respectively (see below).

Analytical chemistry of water samples

Preserved water samples were analyzed for twenty-two PPCPs and other CECs using previously established methods (Arlos et al., 2015). The analyzed chemicals included lipid regulators and metabolites (gemfibrozil, atorvastatin, p-hydroxy atorvastatin, o-hydroxy atorvastatin), anti-epileptic (carbamazepine), analgesic (acetaminophen), stimulant (caffeine), antibacterials (triclosan, sulfamethazine), antibiotics (monensin, trimethoprim, lincomycin, sulfamethoxazole), antidepressants (fluoxetine, norfluoxetine, venlafaxine, desvenlafaxine), non-steroidal anti-inflammatory agents (NSAIDs; ibuprofen, naproxen, diclofenac), herbicide (atrazine), and industrial chemicals (bis-phenol, BPA). Wastewater effluent samples were first divided into 100 mL aliquots, while tank samples divided into 50 mL aliquots. Each sample was then individually spiked with 100 µL of 100 µg/L of isotopically labelled standards prior to extraction. Solid phase extraction (SPE) was performed using the Agilent Bond Elute Plexa cartridge (6cc, 500 mg) on samples adjusted to pH 2, with final extracts reconstituted in 500 µL methanol with internal standards. These extracts were then stored at -20°C until analysis using an Agilent 1260 HPLC with 6460 triple quad mass spectrometer (LC-MS/MS) with Agilent Jet Stream (AJS) electrospray ionization in both positive and negative modes (Mehdi et al., 2020, *unpublished* data). Of all the preserved water samples that are described above, technical issues precluded the

analysis of two samples of clean water tank replicates and three samples of wastewater tank replicates. Some individual data points for acetaminophen and caffeine were excluded due to problems with matrix effects for these particular compounds.

Sampling of fish tissues

The sampled fish were first euthanized with a sharp blow to the head followed by spinal transection, and the tail was severed for blood collection. A portion of blood (5 µL) was stored in Drabkin's reagent for determining haemoglobin concentration according to product instructions (Sigma-Aldrich, St. Louis, MO, USA). The remaining blood was collected in a heparinized capillary tube and centrifuged at 12,700 g for 5 min to determine haematocrit. Brain, liver, and axial white muscle were excised, weighed, freeze-clamped in liquid nitrogen, and then stored at -80°C for later analysis of metabolites. Gills were dissected, placed in 0.2 M PBS (274 mmol/L NaCl, 30.4 mmol/L Na_2HPO_4, 5.4 mmol/L KCl, 3.0 mmol/L KH_2PO_4; pH 7.8) containing fixative (2% paraformaldehyde and, 2% glutaraldehyde) for at least 48 h, then stored in PBS at 4°C for later analysis of gill morphometrics.

Respirometry and hypoxia tolerance

Killifish were subjected to two series of measurements of metabolism and hypoxia tolerance. In one subset of fish (n=4 per tank replicate) from each chronic exposure group, we used stop-flow respirometry in clean water at 4 ppt to measure standard metabolic rate (SMR) and critical oxygen tension (P_{crit}). Fish were fasted for the last 48 h of chronic exposures and were then transferred at ~1700-1900 local time to 90 mL cylindrical acrylic chambers containing normoxic water, which were covered in dark plastic to minimize visual disturbance. These respirometry chambers were connected to two water circulation circuits: a 'flushing circuit' that used a pump to flush the respirometry chamber with water from a surrounding buffer tank; and a 'measurement circuit' that continuously pumped water from the respirometry chamber across a fibre-optic O_2 sensor (FireSting FSO2-4, PyroScience GmbH) in a closed loop. The O_2 level in the respirometry chambers could thus

be set by controlling the O_2 levels in the buffer tank, as described above for the chronic exposures. Measurements of O_2 consumption rate (MO_2) were made overnight in normoxia using an automated respirometry system (AutoResp, Loligo Systems, Viborg, Denmark) by alternating between 4.5 min flush periods (when both flushing and measurement circuits were active) and 4 min measurement periods (when only the measurement circuit was active such that the respirometry chamber acted as a closed system), during which MO_2 was determined from the depletion of water oxygen content over time, and is expressed relative to body mass ($\mu mol\ g^{-1}\ h^{-1}$). Standard metabolic rate (SMR) was calculated as the average of the lowest 10 MO_2 measurements that were made during the night-time inactive phase. Starting at ~1100-1200 the following day, resting metabolic rate (RMR) was calculated as the average MO_2 measured at 100% air saturation. MO_2 was continuously measured throughout a stepwise hypoxia protocol, during which the water O_2 level was reduced by 10% air saturation every 10 min, as previously described (Borowiec et al., 2020). P_{crit} was calculated using the 'Respirometry' package in R, as the breakpoint PO_2 in broken-stick regression models of MO_2 versus PO_2 values. P_{crit} represents the PO_2 below which fish transition from oxyregulating (when PO_2 has no or little effect on MO_2) to oxyconforming (when PO_2 leads to sharp declines in MO_2). In a second subset of fish (n = 4 per tank replicate) from each chronic exposure group, we measured time to loss of equilibrium (t_{LOE}) by exposing fish to an O_2 tension of 0.5 kPa in 5 L aquaria. Fish were monitored continuously, and t_{LOE} was determined when the fish was unable to maintain an upright position in the water column and was unresponsive to a gentle tail pinch.

Gill histology

Gill histology was performed for the normoxic and severe hypoxic exposure groups. After fixation, the first gill arch was separated from one side of the gill basket and stored in cryoprotectant solution (24% sucrose in 0.2 M PBS) for ~24-48 h. Each gill arch was then frozen in embedding medium (Shandon Cryomatrix, Fisher Scientific, Runcorn, Cheshire, UK) and stored at -80°C until sectioning. Frozen blocks were sectioned at 5 µm thickness in a cryostat maintained at -20°C (Leica CM 1860). Slides were air dried

overnight and again stored at -80°C until haematoxylin and eosin staining. The staining procedure involved an initial dehydration step in 95% ethanol for 15 min, incubation in Gills II haematoxylin for 2 min, and then eosin for ~45 s, rinsing in distilled water between each of these steps. After staining, sections were dehydrated through progressively increasing concentrations of ethanol (up to 100%) and then finally in xylene, before being mounted in Permount Mounting Medium (Fisher Scientific). For each individual, 10-15 brightfield images were taken of sections throughout the whole gill arch tissue using a Nikon Eclipse E8000 microscope (Nikon Instruments, Melville, New York, USA). Gill morphometrics were analyzed using ImageJ (v1.52a) software (Rasband, 2008) including lamellar height, lamellae width, and interlamellar cell mass height as previously described (Du et al., 2018). Lamellar density was also quantified as the number of lamellae per length of filament.

Tissue metabolite assays

Lactate, free-glucose, and glycogen levels were determined in brain, muscle, and liver samples using protocols that have been previously described (Borowiec et al., 2018). Muscle and liver samples were first ground into fine powder using insulated mortar and pestle that were pre-cooled using liquid nitrogen and then returned to -80°C until assayed. To prevent the loss of tissue, brain samples were homogenized whole on the same day of the assay. For all tissues, approximately 20 mg of tissue (powdered or whole) was homogenized in ~300 µL of ice-cold 6% $HClO_4$ for 20 s using a PowerGen 125 electric homogenizer (Fisher Scientific, Whitby, ON, Canada). The homogenate was then vortexed and an aliquot of 100 µL was immediately frozen in liquid nitrogen for later analysis of glucose and glycogen. The remainder was centrifuged at 4°C for 10 min at 10,000*g*, and 200 µL of the supernatant was transferred into a new microcentrifuge tube. This sample was neutralized with 25 µL of 3 mol/L K_2CO_3 ($6.8 \leq pH \leq 7.2$) and centrifuged again under the same conditions. The supernatant was then assayed for lactate at 37°C in duplicate under the following conditions: 2.5 mmol/L NAD^+ in glycine buffer (0.6 mol/L glycine, 0.5 mol/L hydrazine sulphate; pH 9.4). An initial NADH absorbance measurement was made at 340

nm, excess (5 U/mL) lactate dehydrogenase was added, and a second absorbance measurement was made after incubation for 30 min. Absorbance measurements were made using a Synergy H1 hybrid multimode microplate reader (Biotek Instruments, Vermont, USA). The lactate content was determined from the change in NADH absorbance induced by the addition of lactate dehydrogenase.

For the glucose and glycogen assays, the frozen aliquot was thawed on ice, and 100 µL of 400 mM Na acetate buffer (pH 4.8) and 50 µL of 1M K_2CO_3 were added. Half of this solution was digested with 7 µl of amyloglycosidase solution (suspended at 4 U/L in 300 mmol/L Tris-HCl, 4.05 mmol/L $MgSO_4$; pH 7.5) for 2 h at 40°C with light agitation (80 rpm), while the remaining half was kept on ice. Both digested and undigested samples were then neutralized with 1 mol/L K_2CO_3 ($6.8 \leq pH \leq 7.2$), and centrifuged at 4°C for 10 min at 10,000 g. Glucose was then assayed at 37°C in both digested and undigested samples under the following assay conditions: 1 mmol/L ATP; 0.5 mmol/L $NADP^+$; 5 mmol/L $MgCl_2$; 20 mmol/L imidazole (pH 7.4). Initial absorbance readings were taken at 340 m, excess hexokinase (5 U/mL) and glucose-6-phosphate dehydrogenase (3 U/mL), and a final reading was taken after 20 min. Glucose content was determined as the change in NADPH absorbance induced by the addition of hexokinase and glucose-6-phosphate dehydrogenase. The free-glucose concentration of the tissue was determined from the undigested samples, while glycogen concentration was determined in glucose equivalents from the difference in glucose content between the digested sample (which contained both endogenous free glucose and the glucose produced from the enzymatic breakdown of glycogen) and undigested samples (which contained only endogenous free-glucose).

Statistical analysis

We used two-way ANOVA to test for main effects of and interactions between chronic hypoxia and wastewater exposure. Holm-Šídák post-hoc tests were then used to identify pairwise differences (a) between clean water and wastewater within each chronic oxygen treatment, (b) between normoxia and hypoxia within the clean water groups, and (c) between normoxia and hypoxia within the wastewater groups. All statistical analyses

were performed using GraphPad Prism (version 8.4.2, San Diego, California USA). Data are presented as means ± SEM, and $p<0.05$ was considered significant.

2.4. Results

Water quality and contaminant concentrations

Several indices of water quality during the exposures were comparable between clean and wastewater treatments (Table 1). Although ammonia and nitrate levels were high in the collected wastewater effluent (Fig. S1), nitrogenous wastes were reduced by the in-tank filters during exposures. We examined the concentrations of several contaminants in the exposure tanks to gain insight on the relative levels of exposure experienced by the killifish (Table 2). Many contaminants were significantly higher in the wastewater, while there were some unexpected observations of moderate caffeine and BPA concentrations in clean water. Although we used BPA-free carboys to transport the wastewater, some of the plastic materials used in husbandry and experimental control (*e.g.*, beakers, transfer pipettes, aquarium filter, PVC refuge tubes, airline tubing, bubble wrap, etc.) may have contained BPA and thus contaminated the tank water.

Over the course of our chronic exposures, water quality seemed to improve and the concentrations of target contaminants declined or remained the same, but our staggered tank replicate design assured that these effects did not vary systematically between treatment groups (Figs. S1-8). However, the concentration of target contaminants were higher in wastewater at the time of collection compared to those measured in fresh tank water samples (Table 1), suggesting that there was degradation of some compounds during the three-day storage period before wastewater was used for tank exposures. Degradation of some, but not all, contaminants continued during the tank exposures, likely as a result of the exposure of contaminants to light and warmer temperatures (Table S1). Therefore, the data in Tables 1 and 2 provide a relative indication of the exposure conditions in clean water versus wastewater, but only approximate the variable concentrations that were likely experienced throughout chronic exposure treatments.

Metabolic rate

Concurrent exposure to wastewater effluent and chronic hypoxia reduced standard metabolic rate (Fig. 1, Table 3). There was a significant interaction between chronic hypoxia and wastewater (p = 0.0021) across killifish that were chronically exposed to normoxia (~20 kPa O_2), moderate hypoxia (5 kPa O_2), or severe hypoxia (2 kPa O_2) in clean water or wastewater. Hypoxia acclimation did not affect SMR among fish held in clean water, but concurrent exposure to hypoxia and wastewater reduced SMR at both levels of hypoxia compared to the normoxic wastewater group (p<0.01; Fig. 1A). Resting metabolic rate (RMR), which was measured later during the daytime in normoxia did not show the same patterns observed in SMR. For this trait, there were no statistically significant effects of chronic hypoxia or wastewater on RMR across treatment groups (Fig. 1B, Table 3), suggesting that the relationship between SMR and RMR may be altered by chronic hypoxia and/or wastewater exposure (Fig. 1A). Variation in metabolic rate data did not appear to result from variation in body mass, as there were no significant differences in body mass across groups, nor were there any differences in indices of condition (Fulton's K, hepatosomatic index; Table 4).

Hypoxia tolerance

In general, killifish regulated MO_2 across a range of higher PO_2, and MO_2 declined at lower PO_2 (Fig. S9). The data for MO_2 as a function of PO_2 for each individual were used to calculate the critical O_2 tension (P_{crit}). There was a reduction in critical oxygen tension (P_{crit}) after chronic exposure to severe hypoxia, as reflected by a significant main effect of hypoxia (p = 0.02), but this trait was unaffected by wastewater exposure (p = 0.59; Fig. 2A; Table 3).

Wastewater exposure impeded the improvement with hypoxia acclimation in a key metric of hypoxia tolerance, the time to loss of equilibrium (t_{LOE}) in near anoxia (0.5 kPa) (Fig. 2B; Table 3). There was a significant main effect of chronic hypoxia on t_{LOE} (p<0.0001), driven largely by fish in clean water, which increased t_{LOE} 1.79-fold after acclimation to moderate hypoxia and 3.56-fold after acclimation to severe hypoxia. There

was also a significant effect of wastewater on t_{LOE} ($p<0.01$), which completely eliminated improvements in t_{LOE} in fish acclimated to moderate hypoxia and appeared to reduce the improvements in those acclimated to severe hypoxia.

Gill histology

Wastewater exposure disrupted changes in gill morphology that were associated with hypoxia acclimation (Figs. 3-4; Table 3). Severe chronic hypoxia in clean water led to growth in the height of lamellae along with reduction in the proportion of lamellae that were covered by interlamellar cell mass. The latter change appeared to drive a significant main effect of chronic hypoxia ($p<0.01$). These responses are likely advantageous in hypoxia, as increases in the surface area of the gills allows for increased O_2 diffusion. However, there were significant interactions between wastewater and hypoxia on both lamellar height ($p<0.01$) and the proportional height of the ILCM ($p = 0.02$). In fact, among wastewater exposed fish, lamellar height shrunk slightly in hypoxia and the proportional ILCM height was unchanged.

Haematology

Wastewater exposure also altered haematology (Fig. 5; Table 3). Fish responded to chronic hypoxia in clean water with increases in haematocrit, contributing to a significant main effect of hypoxia acclimation ($p<0.01$). This was not associated with increases in blood haemoglobin content, for which there was not a significant main effect of chronic hypoxia ($p = 0.08$), such that mean corpuscular haemoglobin concentration (MCHC) tended to decline ($p = 0.05$). Wastewater affected these haematological responses, as reflected by a significant main effect on haematocrit ($p<0.01$) and a significant hypoxia×wastewater interaction for blood haemoglobin content ($p = 0.03$), which appeared to result from increases in these traits in normoxia and/or moderate hypoxia.

Tissue metabolites

Exposure to wastewater affected glycogen stores in fish exposed to normoxia and hypoxia (Fig. 6; Table 3). The general pattern of variation across tissues suggested that exposure to chronic hypoxia in clean water reduced glycogen stores in muscle and liver but had no effect on brain glycogen stores. However, in the muscle, there was a significant hypoxia×wastewater interaction on glycogen content ($p = 0.05$), driven primarily from a depletion of muscle glycogen during wastewater exposure in normoxia (Fig. 6A). There was also a significant hypoxia×wastewater interaction on glycogen content in liver ($p = 0.01$), but in this tissue, wastewater exposure appeared to mitigate glycogen depletion in severe hypoxia (Fig. 6B). Wastewater had a particularly detrimental effect in brain tissue, in which concurrent exposure to wastewater and severe hypoxia led to a near complete depletion of glycogen stores (Fig. 6C), which appeared to drive the significant main effect of hypoxia ($p = 0.03$) and the nearly significant hypoxia×wastewater interaction ($p = 0.06$). These changes in glycogen concentration occurred without any significant variation in tissue glucose or lactate contents (Tables 3, 5).

2.5 Discussion

Aquatic hypoxia is prevalent in many ecosystems that are heavily burdened by contaminants, including those that receive effluent discharge from municipal wastewater treatment plants. This study shows that exposure to wastewater effluent can disrupt many key physiological adjustments for coping with chronic hypoxia. Wastewater exposure had relatively modest effects on killifish in normoxia, but it impeded the improvements in hypoxia tolerance in response to chronic hypoxia and disrupted the plasticity of several associated physiological traits. This suggests that the ability of fish to cope with other natural or anthropogenic stressors may be impaired in environments contaminated with wastewater effluent.

Wastewater exposure had modest physiological effects in normoxia

The evidence emerging from our findings here in killifish and those from previous studies suggest that the metabolic impacts of wastewater exposure may differ between species. The lack of any effect on metabolic rate in killifish from exposure to wastewater in normoxia is similar to previous findings in round goby that were cage exposed to the receiving waters near a WWTP (McCallum et al., 2017). In contrast, some other species exhibit a prominent increase in metabolic rate in response to wastewater exposure, including bluegill sunfish (*Lepomis macrochirus*; Du et al., 2019, 2018) and rainbow darter (*Etheostoma caeruleum*; Mehdi et al., 2018). This could indicate that some species are more sensitive and experience a greater metabolic cost of wastewater exposure than others. Killifish are often found in many contaminated sites and are considered to be a relatively tolerant species, and several populations have evolved especially high resistance to persistent organic pollutants (Crawford et al., 2020; Reid et al., 2016; Weis, 2002). Killifish may therefore experience less metabolic stress than other less tolerant species when exposed to wastewater effluent, and thus avoid increases in whole-animal energy demands.

Exposure to wastewater effluent in normoxia had some subtle effects on gill structure and glycogen content that could reflect metabolic impacts that were not manifested as increases in whole-organism metabolic rate. We found that wastewater exposure increased lamellar height (Fig. 4A), consistent with similar findings in bluegill sunfish that were subjected to caged exposures to the receiving waters near a WWTP (Du et al., 2018), and without any obvious signs of the histopathological changes (*i.e.*, edema, lifting of lamellar epithelium) that have been observed after sewage exposure in a study on Nile tilapia (*Oreochromis niloticus*; Fontaínhas-Fernandes et al., 2008). We also observed muscle glycogen depletion after exposure to wastewater in normoxia (Fig. 6A), as previously observed in the muscle and liver of some other species caged downstream of a wastewater treatment plant (Cazenave et al., 2014; Du et al., 2019). These changes could reflect greater energy demands in a subset of tissues as a result of wastewater exposure, which are either too small to increase the metabolic rate of the whole organism or are offset by reductions in the energy demands of some other tissues.

Wastewater exposure disrupts responses to chronic hypoxia

One of the most significant impacts of wastewater exposure was the attenuation of plastic improvements in hypoxia tolerance, as reflected by increased time to loss of equilibrium (t_{LOE}) in near anoxia (0.5 kPa), which normally occurs in response to chronic hypoxia (Fig. 2B). Killifish in clean water responded to chronic hypoxia with increases in t_{LOE}, consistent with previous findings in several killifish species (Borowiec et al., 2015, 2020), which could reflect an improved ability to match O_2 supply and O_2 demand during hypoxia. The reduction in t_{LOE} in chronic hypoxia due to wastewater could not be explained by a metabolic cost of exposure that increased O_2 demands, because combined exposure to wastewater and hypoxia reduced, rather than increased SMR (Fig. 1). Wastewater exposure could have instead reduced t_{LOE} by impairing tissue O_2 supply. This possibility is supported by our observation that wastewater exposure blunted the plastic responses of gill morphology to chronic hypoxia, eliminating the lengthening of lamellae and regression of the ILCM that occurred in response to chronic hypoxia in clean water (Figs. 3-4). Wastewater exposure could have also reduced t_{LOE} by reducing the ability of killifish to use anaerobic metabolism. Glycogen stores in the brain are a key fuel for anaerobic metabolism that help determine the ability to maintain ATP levels and avoid losing equilibrium in severe hypoxia (Speers-Roesch et al., 2013). Therefore, the low brain glycogen reserves in killifish exposed to wastewater and hypoxia (Fig. 6C) likely reduced the ability to fuel anaerobic metabolism and thus constrained t_{LOE}.

Exposure to wastewater effluent had no effects on P_{crit}. Killifish responded to chronic hypoxia in clean water with decreases in P_{crit}, consistent with previous findings in killifish and other species (Borowiec et al., 2015, 2020; Fu et al., 2011), but this plastic response was unaffected by wastewater (Fig. 2A). This contrasts recent findings in threespine stickleback (*Gasterosteus aculeatus*), in which copper exposure impaired the improvements in P_{crit} that result from hypoxia acclimation (Fitzgerald et al., 2019). P_{crit} is often considered to reflect the ability to extract oxygen from the environment during hypoxia, and can be a useful indicator of hypoxia tolerance in many species (Regan et al.,

2019; Rogers et al., 2016; Speers-Roesch et al., 2013). However, in hypoxia-tolerant species such as killifish that can depress metabolic rate (Borowiec et al., 2018), the reductions in MO_2 below P_{crit} (Fig. S9) could reflect a facultative reduction in O_2 demands rather than a hypoxia-induced constraint on O_2 extraction. This may explain why wastewater exposure impaired gill plasticity but had no effect on the decreases in P_{crit} in response to chronic hypoxia. Furthermore, species differences in the effects of contaminant exposure on P_{crit} could result from differences in whether MO_2 depression below P_{crit} is facultative or a result of O_2 supply limitation. For example, many populations of threespine stickleback do not employ metabolic depression in hypoxia (Regan et al., 2017), which may make P_{crit} more susceptible to contaminant exposure in this species than it is in killifish.

The disruption of hypoxic responses could be attributed to several of the chemical contaminants in wastewater effluent. Selective serotonin reuptake inhibitors (SSRIs) like fluoxetine could inhibit serotonin signaling by oxygen sensing neuroepithelial cells in the gills, which may have disrupted key cardiorespiratory adjustments to hypoxia exposure. Indeed, in gulf toadfish, short-term exposure to fluoxetine (intraperitoneal injection) has been shown to reduce the ventilatory response to hypoxia and to accentuate oxyconformation in hypoxia (*i.e.*, decreased regulation index; Amador et al., 2018; Panlilio et al., 2016). β-blockers such as propranolol can also disrupt oxygen chemoreception by NECs in the gills (Burleson & Milsom, 1990), and can affect heart rate (Fraysse et al., 2006) and blood-flow distribution through the gills (Payan & Girard, 1977). Ligands of the aryl hydrocarbon receptor (AHR), such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and other persistent organic pollutants, could also disrupt cellular responses to hypoxia (Fleming et al., 2009; Kraemer & Schulte, 2004). This is because many of the biochemical and physiological responses to hypoxia result from activation of the hypoxia-inducible factor (HIF) pathway (Pelster & Egg, 2018), and there can be crosstalk between HIF-α and AHR signaling as they require the same dimerization partner (HIF-1β, also called the AHR Nuclear Translocator or ARNT) to drive the expression of target genes. Given that many of the cellular responses to hypoxia lead to

adjustments in metabolic pathways (*e.g.*, metabolic depression, increased use of anaerobic metabolism, shifts in fuel use between lipids and carbohydrates, etc.), it is also possible that such changes could be disrupted by the various pharmaceuticals that target these pathways (*e.g.*, statins such as atorvastatin, diabetes medications such as metformin, etc.). It is important to pursue molecular and cellular events that are linked to whole-organism effects in multiple stressor environments. Further studies should prioritize the confirmation of mechanisms underlying the combined effects of the contaminants found in wastewater effluent and other abiotic stressors (particularly those that are projected to become more prevalent with climate change, such as warming temperature, reduced pH).

Conclusions

Our findings suggest that the interaction between the chemical contaminants in wastewater and the other abiotic stressors near WWTPs may have significant detrimental effects on the physiology and health of fish. Wastewater exposure disrupted the physiological adjustments employed by killifish to cope with chronic hypoxia and thus impaired hypoxia tolerance. Chronic exposure to wastewater and hypoxia combined also led to the near depletion of brain glycogen reserves, which could put animals at extreme risk if faced with an additional metabolic stress (*e.g.*, heat waves, predator evasion). Our results support growing evidence that wastewater exposure can have significant disruptive effects on respiratory and metabolic processes in fish. Lab exposures to whole wastewater effluent are relevant to what aquatic biota experience in the natural environment and captures responses to real world complex mixtures of pollutants. Mechanistic approaches to better understand these effects can therefore improve our capacity to predict the impacts of aquatic pollution at organismal and population levels.

2.6 Tables and Figures

Table 1: Water quality measurements during chronic exposures.

Parameters	**Clean water**	**Wastewater**
pH	8.88 (±0.03)	8.93 (±0.04)
Conductivity (μS)	7.62 (±0.17)	7.77 (±0.14)
Total dissolved solids (ppt)	5.26 (±0.16)	5.40 (±0.12)
Salinity (ppt)	4.25 (±0.06)	4.21(±0.04)
Temperature (°C)	17.78 (±0.20)	17.88 (±0.17)
NH_3 (ppm)	0.69 (±0.14)	0.90 (±0.17)
NO_3 (ppm)	11.18 (±3.43)	11.18 (±2.76)
NO_2 (ppm)	0.54 (±0.15)	0.68 (±0.13)

Measurements were made immediately before water changes, which were conducted every 3 days. Data are means (± SEM) of all measurements made in clean water or wastewater (n = 28-38).

Table 2: Average concentrations (ng/L) of various classes of contaminants and some associated metabolites measured in tank water during exposures.

Class	Chemical	Clean water (n = 10)	Wastewater (n = 13-14)
Lipid Regulator	Gemfibrozil	0.3 (±0.1)	**10.0 (±1.4)**
	Atorvastatin	0.3 (±0.2)	**10.5 (±3.4)**
	p-hydroxy Atorvastatin	0.0 (±0.0)	**26.6(±5.9)**
	o-hydroxy Atorvastatin	0.3 (±0.1)	**20.5 (±6.1)**
Antiepileptic	Carbamazepine	2.1 (±1.1)	**44.4 (±3.0)**
Analgesic	Acetaminophen	0.0 (±0.0)	27.2 (±26.2)
Stimulant	Caffeine	122.2 (±25.9)	266.1 (±150.8)
Antibacterial	Triclosan	8.4 (±1.9)	12.6 (±2.8)
	Sulfamethazine	1.8 (±1.0)	**16.0 (±1.2)**
Antibiotic	Monensin	0.0 (±0.0)	0.0 (±0.0)
	Trimethoprim	0.0 (±0.0)	**22.6 (±4.1)**
	Lincomycin	0.2 (±0.2)	**7.0 (±1.1)**
	Sulfamethoxazole	0.0 (±0.0)	**63.9 (±5.5)**
Antidepressant	Fluoxetine	0.2 (±0.1)	**2.2 (±0.6)**
	Norfluoxetine	0.6 (±0.2)	2.2 (±0.6)
	Venlafaxine	0.5 (±0.2)	**89.2 (±6.8)**
	Desvenlafaxine	0.9 (±0.3)	**155.3 (±12.9)**
NSAID	Ibuprofen	1.1 (±0.4)	**152.5 (±51.1)**
	Naproxen	1.5 (±0.4)	**131.2 (±41.5)**
	Diclofenac	30.7 (±9.6)	**154.4 (±9.3)**
Herbicide	Atrazine	1.1 (±0.1)	**4.6 (±0.2)**
Industrial	Bisphenol A	27.8 (±4.7)	138.3 (±48.0)

Data above represent measurements of samples taken once from each tank replicate in each treatment group immediately after the renewal of tank water with fresh effluent (see Materials and Methods), shown as means (± SEM) of all measurements made in clean water or wastewater (n = 10-14). Zeros indicate measurements that were below the detection limit, while bolded values indicate concentrations that were significantly different from clean water ($p<0.05$). NSAID: Nonsteroidal anti-inflammatory drug.

Table 3: Statistical results of two-way ANOVA.

	Hypoxia effect			**Wastewater effect**			**Hypoxia × wastewater**		
	F	*df*	*p*	*F*	*df*	*p*	*F*	*df*	*p*
SMR	1.19	2	0.31	2.89	1	0.10	7.00	2	**<0.01**
RMR	0.65	2	0.53	0.0007	1	0.98	3.03	2	0.06
t_{LOE}	33.14	2	**<0.01**	7.71	1	**<0.01**	2.04	2	0.14
P_{crit}	4.36	2	**0.02**	0.30	1	0.59	0.53	2	0.60
Total Lamellar Height	1.31	1	0.27	0.002	1	0.96	20.23	**1**	**<0.01**
Lamellar Width	0.35	1	0.56	0.004	1	0.95	1.51	1	0.23
Lamellar Density	0.33	1	0.57	0.21	1	0.65	1.73	1	0.20
Proportion ILCM	11.13	1	**<0.01**	0.037	1	0.85	6.38	**1**	**0.02**
Haematocrit	8.35	2	**<0.01**	10.33	1	**<0.01**	1.08	2	0.35
[Hb]	2.69	2	0.08	1.99	1	0.16	3.61	2	**0.03**
MCHC	3.14	2	0.05	0.04	1	0.84	0.38	2	0.68
Muscle Glycogen	0.79	2	0.46	2.73	1	0.10	3.17	2	**0.05**
Liver Glycogen	0.40	2	0.67	2.25	1	0.14	4.89	2	**0.01**
Brain Glycogen	3.95	2	**0.03**	0.09	1	0.77	3.00	2	0.06
Muscle Glucose	1.66	2	0.20	0.27	1	0.60	0.02	2	0.98
Liver Glucose	0.39	2	0.68	0.05	1	0.83	0.74	2	0.48
Brain Glucose	0.88	2	0.423	0.76	1	0.39	0.75	2	0.48
Muscle Lactate	0.76	2	0.47	1.21	1	0.28	1.68	2	0.19
Liver Lactate	1.30	2	0.28	0.55	1	0.46	2.19	2	0.12
Brain Lactate	0.79	2	0.46	0.35	1	0.56	0.09	2	0.91

df, degrees of freedom; SMR, standard metabolic rate; RMR, resting metabolic rate; t_{LOE}, time to loss of equilibrium; P_{crit}, critical oxygen tension; ILCM, interlamellar cell mass; [Hb], blood hemoglobin content; MCHC, mean corpuscular haemoglobin concentration.

Table 4: Fish body mass, standard length, Fulton's condition factor, and hepatosomatic index.

	Normoxia (20 kPa)		**Moderate hypoxia (5 kPa)**		**Severe hypoxia (2 kPa)**	
	Clean	**WW**	**Clean**	**WW**	**Clean**	**WW**
Fish used to measure SMR and P_{crit}						
Body Mass (g)	3.77±0.12 (8)	3.48±0.50 (8)	3.19±0.22 (8)	3.90±0.25 (12)	3.28±0.21 (8)	3.50±0.31 (12)
Standard length (mm)	57.64±0.8 (8)	57.53±2.11 (8)	55.39±1.47 (8)	59.21±1.51 (12)	57.91±1.22 (8)	56.18±1.42 (12)
K (g/cm^3)	1.02±0.03 (8)	0.96±0.05 (6)	0.96±0.04 (8)	0.94±0.03 (12	0.86±0.04 (8)	0.97±0.02 (12)
HSI (%)	1.48±0.10 (8)	1.56±0.13 (8)	1.52±0.20 (8)	1.56±0.21 (12)	1.23±0.50 (8)	1.73±0.15 (12)
Fish used to measure t_{LOE}						
Body Mass (g)	3.32±0.22 (8)	2.50±0.10 (8)	2.75±0.26 (8)	2.57±0.18 (12)	2.88±0.23 (8)	2.93±0.20 (12)
Standard length (mm)	57.18±1.5 5 (8)	51.81±0.79 (8)	52.49±2.05 (8)	52.66±0.98 (12)	54.06±1.11 (8)	54.25±1.14 (12)
K (g/cm^3)	0.97±0.04 (8)	0.95±0.05 (7)	0.91±0.05 (8)	0.92±0.03 (10)	0.90±0.03 (8)	0.92±0.04 (12)
HSI (%)	1.39±0.07 (8)	1.71±0.25 (8)	1.750.16 (8)	1.73±0.20 (12)	1.34±0.10 (8)	1.75±0.25 (12)
Sampled Fish						
Body Mass (g)	3.53±0.18 (8)	3.30±0.26 (13)	2.58±0.17 (11)	3.03±0.15 (19)	3.20±0.20 (13)	2.67±0.22 (13)
Standard length (mm)	58.46±1.5 6 (8)	56.95±1.52 (13)	51.48±1.27 (11)	55.11±1.12 (19)	56.30±1.42 (13)	52.54±1.47 (13)
K (g/cm^3)	1.05±0.14 (7)	0.94±0.03 (8)	1.12±0.12 (9)	0.97±0.04 (18)	0.86±0.03 (13)	0.94±0.03 (12)
HSI (%)	1.48±0.15 (8)	1.60±0.20 (13)	1.57±0.22 (10)	1.86±0.18 (19)	1.10±0.10 (13)	1.40±0.16 (13)

Data are means ± SEM (n). WW, wastewater; K, Fulton's condition factor (g/cm^3); HSI, hepatosomatic index, equals liver mass relative to body mass (%); SMR, standard metabolic rate; P_{crit}, critical O_2 tension; t_{LOE}, time to loss of equilibrium at 0.5 kPa O_2.

Table 5: Glucose and lactate content in muscle, liver, and brain tissues.

	Normoxia (20 kPa)		**Moderate hypoxia (5 kPa)**		**Severe hypoxia (2 kPa)**	
	Clean	**WW**	**Clean**	**WW**	**Clean**	**WW**
Muscle Glucose	3.34±0.89 (11)	3.12±0.81 (12)	4.81±0.85 (11)	4.40±0.68 (12)	4.99±1.07 (11)	4.46±1.11 (12)
Liver Glucose	15.71±3.89 (12)	18.44±2.91 (13)	19.88±4.38 (10)	15.83±2.95 (15)	13.50±3.00 (11)	15.53±2.55 (12)
Brain Glucose	4.13±0.41 (6)	3.13±0.59 (6)	3.25±0.68 (6)	2.87±0.43 (8)	3.52±0.58 (6)	3.80±0.41 (8)
Muscle Lactate	13.33±1.55 (11)	9.86±1.21 (12)	12.42±1.55 (11)	12.01±0.86 (13)	12.69±1.03 (11)	13.40±0.76 (12)
Liver Lactate	5.32±0.85 (8)	5.29±0.60 (12)	6.49±0.78 (11)	5.78±0.58 (15)	5.43±0.54 (11)	7.47±0.86 (12)
Brain Lactate	15.64±0.32 (6)	15.69±0.68 (6)	16.82±2.65 (6)	17.38±0.56 (8)	15.56±0.84 (6)	16.64±0.88 (8)

WW, wastewater. Data are expressed in units μmol/g tissue as means ± SEM (n).

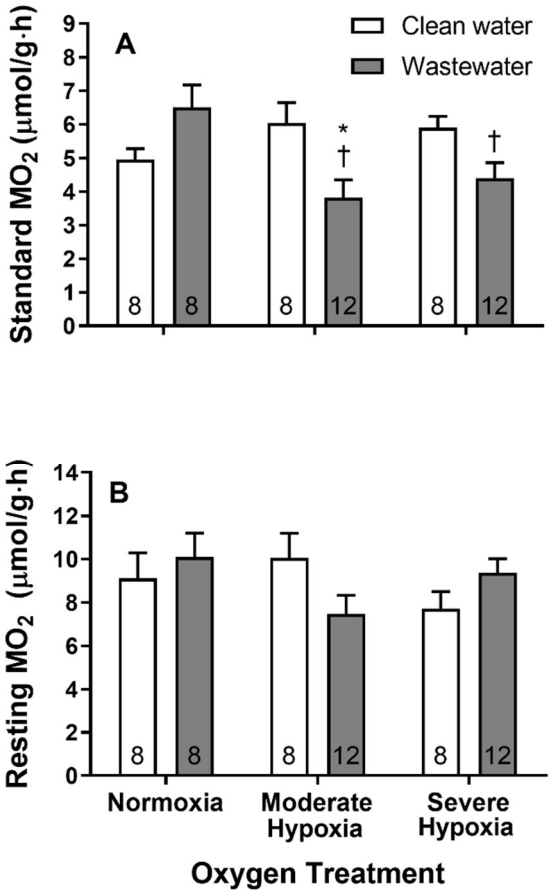

A
Clean water
Wastewater
Standard MO_2 (μmol/g·h)
9
8
7
6
5
4
3
2
1
0
*
†
†
8
8
8
12
8
12
B
Resting MO_2 (μmol/g·h)
14
12
10
8
6
4
2
0
8
8
8
12
8
12
Normoxia
Moderate Hypoxia
Severe Hypoxia
Oxygen Treatment

Figure 1: Chronic exposure to the combination of hypoxia and wastewater reduced (A) standard metabolic rate (MO_2) but not (B) resting MO_2. *Significant pairwise difference from clean water controls within an oxygen treatment ($p<0.05$). † Significant pairwise difference from normoxic controls within clean water or wastewater groups ($p<0.05$). Data are presented as means ± SEM, with sample sizes indicated within each bar.

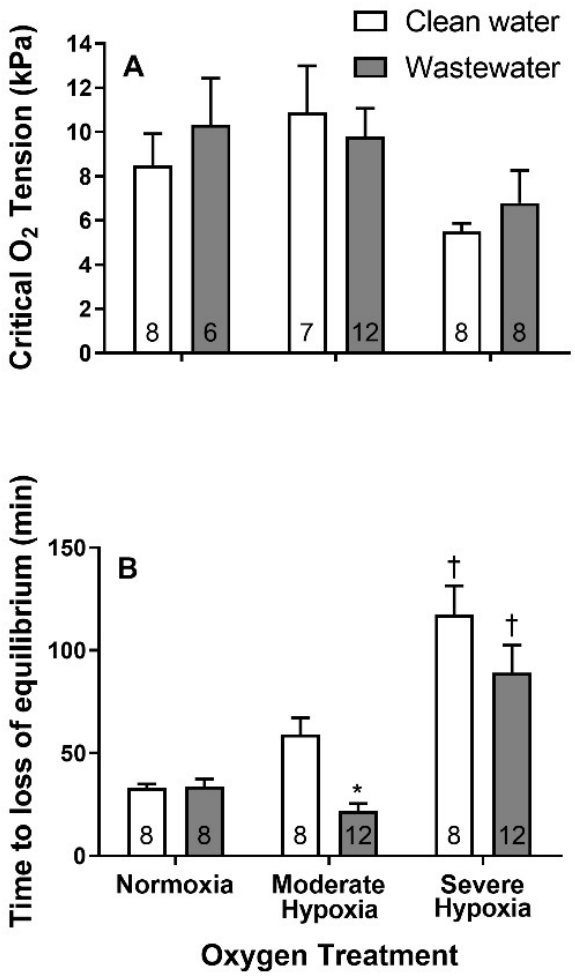

A
Clean water
Wastewater
Critical O_2 Tension (kPa)
0
2
4
6
8
10
12
14
8
6
7
12
8
8
B
Time to loss of equilibrium (min)
0
50
100
150
8
8
8
12
8
12
*
†
†
Normoxia
Moderate Hypoxia
Severe Hypoxia
Oxygen Treatment

Figure 2: Wastewater exposure attenuated the improvement in hypoxia tolerance in response to chronic hypoxia without affecting critical O_2 tension. (A) Critical oxygen tension (P_{crit}); (B) time to loss of equilibrium during acute exposure to 0.5 kPa O_2. *Significant pairwise difference from clean water controls within an oxygen treatment (p<0.05). †Significant pairwise difference from normoxic controls within clean water or wastewater groups (p<0.05). Data are presented as means ± SEM, with sample sizes indicated within each bar.

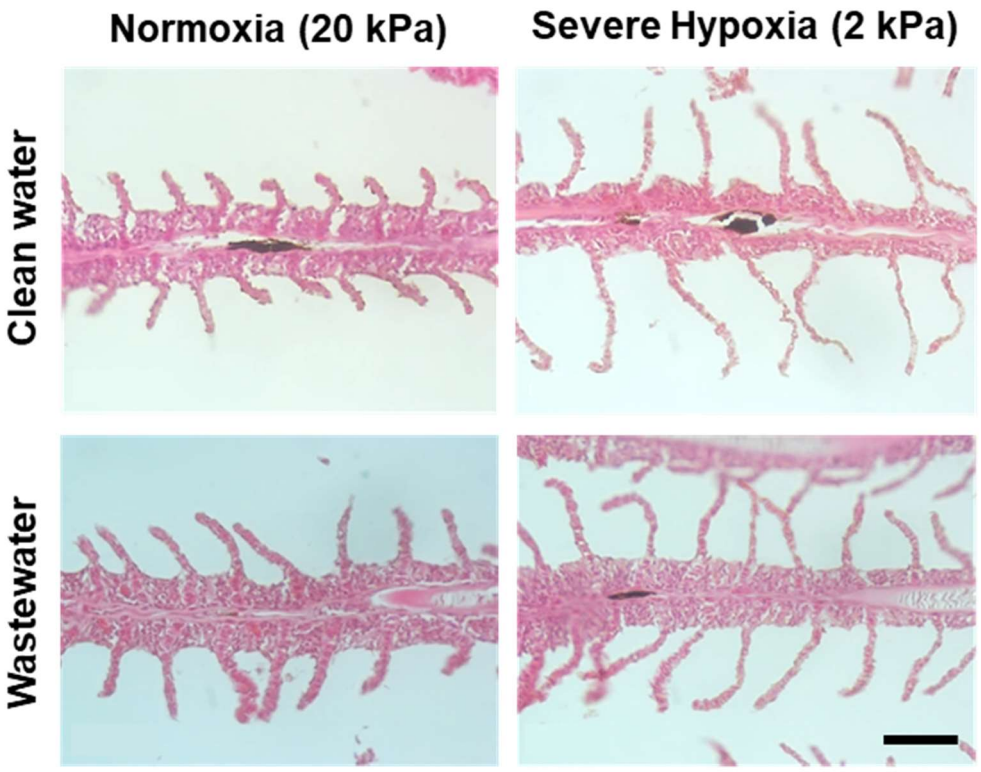

Normoxia (20 kPa)
Severe Hypoxia (2 kPa)
Clean water
Wastewater

Figure 3: Representative images of the gills of killifish exposed to (A) normoxia in clean water, (B) severe hypoxia in clean water, (C) normoxia in wastewater, and (D) severe hypoxia in wastewater. Slides are stained with haematoxylin and eosin. Scale bar represents 50 μm and all images are shown at the same scale.

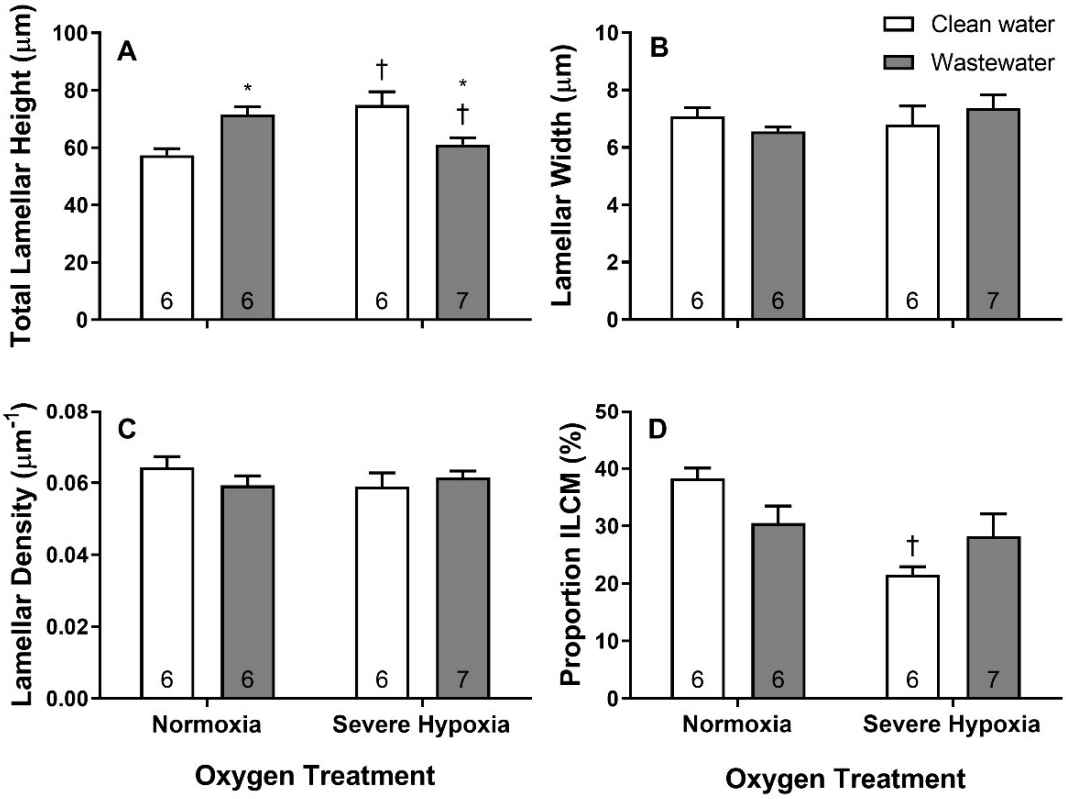

A
B
C
D
Total Lamellar Height (μm)
Lamellar Width (μm)
Lamellar Density (μm-1)
Proportion ILCM (%)
Clean water
Wastewater
Normoxia
Severe Hypoxia
Oxygen Treatment
Oxygen Treatment

Figure 4: Morphological changes in the gills of killifish in response to exposure to hypoxia and/or wastewater. *Significant pairwise difference from clean water controls within an oxygen treatment (p<0.05). †Significant pairwise difference from normoxic controls within clean water or wastewater groups (p<0.05). Data are presented as means ± SEM, with sample sizes indicated within the bars.

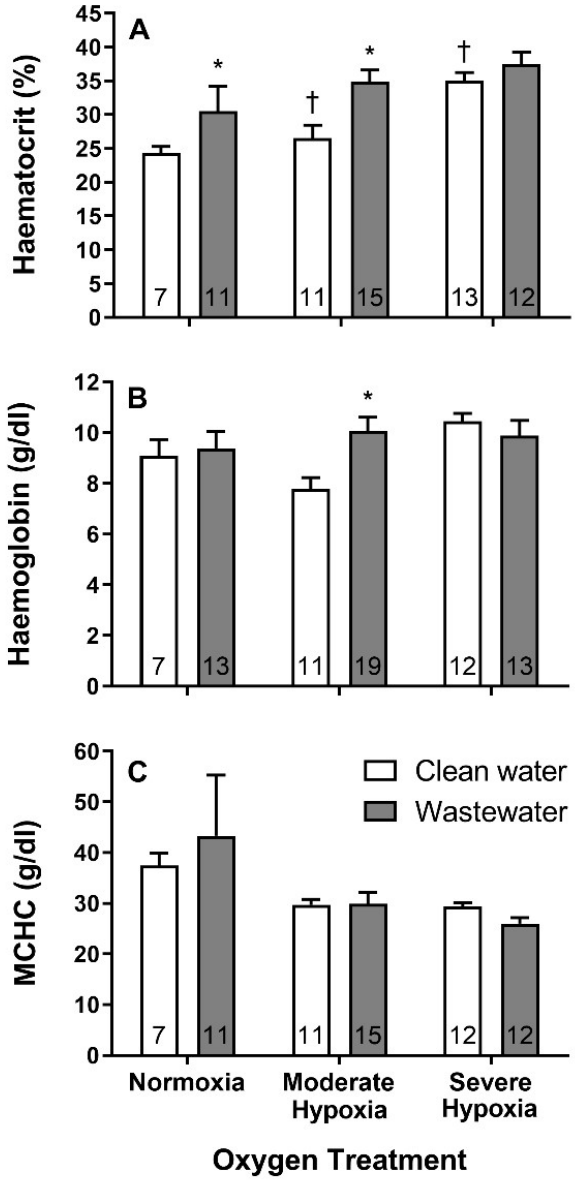

A
Haematocrit (%)
45
40
35
30
25
20
15
10
5
0
*
†
*
†
7
11
11
15
13
12
B
Haemoglobin (g/dl)
12
10
8
6
4
2
0
*
7
13
11
19
12
13
C
MCHC (g/dl)
60
50
40
30
20
10
0
Clean water
Wastewater
7
11
11
15
12
12
Normoxia
Moderate Hypoxia
Severe Hypoxia
Oxygen Treatment

Figure 5: The effects of hypoxia and/or wastewater exposure on (A) haematocrit, (B) blood haemoglobin content, and (C) mean corpuscular haemoglobin concentration (MCHC). *Significant pairwise difference from clean water controls within an oxygen treatment ($p<0.05$). †Significant pairwise difference from normoxic controls within clean water or wastewater groups ($p<0.05$). Data are presented as means ± SEM, with sample sizes indicated within the bars.

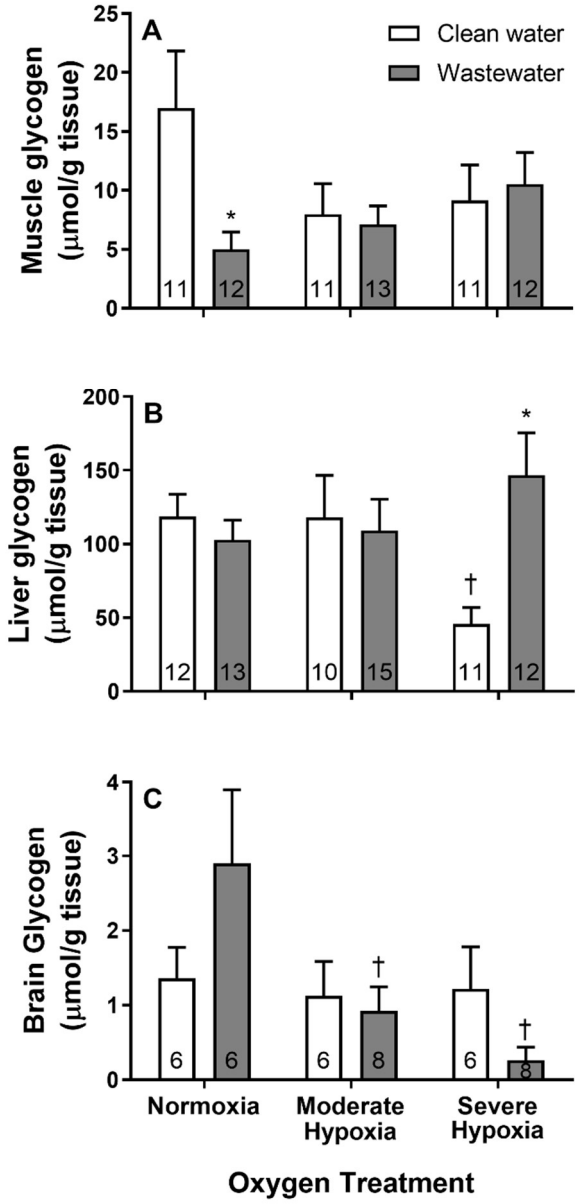

A
Clean water
Wastewater
Muscle glycogen (μmol/g tissue)
25
20
15
10
5
0
*
11
12
11
13
11
12
B
Liver glycogen (μmol/g tissue)
200
150
100
50
0
*
†
12
13
10
15
11
12
C
Brain Glycogen (μmol/g tissue)
4
3
2
1
0
†
†
6
6
6
8
6
8
Normoxia
Moderate Hypoxia
Severe Hypoxia
Oxygen Treatment

Figure 6: The effects of hypoxia and/or wastewater exposure on glycogen content in muscle, liver, and brain. *Significant pairwise difference from clean water controls within an oxygen treatment (p<0.05). †Significant pairwise difference from normoxic controls within clean water or wastewater groups (p<0.05). Data are presented as means ± SEM, with sample sizes indicated within the bars.

2.7 Supplementary Materials

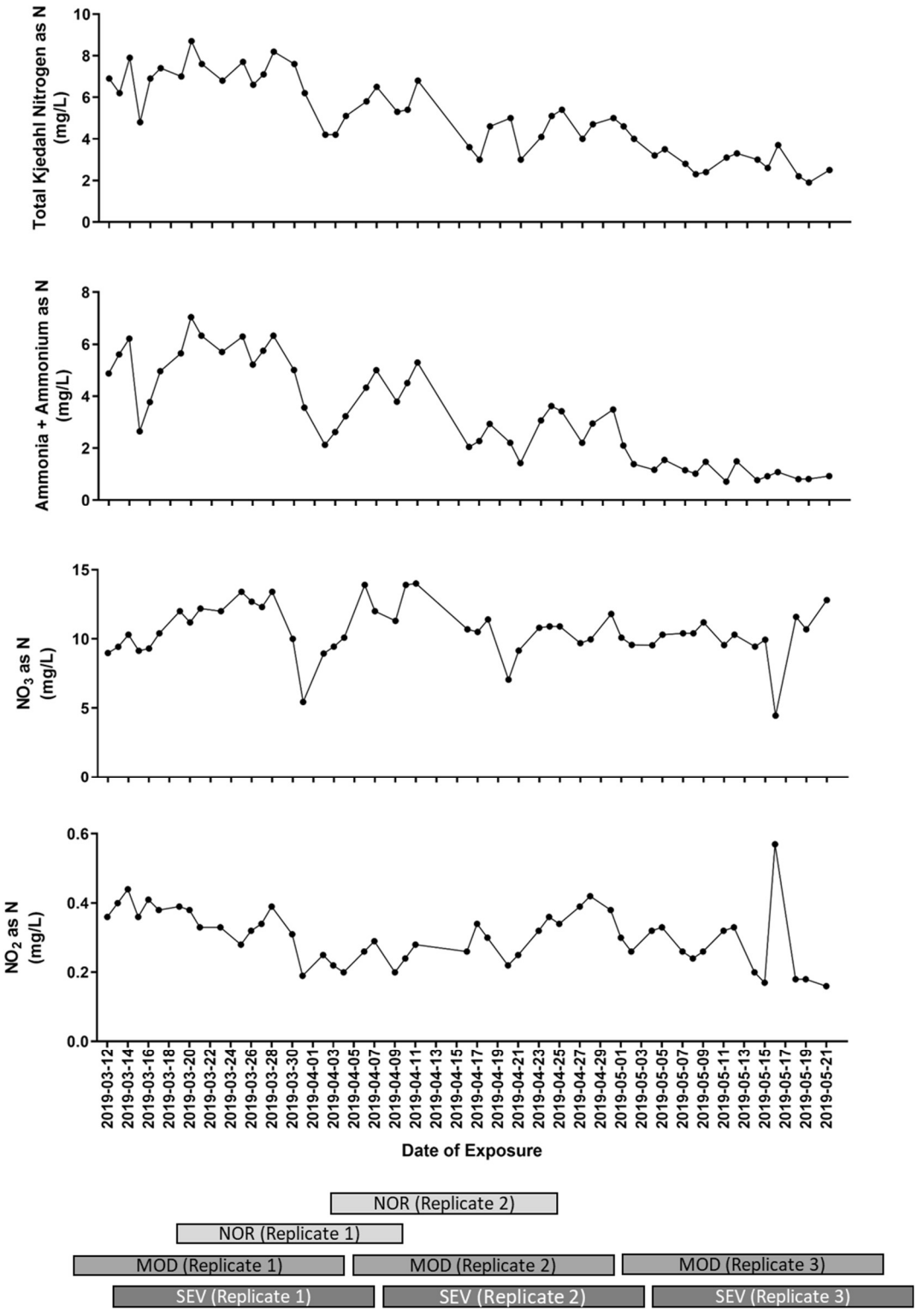
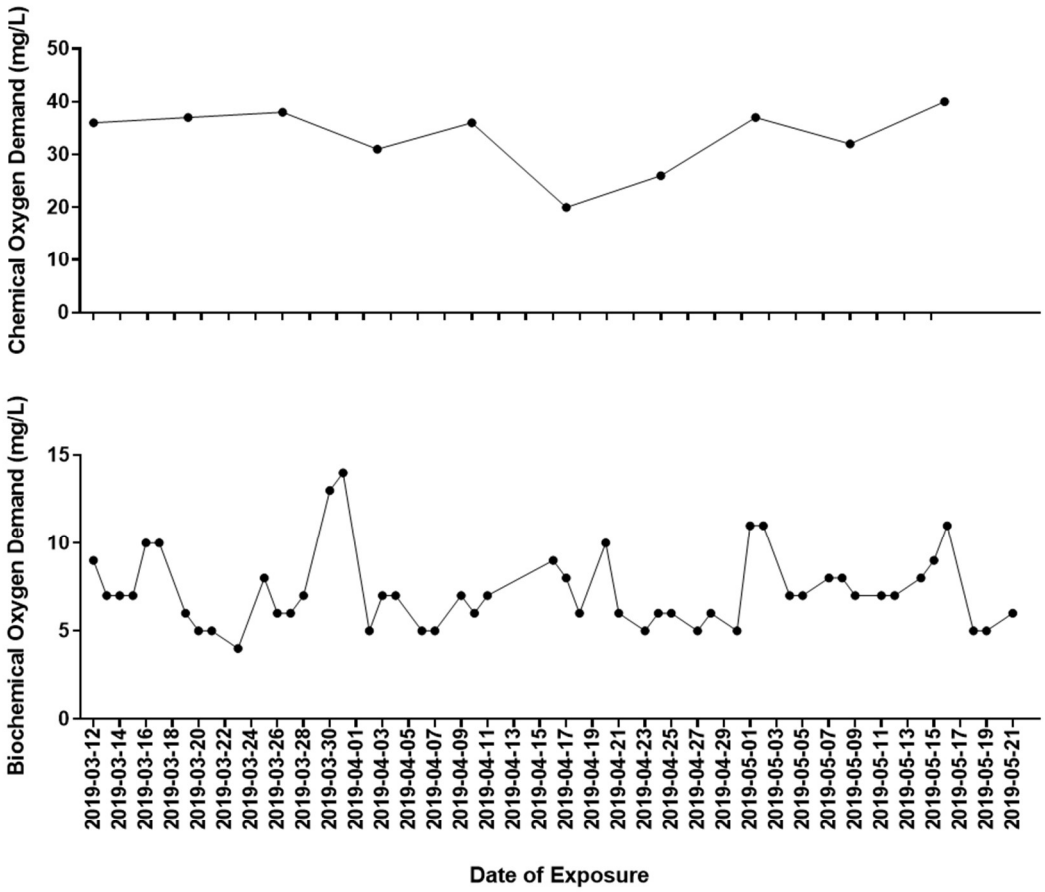


Figure S1: Changes in nitrogen compounds in final treated effluent during the chronic exposure period. Final treated effluent data was provided by the Hamilton Water Environmental Laboratory. Staggered schedule of wastewater exposed tank replicates is shown below. NOR = Normoxia, MOD = Moderate hypoxia, SEV = Severe hypoxia.


Chemical Oxygen Demand (mg/L)
50
40
30
20
10
0
Biochemical Oxygen Demand (mg/L)
15
10
5
0
2019-03-12
2019-03-14
2019-03-16
2019-03-18
2019-03-20
2019-03-22
2019-03-24
2019-03-26
2019-03-28
2019-03-30
2019-04-01
2019-04-03
2019-04-05
2019-04-07
2019-04-09
2019-04-11
2019-04-13
2019-04-15
2019-04-17
2019-04-19
2019-04-21
2019-04-23
2019-04-25
2019-04-27
2019-04-29
2019-05-01
2019-05-03
2019-05-05
2019-05-07
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2019-05-13
2019-05-15
2019-05-17
2019-05-19
2019-05-21
Date of Exposure

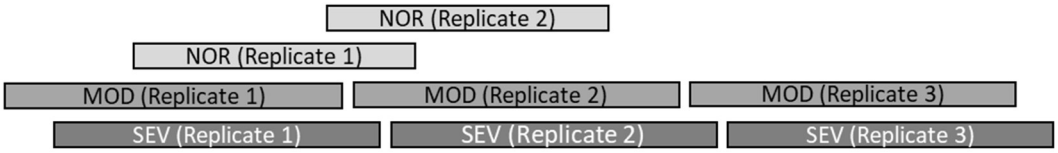
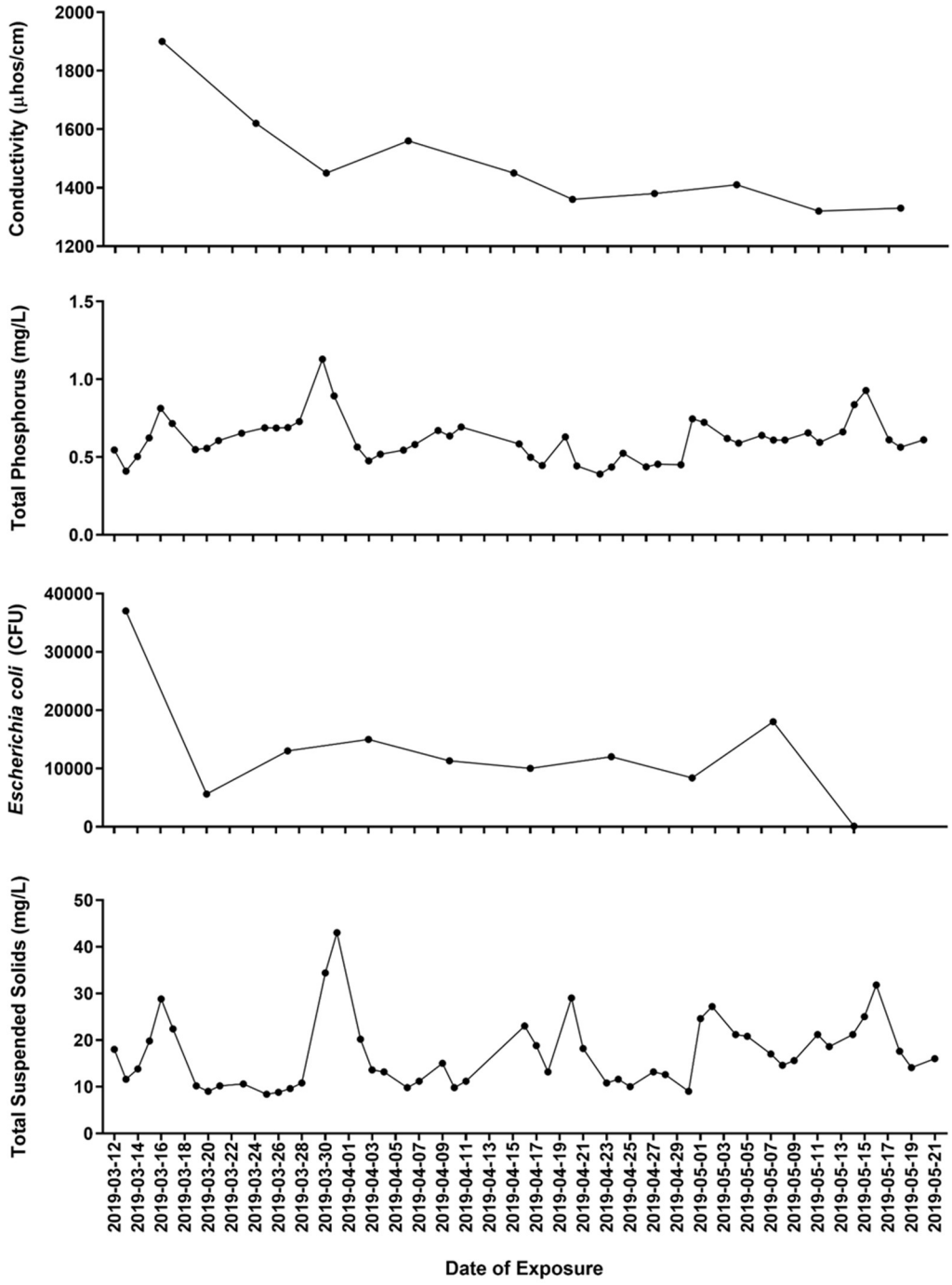

NOR (Replicate 2)
NOR (Replicate 1)
MOD (Replicate 1)
MOD (Replicate 2)
MOD (Replicate 3)
SEV (Replicate 1)
SEV (Replicate 2)
SEV (Replicate 3)

Figure S2: Changes in biochemical and chemical oxygen demand in final treated effluent during the exposure period. Final treated effluent data was provided by the Hamilton Water Environmental Laboratory. Staggered schedule of wastewater exposed tank replicates is shown below. NOR = Normoxia, MOD = Moderate hypoxia, SEV = Severe hypoxia.


Conductivity (µhos/cm)
2000
1800
1600
1400
1200
Total Phosphorus (mg/L)
1.5
1.0
0.5
0.0
Escherichia coli (CFU)
40000
30000
20000
10000
0
Total Suspended Solids (mg/L)
50
40
30
20
10
0
2019-03-12
2019-03-14
2019-03-16
2019-03-18
2019-03-20
2019-03-22
2019-03-24
2019-03-26
2019-03-28
2019-03-30
2019-04-01
2019-04-03
2019-04-05
2019-04-07
2019-04-09
2019-04-11
2019-04-13
2019-04-15
2019-04-17
2019-04-19
2019-04-21
2019-04-23
2019-04-25
2019-04-27
2019-04-29
2019-05-01
2019-05-03
2019-05-05
2019-05-07
2019-05-09
2019-05-11
2019-05-13
2019-05-15
2019-05-17
2019-05-19
2019-05-21
Date of Exposure

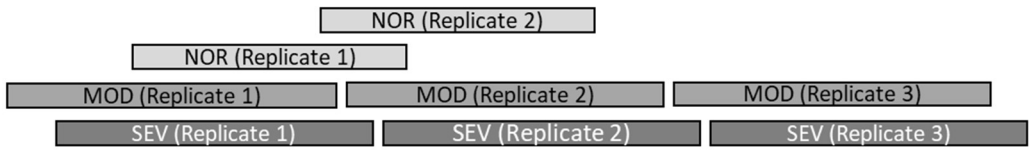

NOR (Replicate 2)
NOR (Replicate 1)
MOD (Replicate 1)
MOD (Replicate 2)
MOD (Replicate 3)
SEV (Replicate 1)
SEV (Replicate 2)
SEV (Replicate 3)

Figure S3: Changes in other water quality measures of the final treated effluent during the exposure period. Final treated effluent data was provided by the Hamilton Water Environmental Laboratory. Staggered schedule of wastewater exposed tank replicates is shown below. NOR = Normoxia, MOD = Moderate hypoxia, SEV = Severe hypoxia.

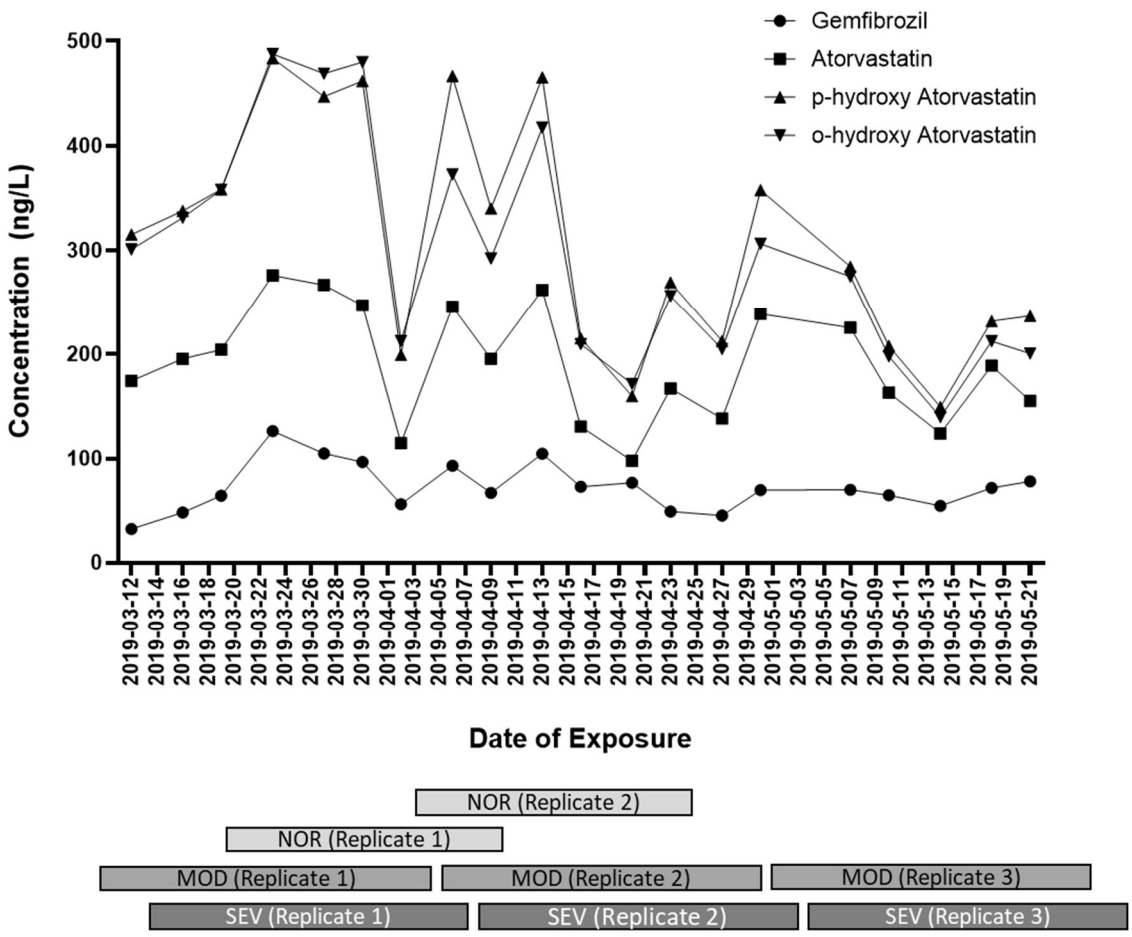

Gemfibrozil
Atorvastatin
p-hydroxy Atorvastatin
o-hydroxy Atorvastatin
Concentration (ng/L)
500
400
300
200
100
0
2019-03-12
2019-03-14
2019-03-16
2019-03-18
2019-03-20
2019-03-22
2019-03-24
2019-03-26
2019-03-28
2019-03-30
2019-04-01
2019-04-03
2019-04-05
2019-04-07
2019-04-09
2019-04-11
2019-04-13
2019-04-15
2019-04-17
2019-04-19
2019-04-21
2019-04-23
2019-04-25
2019-04-27
2019-04-29
2019-05-01
2019-05-03
2019-05-05
2019-05-07
2019-05-09
2019-05-11
2019-05-13
2019-05-15
2019-05-17
2019-05-19
2019-05-21
Date of Exposure
NOR (Replicate 2)
NOR (Replicate 1)
MOD (Replicate 1)
MOD (Replicate 2)
MOD (Replicate 3)
SEV (Replicate 1)
SEV (Replicate 2)
SEV (Replicate 3)

Figure S4: Changes in the concentrations of lipid regulator compounds and metabolites in wastewater effluent at the time of collection. Staggered schedule of wastewater exposed tank replicates is shown below. NOR = Normoxia, MOD = Moderate hypoxia, SEV = Severe hypoxia.

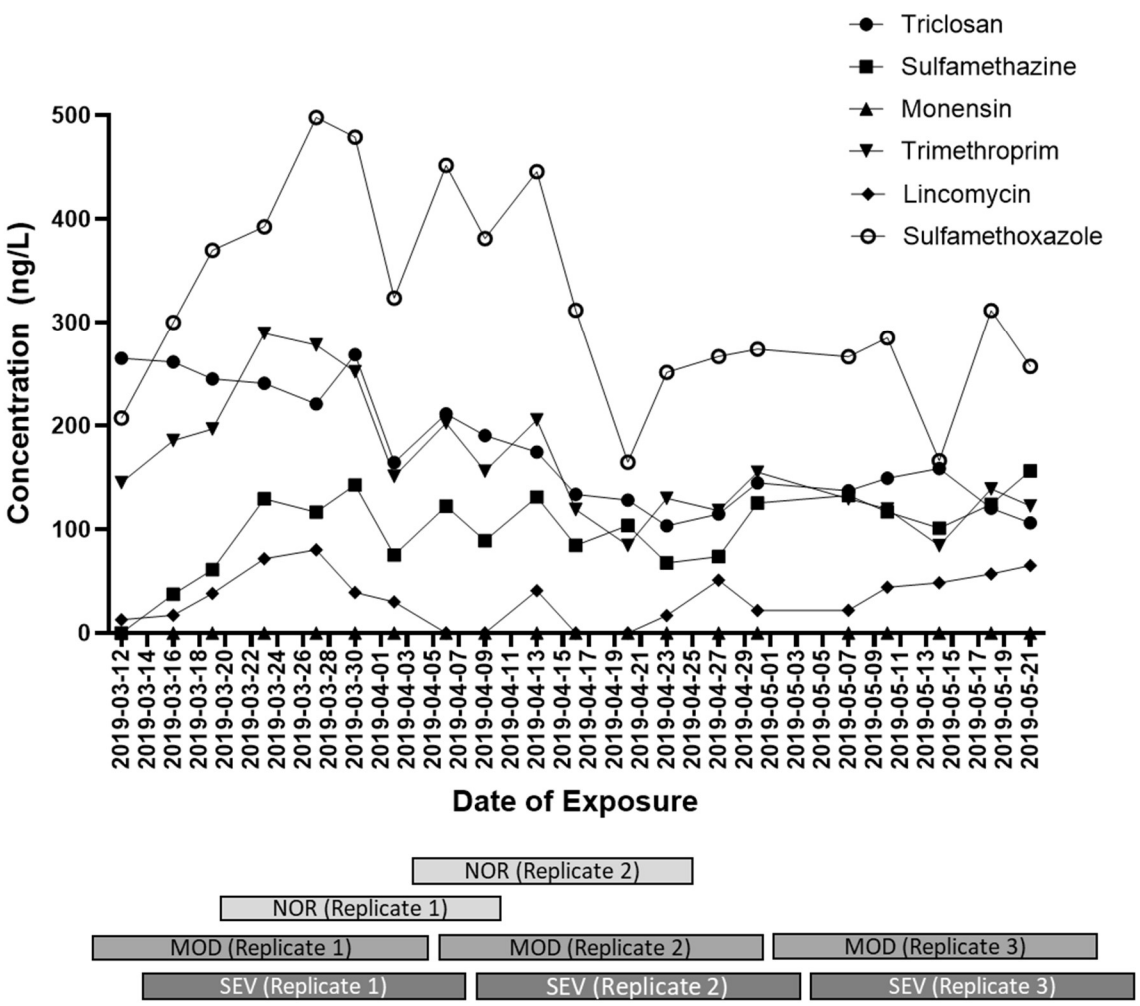

Triclosan
Sulfamethazine
Monensin
Trimethroprim
Lincomycin
Sulfamethoxazole
Concentration (ng/L)
500
400
300
200
100
0
2019-03-12
2019-03-14
2019-03-16
2019-03-18
2019-03-20
2019-03-22
2019-03-24
2019-03-26
2019-03-28
2019-03-30
2019-04-01
2019-04-03
2019-04-05
2019-04-07
2019-04-09
2019-04-11
2019-04-13
2019-04-15
2019-04-17
2019-04-19
2019-04-21
2019-04-23
2019-04-25
2019-04-27
2019-04-29
2019-05-01
2019-05-03
2019-05-05
2019-05-07
2019-05-09
2019-05-11
2019-05-13
2019-05-15
2019-05-17
2019-05-19
2019-05-21
Date of Exposure
NOR (Replicate 2)
NOR (Replicate 1)
MOD (Replicate 1)
MOD (Replicate 2)
MOD (Replicate 3)
SEV (Replicate 1)
SEV (Replicate 2)
SEV (Replicate 3)

Figure S5: Changes in the concentrations of antibacterial and antibiotic compounds in wastewater effluent at the time of collection. Zeros indicate concentrations measured below the detection limit. Staggered schedule of wastewater exposed tank replicates is shown below. NOR = Normoxia, MOD = Moderate hypoxia, SEV = Severe hypoxia.

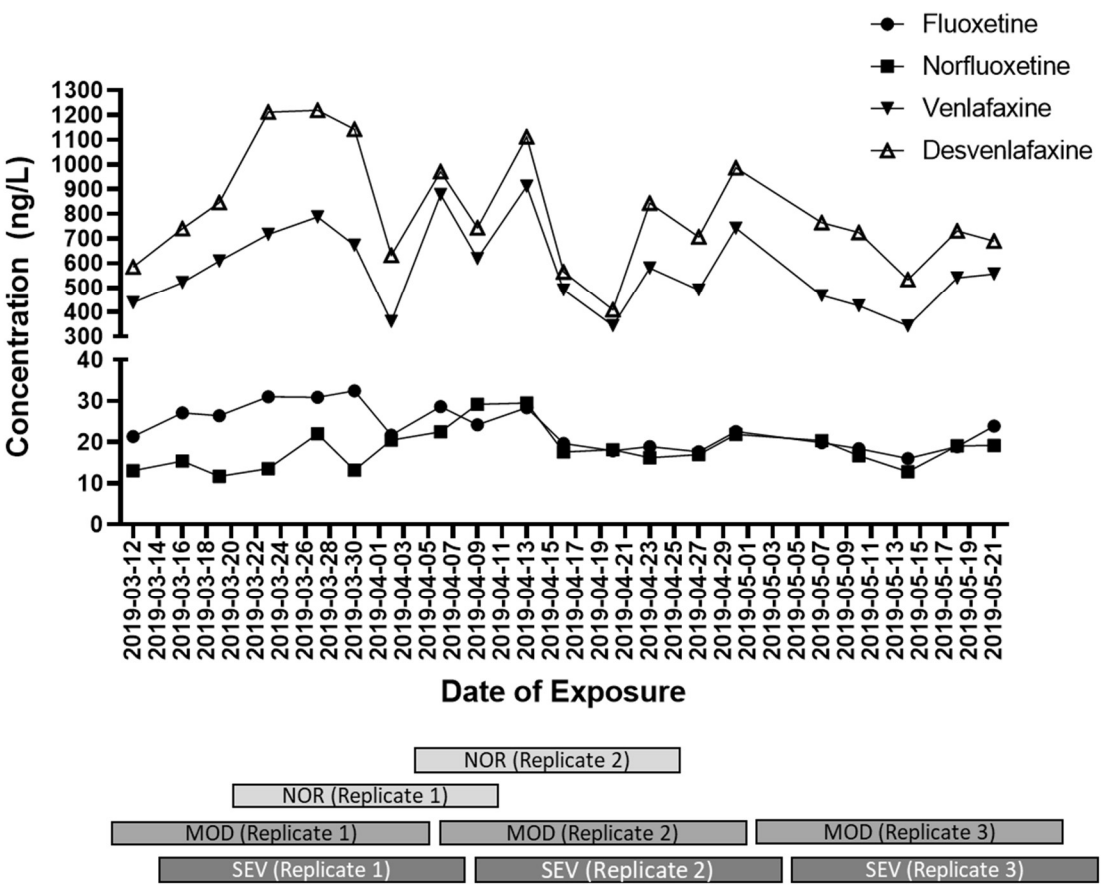

Fluoxetine
Norfluoxetine
Venlafaxine
Desvenlafaxine
Concentration (ng/L)
1300
1200
1100
1000
900
800
700
600
500
400
300
40
30
20
10
0
2019-03-12
2019-03-14
2019-03-16
2019-03-18
2019-03-20
2019-03-22
2019-03-24
2019-03-26
2019-03-28
2019-03-30
2019-04-01
2019-04-03
2019-04-05
2019-04-07
2019-04-09
2019-04-11
2019-04-13
2019-04-15
2019-04-17
2019-04-19
2019-04-21
2019-04-23
2019-04-25
2019-04-27
2019-04-29
2019-05-01
2019-05-03
2019-05-05
2019-05-07
2019-05-09
2019-05-11
2019-05-13
2019-05-15
2019-05-17
2019-05-19
2019-05-21
Date of Exposure
NOR (Replicate 2)
NOR (Replicate 1)
MOD (Replicate 1)
MOD (Replicate 2)
MOD (Replicate 3)
SEV (Replicate 1)
SEV (Replicate 2)
SEV (Replicate 3)

Figure S6: Changes in the concentrations of antidepressant compounds and metabolites in wastewater effluent at the time of collection. Staggered schedule of wastewater exposed tank replicates is shown below. NOR = Normoxia, MOD = Moderate hypoxia, SEV = Severe hypoxia.

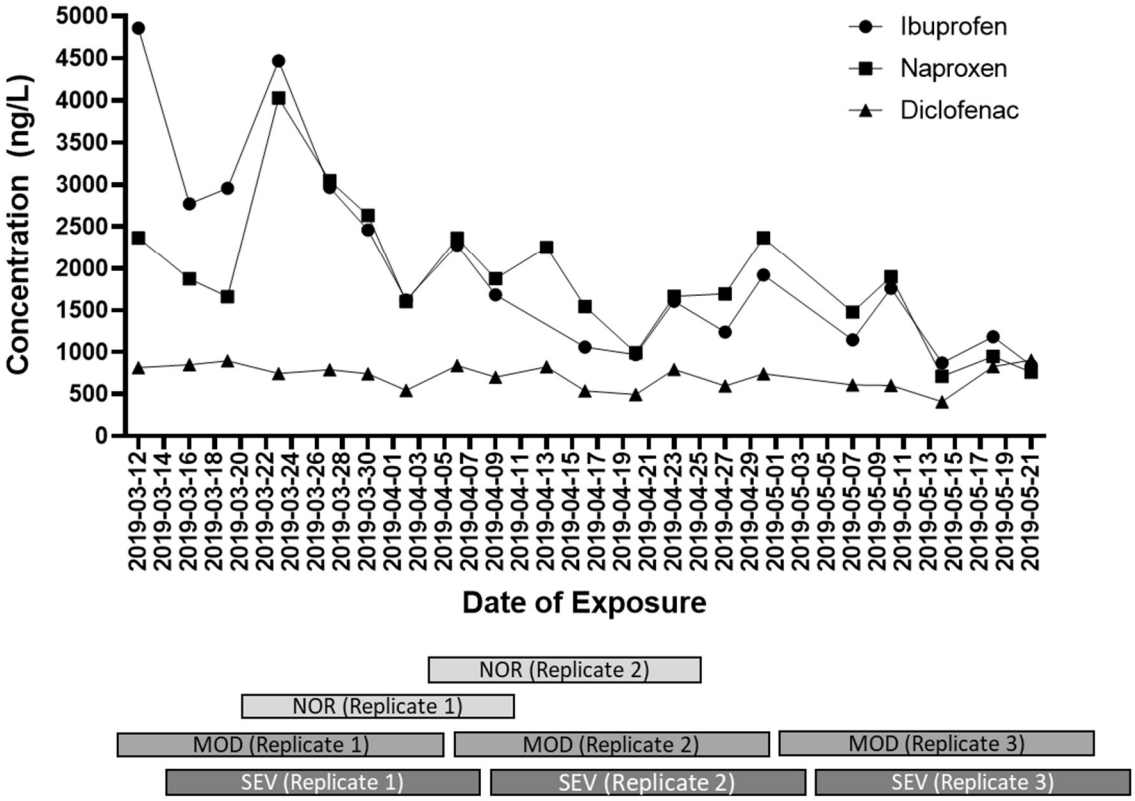

Ibuprofen
Naproxen
Diclofenac
Concentration (ng/L)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
2019-03-12
2019-03-14
2019-03-16
2019-03-18
2019-03-20
2019-03-22
2019-03-24
2019-03-26
2019-03-28
2019-03-30
2019-04-01
2019-04-03
2019-04-05
2019-04-07
2019-04-09
2019-04-11
2019-04-13
2019-04-15
2019-04-17
2019-04-19
2019-04-21
2019-04-23
2019-04-25
2019-04-27
2019-04-29
2019-05-01
2019-05-03
2019-05-05
2019-05-07
2019-05-09
2019-05-11
2019-05-13
2019-05-15
2019-05-17
2019-05-19
2019-05-21
Date of Exposure
NOR (Replicate 2)
NOR (Replicate 1)
MOD (Replicate 1)
MOD (Replicate 2)
MOD (Replicate 3)
SEV (Replicate 1)
SEV (Replicate 2)
SEV (Replicate 3)

Figure S7: Changes in the concentrations of nonsteroidal anti-inflammatory drugs (NSAID) in wastewater effluent at the time of collection. Staggered schedule of wastewater exposed tank replicates is shown below. NOR = Normoxia, MOD = Moderate hypoxia, SEV = Severe hypoxia.

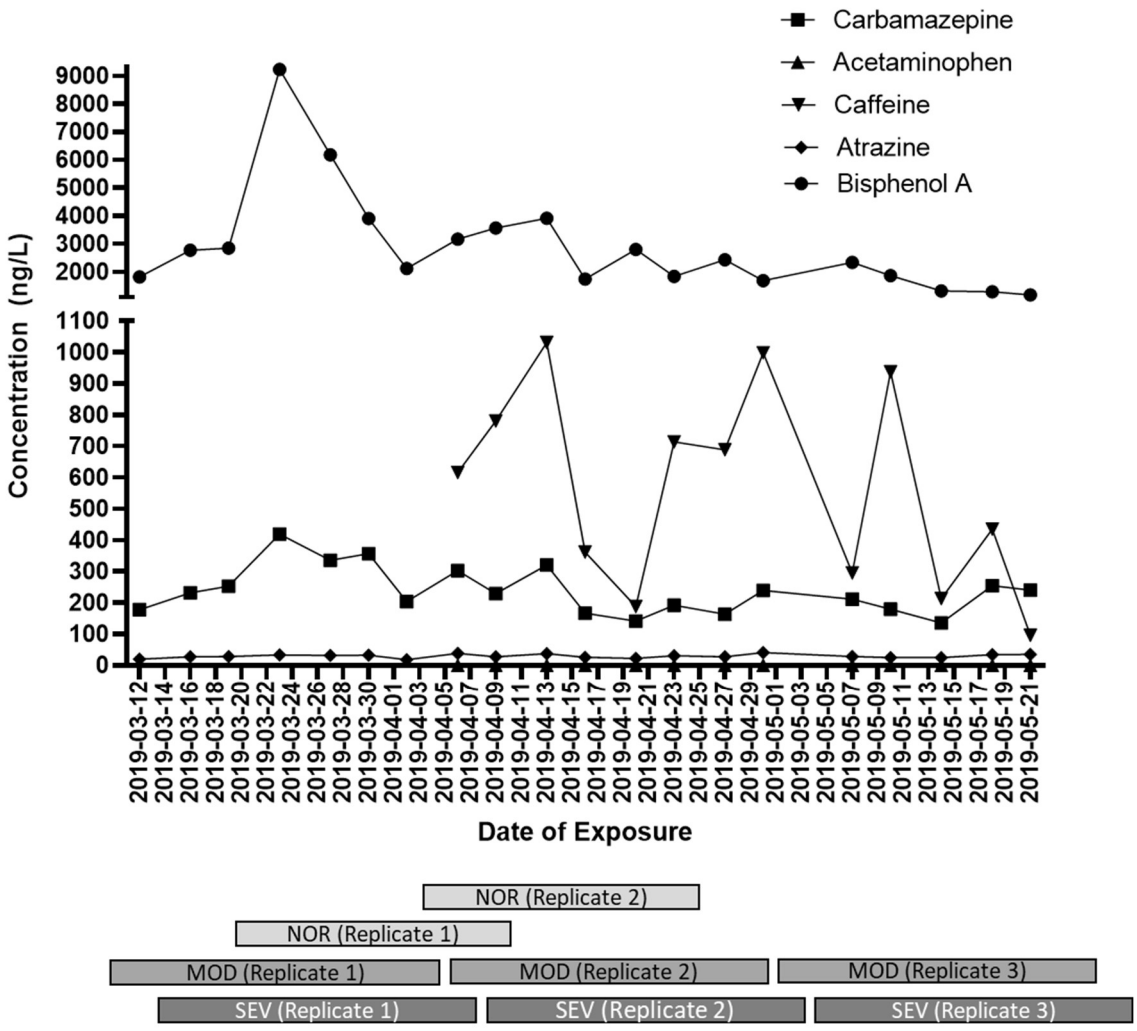

Carbamazepine
Acetaminophen
Caffeine
Atrazine
Bisphenol A
Concentration (ng/L)
Date of Exposure
NOR (Replicate 2)
NOR (Replicate 1)
MOD (Replicate 1)
MOD (Replicate 2)
MOD (Replicate 3)
SEV (Replicate 1)
SEV (Replicate 2)
SEV (Replicate 3)

Figure S8: Changes in the concentrations of carbamazepine (anti-epileptic), acetaminophen (analgesic), caffeine (dietary), and atrazine (herbicide) in wastewater effluent at the time of collection. Staggered schedule of wastewater exposed tank replicates is shown below. NOR = Normoxia, MOD = Moderate hypoxia, SEV = Severe hypoxia.

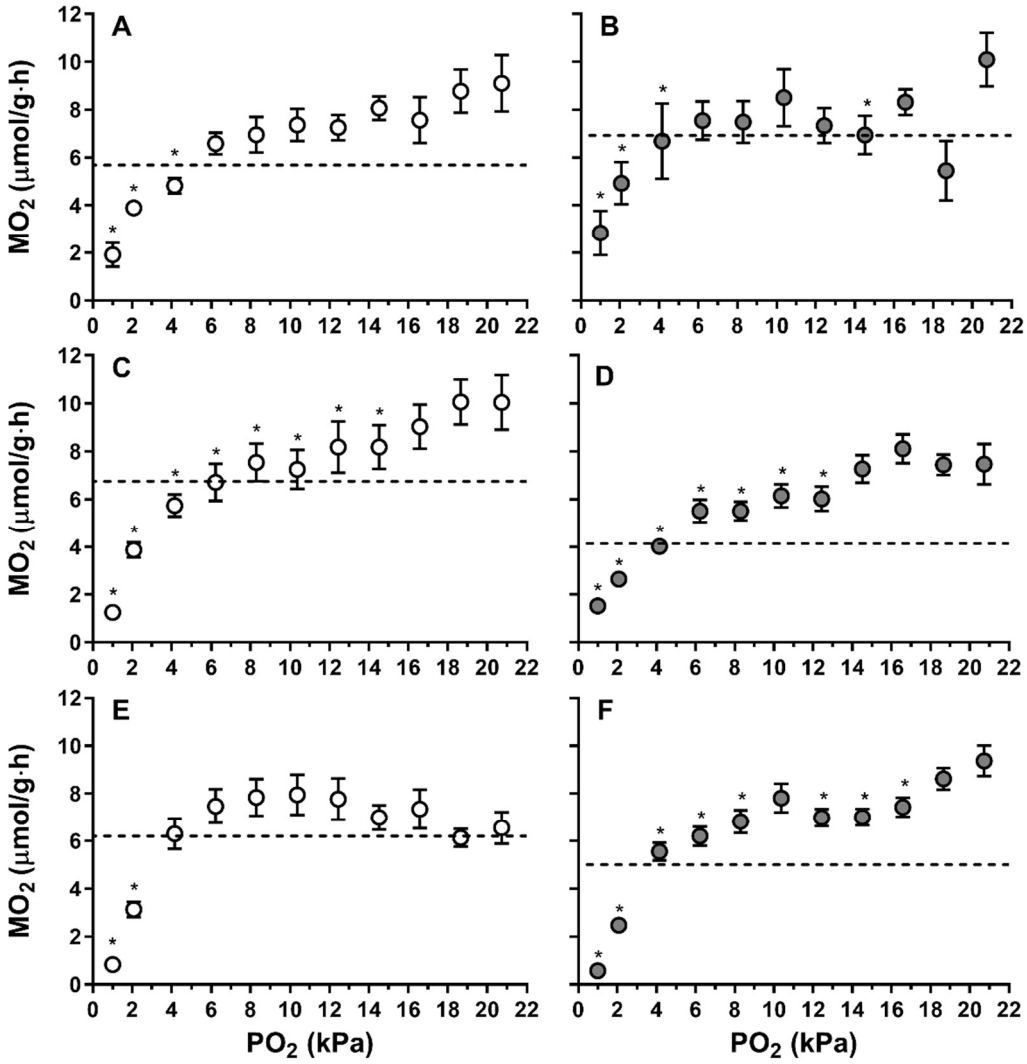

A
B
C
D
E
F
MO_2 (μmol/g·h)
PO_2 (kPa)

Figure S9: Changes in O_2 consumption rate (MO_2) with oxygen tension (PO_2). The effects of PO_2 on MO_2 were examined within each treatment group using one-way ANOVA, followed by Dunnett post-hoc tests to make pairwise comparisons to RMR in normoxia. Each panel represents a different chronic exposure group: (A) normoxia in clean water (main effect of PO_2, $p<0.0001$); (B) normoxia in wastewater ($p = 0.0002$); (C) moderate hypoxia in clean water ($p<0.0001$); (D) moderate hypoxia in wastewater ($p<0.0001$); (E) severe hypoxia in clean water ($p<0.0001$); (F) severe hypoxia in wastewater ($p<0.0001$). The mean standard metabolic rates from Fig. 1 are shown as dashed lines. Data are presented as means ± SEM, with the *N* of each group shown in Fig. 1. *Significant pairwise difference from MO_2 measured in normoxic water ($p<0.05$).

Table S1: Average percentage of initial dosage concentration (%) remaining in tank water during exposure in wastewater treatment groups.

Class	Chemical	Normoxia (20 kPa)	Moderate Hypoxia (5 kPa)	Severe Hypoxia (2 kPa)
Lipid Regulator	Gemfibrozil	92.79	77.56	99.15
	Atorvastatin			3.79
	p-hydroxy Atorvastatin	30.16	43.63	55.10
	o-hydroxy Atorvastatin	7.10	45.59	16.26
Antiepileptic	Carbamazepine	102.62	105.20	97.38
Analgesic	Acetaminophen	-	-	0.00
Stimulant	Caffeine	42.27	61.59	49.53
Antibacterial	Triclosan	40.63	89.40	23.03
	Sulfamethazine	118.00	104.88	74.60
Antibiotic	Monensin			-
	Trimethoprim	93.86	64.62	101.03
	Lincomycin	68.58	108.18	64.16
	Sulfamethoxazole	118.40	105.70	68.96
Antidepressant	Fluoxetine	94.81	58.43	53.28
	Norfluoxetine	1432.93	0.00	0.00
	Venlafaxine	78.87	96.32	80.49
	Desvenlafaxine	77.12	92.21	87.14
NSAID	Ibuprofen	26.44	19.64	83.89
	Naproxen	14.19	34.58	83.58
	Diclofenac	125.46	101.02	113.50
Herbicide	Atrazine	73.02	103.35	157.43
Industrial	Bisphenol A	20.92	61.07	20.16

Values are the average of the measurements made across the tank replicates in each wastewater treatment group. Paired samples were collected once for each tank replicate over the duration between water changes – one for the fresh tank water immediately after a water change and one for the water just prior to the next water change (of which we obtained both measurements from 2 tank replicates in normoxia, 3 replicates in moderate hypoxia, and 1 replicate in severe hypoxia). Dashes indicate concentrations measured at 0.00 ng/L in both samples. NSAID: Nonsteroidal anti-inflammatory drug.

CHAPTER THREE: GENERAL DISCUSSION

3.1 Overview

The **aim** of my thesis was to examine the effects of wastewater exposure on the adaptive responses to chronic hypoxia in mummichog killifish. I used a full factorial exposure design to complete my **objectives**, which were two-fold**:** 1) To determine whether wastewater exposure disrupts the changes in metabolism and hypoxia tolerance in response to hypoxia acclimation, and 2) To assess the underlying mechanisms along the oxygen transport cascade to explain these changes in metabolism and hypoxia tolerance. The **overarching hypothesis** stated that strategies fish use to cope with hypoxia in clean water may be disturbed with exposure to wastewater pollution. I predicted that mummichog killifish exposed to chronic hypoxia in wastewater would have impaired hypoxia tolerance and disruptions within the natural respiratory and metabolic adjustments made to cope with chronic hypoxia. My results show that 1) combined exposure led to metabolic depression and impeded the improvement of hypoxia tolerance, associated with 2) blunted gill plasticity, changes in haematology, and depletion of glycogen stores. Overall, this work demonstrates that wastewater exposure disrupted the physiological mechanisms mummichog killifish use to cope with chronic hypoxia and impaired hypoxia tolerance. This thesis contributes to the body of work that examines the combination of stressors near WWTPs, and their interactive effects on the physiology and health of fish.

3.2 Effects of exposure to wastewater in normoxia

Wastewater had no effect on standard metabolic rate

The results in **Chapter 2** showed that wastewater exposure in normoxia had no effect on standard metabolic rate in mummichog killifish. The lack of any effect on metabolic rate in killifish from exposure to wastewater in normoxia is similar to previous studies on round goby (*Neogobius melanostomus*), where fish cage exposed to the receiving waters near a WWTP displayed similar resting metabolism to fish caged at a reference site (McCallum et al., 2017). Round goby is an invasive fish species within the Laurentian Great Lakes, and found in high abundance near wastewater outfalls, along with other tolerant,

non-native species (McCallum et al., 2019; Mehdi et al., 2020, *unpublished data*). In contrast, some other species exhibit a prominent increase in metabolic rate in response to wastewater exposure, including bluegill sunfish (*Lepomis macrochirus*; Du et al., 2019, 2018) and rainbow darter (*Etheostoma caeruleum*; Mehdi et al., 2018). These findings contribute to the emerging evidence suggesting that the metabolic costs of wastewater exposure can vary between species.

Killifish are considered to be relatively tolerant of environmental pollution as a species, and some exceptionally tolerant populations have been identified in heavily contaminated sites have evolved resistance to pollutants, like methylmercury, kepones, dioxins, polychlorinated biphenyls (PCBs), and polyaromatic hydrocarbons (PAHs; Crawford et al., 2020; Reid et al., 2016; Weis, 2002). Some studies have shown that F1 and F2 embryos from populations at polluted sites still possess resistance to organic pollutants, even when reared in clean water conditions (Crawford et al., 2020). Evolutionary physiologists attribute their rapid evolutionary responses to changing environments and pollution to their polygynous mating system, high genetic variability and reproductive potential, and limited home range reducing gene flow between populations (Crawford et al., 2020; Weis, 2002). The genes in the aromatic hydrocarbon receptor (AHR) pathway have been identified as key, repeated targets of selection in tolerant *F. heteroclitus* populations (Reid et al., 2016). This ability to tolerate polluted environments may contribute to the lowered metabolic stress experienced by killifish exposed to wastewater effluent, thus avoiding increases in whole-animal energy demands compared to what is experienced by less tolerant species.

Wastewater increased the height of gill lamellae

Exposure to wastewater effluent in normoxia resulted in increases in gill lamellar height. These modest changes may reflect a response to respiratory and metabolic impacts that did not manifest as increases in whole-organism metabolic rate. Killifish exposed to wastewater exhibited a 23% increase in lamellar height compared to clean water controls. This is similar to previous field studies on bluegill sunfish, where fish caged at the outfall

site downstream of a WWTP in Dundas, Ontario also exhibited a 20-45% increase in total gill lamellar height linked with the reduction interlamellar cell mass (Du et al., 2018). Histopathological damage, like edema, lifting of lamellar and filamentary epithelium, and lamellar fusion have been associated with exposure to sewage, as shown in a study on Nile tilapia (*Oreochromis niloticus*; Fontaínhas-Fernandes et al., 2008). Although this was not formally quantified in my results, I observed evidence of some modest histopathological changes in the wastewater treatment groups, including epithelial lifting and fusion of lamellae.

Wastewater depleted muscle glycogen stores

Killifish exposed to wastewater in normoxia showed greater muscular glycogen depletion, suggesting an increased reliance on endogenous carbohydrate stores to fuel energy metabolism. This depletion of muscular and hepatic glycogen was also observed in curimbata (*Prochilodus lineatus*) and bluegill sunfish caged downstream of a wastewater treatment plant (Cazenave et al., 2014; Du et al., 2019). These changes may reflect greater energy demands in a subset of tissues, but also that they are offset by reductions in energy demands in other tissues, as glycogen reserves were not altered in liver or brain in normoxia, nor were they manifested as an increase in whole-animal metabolism.

3.3 Exposure to wastewater effluent disrupts physiological responses to chronic hypoxia

Wastewater limits improvements in hypoxia tolerance

One of the key impacts of wastewater exposure that I observed was the attenuation of plastic improvements in hypoxia tolerance, as reflected by time to loss of equilibrium (t_{LOE}) during acute exposure to near anoxia (0.5 kPa). Exposure to other stressors, such as acute heat exposure, also resulted in decreased t_{LOE} in mummichog killifish (McBryan et al., 2013; McBryan et al., 2016). It is thought that LOE is linked with the loss of ATP homeostasis, the depletion of glycogen stores, and/or the accumulation of lactate and associated metabolic acidosis in the brain (Speers-Roesch et al., 2013; van Raaij et al.,

1994). In clean water, killifish responded to chronic hypoxia with increases in t_{LOE}, consistent with previous findings in fundulid killifish species (Borowiec et al., 2015, 2020), which could reflect an improved ability to match O_2 supply and O_2 demand in hypoxia and thus avoid these problems. The reduction in t_{LOE} in chronic hypoxia due to wastewater could not be explained by a metabolic cost of exposure that increased O_2 demands, which could lead to an imbalance whereby O_2 demands exceeded O_2 supply, because combined exposure to wastewater and hypoxia reduced, rather than increased, SMR. The reduction in t_{LOE} may have instead arisen from an impairment in tissue O_2 supply.

Support for the possibility that wastewater exposure impaired tissue O_2 supply in chronic hypoxia comes from my observation that plastic responses in gill morphology were blunted. Specifically, wastewater prevented the lengthening of lamellae and regression of the ILCM that occurred in response to chronic hypoxia in clean water. Various fish, including hypoxia-tolerant species like killifish, crucian carp (*Carassius carassius*), and goldfish (*Carassius auratus*), possess the ability to remodel their gill structure to increase surface area for oxygen uptake (Abdel-Tawwab et al., 2019; McBryan et al., 2016; Sollid et al., 2003; Sollid & Nilsson, 2006). This hypoxia-induced remodelling of the ILCM is a reversible morphological change, and occurs due to increased apoptotic and decreased mitotic activity in the cells of the ILCM, resulting in protruding lamellae and increased respiratory surface area (Sollid et al., 2003; Sollid & Nilsson, 2006). Plastic gill remodelling along with changes in gill ventilation and blood flow patterns have consequential trade-offs in osmoregulation (Giacomin et al., 2019), which may be maladaptive in polluted settings because increases in the capacity of the gill for exchange with the environment could augment contaminant uptake. Such an effect is supported by previous work in Brook trout (*Salvelinus fontinalis*), as hypoxia-induced increases in ventilation volume correlated with xenobiotic uptake efficiency (McKim & Goeden, 1982). The blunted morphological response in killifish might be a protective mechanism to reduce contaminant uptake, such as is seen in gills of Antarctic fish (*Trematomus bernacchii*) in which wastewater exposure induced epithelial lifting, hyperplasia, and proliferation and

hypertrophy of mucous cells that reduced surface area and may have limited the uptake of waterborne toxicants (Corbett et al., 2014).

Wastewater exposure may have also impaired improvements in hypoxia tolerance (t_{LOE}) by reducing the ability of killifish to use anaerobic metabolism. Glycogen stores in the brain are a key fuel for anaerobic metabolism that help maintain ATP levels and avoid losing equilibrium in severe hypoxia (Speers-Roesch et al., 2013). The low brain glycogen reserves in killifish that were chronically exposed to wastewater and hypoxia likely reduced the ability to fuel anaerobic metabolism and thus constrained t_{LOE}. In wastewater, the costs of xenobiotic detoxification and repair process may have contributed to the depletion of glycogen reserves (McKenzie et al., 2007).

Wastewater exposure does not affect critical oxygen tension

Critical oxygen tension (P_{crit}) reflects the ability to maintain metabolic rate in hypoxia and is another indicator of hypoxia tolerance (Regan et al., 2019; Rogers et al., 2016; Speers-Roesch et al., 2013). Mummichog killifish responded to chronic hypoxia with decreases in P_{crit}, consistent with previous findings in various killifish species and in other species (Borowiec et al., 2015, 2020; Fu et al., 2011), but unlike t_{LOE}, this response was unaffected by wastewater. This evidence contrasts recent findings in threespine stickleback (*Gasterosteus aculeatus*), in which copper exposure impaired the improvements in P_{crit} that result from hypoxia acclimation (Fitzgerald et al., 2019). P_{crit} is often considered to reflect the ability to extract oxygen from the environment during hypoxia, but in hypoxia-tolerant species such as killifish that can depress metabolic rate (Borowiec et al. 2018), the reductions in MO_2 below P_{crit} could reflect a facultative reduction in O_2 demands rather than a hypoxia-induced constraint on O_2 extraction. This may explain why wastewater exposure impaired gill plasticity but had no effect on the decreases in P_{crit} in response to chronic hypoxia. Furthermore, species differences in the effects of contaminant exposure on P_{crit} could result from differences in whether MO_2 depression below P_{crit} is facultative or a result of O_2 supply limitation. For example, many populations of threespine stickleback

do not employ metabolic depression in hypoxia (Regan et al., 2017), which may make P_{crit} more susceptible to contaminant exposure in this species than it is in killifish.

Potential effects of wastewater on cellular mechanisms of hypoxia responses

The disruption of hypoxic responses could be attributed to several of the chemical contaminants in wastewater effluent. Given that many of the cellular responses to hypoxia lead to adjustments in metabolic pathways (*e.g.*, metabolic depression, increased use of anaerobic metabolism, shifts in fuel use between lipids and carbohydrates, etc.), it is also possible that such changes could be disrupted by the various pharmaceuticals that target these pathways.

Selective serotonin reuptake inhibitors (SSRIs) like the anti-depressant fluoxetine could inhibit serotonin signaling by oxygen sensing neuroepithelial cells in the gills, which may have disrupted key cardiorespiratory adjustments to hypoxia exposure. Indeed, in gulf toadfish (*Opsanus beta*), short-term exposure to fluoxetine has been shown to reduce regulation index (*i.e.* decrease degree of metabolic regulation) as well as interfere with the cardiovascular and ventilatory responses to hypoxia (Amador et al., 2018; Panlilio et al., 2016). Chemoreception by neuroepithelial cells (NECs) in the gill filament epithelium is the initial step in the hypoxic chemoreflex that leads to the metabolic and cardiorespiratory adjustments in response to hypoxia (Perry & Tzaneva, 2016). Afferent signalling by oxygen sensing NECs relies on the neurotransmitter serotonin (5-HT; Perry & Tzaneva, 2016), so exposure to SSRIs could disrupt the stimulation of afferent neurons containing serotonin receptors (SERT) that send information from NECs to cardiorespiratory integration sites in the brain. Exposure to SSRIs has been shown to decrease in the levels of 5-HT circulating in the blood (Prasad et al., 2015), and to elicit downstream organ and organ system changes that disrupt the hypoxia response (McDonald, 2017)

β-blockers could also affect external oxygen sensing and cardiovascular responses in hypoxia (Burleson & Milsom, 1990; Fraysse et al., 2006; Payan & Girard, 1977). Catecholamine signalling via β-adrenergic receptors, which are relatively conserved across vertebrates, is involved in regulating cardiac output, ventilation rates, metabolism, and

oxygen chemoreception by gill NECs (Corcoran et al., 2010; Owen et al., 2007). Discharge from O_2 sensing NECs in the gills were inhibited by exposure to the β-blocker propranolol in rainbow trout (*Oncorhynchus mykiss*) gills, leading to blunted hypoxic ventilatory reflexes (Burleson & Milsom, 1990). Exposure to propranolol was also observed to induce concentration-dependent bradycardia in zebrafish (*Danio rerio;* Fraysse et al., 2006) and to disrupt blood flow distribution in the gills of rainbow trout (Payan & Girard, 1977).

Cellular metabolism and energy turnover could also be affected by exposure to diabetes medications like metformin, which has been shown to affect signalling by AMP-activated protein kinase (AMPK). Metformin has been shown to affect gene expression, metabolism, glucose metabolism, and protein synthesis (Hertz et al., 1989; Ussery et al., 2018). AMPK signalling pathways are important for initiating some of the cellular mechanisms fish use to respond to chronic hypoxia (Jibb & Richards, 2008; Stensløkken et al., 2008), so metformin could impact responses to chronic hypoxia by disrupting these mechanisms.

PAHs, PCBs, and other persistent organic pollutants are ligands of the AHR and can therefore disrupt cellular responses to hypoxia (Fleming et al., 2009; Kraemer & Schulte, 2004). Critical biochemical and physiological responses to hypoxia are controlled by the activation of the hypoxia-inducible factor (HIF) pathway through stabilization of HIF-α isoforms in hypoxic conditions (Pelster & Egg, 2018). There can be reciprocal crosstalk between HIF-α and AHR signaling as they share an identical dimerization partner (HIF-1β, also known as AHR Nuclear Translocator or ARNT; Chan et al., 1999; Vorrink & Domann, 2014). Due to this antagonistic relationship, the simultaneous activation of both pathways by contaminants and hypoxia expression may exacerbate energy reserves (Fleming et al., 2009; Kraemer & Schulte, 2004; Vorrink & Domann, 2014)

3.4 Effects of hypoxia and wastewater on behaviour

The results in **Appendix A** show that exposure to chronic hypoxia had a variety of effects on behaviour in mummichog killifish. Chronic hypoxia decreased boldness and activity, but also attenuated the response to predation cue. The reduction in locomotory

activity is likely attributed to energy conservation under hypoxic conditions (Magnuson et al., 1985; Pollock et al., 2007). However, there were only modest effects of wastewater exposure on these behavioural changes in response to chronic hypoxia, contrasting the findings in **Chapter 2**. The only appreciable effect of wastewater was the elimination of the reduction in boldness induced by moderate hypoxia (but not severe hypoxia). Therefore, although we see significant effects of wastewater exposure on physiology, the behavioural effects of wastewater exposure in killifish are relatively minor.

3.5 Conclusions

Overall, my findings suggest that the interaction between the chemical contaminants in wastewater and the other abiotic stressors near WWTPs may have significant detrimental effects on the physiology and health of fish. This thesis showed that wastewater exposure: 1) had modest effects on physiology and behaviour in normoxia, and 2) disrupted responses to chronic hypoxia. Hypoxia is a natural abiotic stressor to which fish have evolved a range of adaptive physiological and behavioural responses, but the prevalence of hypoxia is increasing in both freshwater and marine systems (Diaz, 2001), so the impairment of hypoxia tolerance in fish near WWTPs may become an increasingly important issue in the future. More generally, my results suggest that wastewater exposure may reduce the ability of fish to respond to metabolic stress by disrupting various respiratory and metabolic processes. Future studies should focus on the interactive effects of wastewater combined with other metabolic stressors, particularly those that are projected to become more prevalent with climate change (*e.g.* warming temperature, reduced pH).

The findings from my thesis support growing evidence that mechanistic approaches can improve our capacity to predict the impacts of aquatic pollution at organismal and population levels. This expanding area of research is important as anthropogenic sources of pollution are making aquatic environments increasingly stressful by changing physico-chemical factors and may constrain existing mechanisms for coping with abiotic stressors.

APPENDIX A

The following describes the methods and results for an additional series of experiments that were conducted during my thesis but are not included as a full data chapter. The subset of experiments carried out in clean water will be submitted for publication in the near future, but I have also included the subset of experiments carried out in wastewater to make them available.

A.1 Materials and methods

Refer to **Chapter 2.3** for details regarding study animals and housing wastewater effluent collection, and chronic exposure design. Fish were held in the chronic exposure condition for a total of 21 days. Chronic exposure tanks contained an opaque PVC tube that provided environmental enrichment and acted as a refuge. Individuals were removed once from the chronic exposure tank on days 19, 20, and 21 for behavioural assays (see below), and after completing these assays they were returned to the appropriate exposure tank. A subset of fish (n=4 per treatment) were used for respirometry trials after the 21-day exposure.

Behavioural assays

We conducted a single trial of four consecutive assays to tests key behavioural traits: (1) boldness, (2) exploration, (3) sociality/shoaling, and (4) response to predator cue. The behavioural assays were conducted between 0900 and 1300 in 40 L aquaria with similar dimensions to the chronic exposure tanks. These experimental tanks were separated into three sealed compartments using plexiglass, each with its own airstone: the main focal fish was situated in an 'arena', flanked by a zone housing three shoal fish of similar size, and an identical zone on the opposite end without shoals. These compartments were initially obscured from the focal fish by opaque barriers. To minimize changes between exposure and testing conditions, all assays were conducted within the respective treatment, clean water or 100% wastewater effluent. We could not maintain hypoxia during the behavioural trials, as the bubble wrap needed to maintain hypoxic conditions would obscure

video recording, so all trials were conducted in normoxia (20 kPa). All experimental tanks were thoroughly cleaned between testing days.

The behavioural trial would begin when a focal fish was transferred from the exposure tank to the experimental arena within an opaque PVC tube (identical to what was provided in the exposure tanks) that acted as an initial 'refuge'. The tube was placed in the centre of the arena and the fish was held within the tube for a 10 min adjustment period. The boldness assay was initiated by remotely removing the black opaque door that held the focal fish within the refuge, revealing the arena. The focal fish was given 10 min to exit the refuge, during which activity in the arena was recorded. After the 10 min, the exploratory assay was initiated by remotely removing the refuge such that the focal fish was left in the empty arena, and activity was recorded for 5 min. The shoaling assay was then initiated by remotely removing the opaque barriers at the ends of the tank, revealing to the focal fish the shoal fish compartment on the right side and an empty but otherwise visually identical compartment on the left side. The focal fish was then allowed to interact with these new stimuli for 10 min. Shoal fish were previously acclimated in the compartment 1 h prior to the start of the behavioural trial. The predator response assay was then conducted by striking the centre of the arena 5 times with a large model fish predator (a silicone salmonid attached to a wooden rod), and then recording the focal fish's response and activity for 5 min. During all of the above assays, movable elements of the assay were controlled remotely by an experimenter hidden behind an opaque curtain to limit any influence of the experimenter on fish behaviour. After the behavioural trial, fish were returned to the respective chronic exposure tank. The entire 45-min trial was recorded on a waterproof digital action camera (GoPro HERO5 Session) and analyzed by an observer who was blind to exposure conditions. Video files were imported into Behavioural Observation Research Interactive Software (BORIS v.7.9.6; Friard & Gamba, 2016) for analysis of the sociality assay.

Video analysis

For the boldness assay, we recorded 1a) ***latency to exit refuge***, measured as time elapsed from barrier removal until the fish exited the refuge. This was used to indicate a decision to explore a novel area. If the fish exited the refuge, we measured 1b) ***activity***, which we defined as the percentage of time when movement of at least half a body length per second was recorded. For the exploratory assay, we continued to measure 2) ***activity*** after the refuge was removed. The effects of chronic treatments on activity that are reported here is the activity measured during both the boldness and exploratory assays combined. For the sociality assay, we recorded 3a) ***time nosing*** (as a percentage of total time during the sociality assay) against the clear barrier separating the focal fish from the visible shoals. We also measured 3b) the time spent in each of 5 equal volume vertical compartments within the arena, from the compartment furthest from the shoals to immediately beside the shoals. We used these measurements to calculate 3c) ***sociality index***, by giving a number to each compartment (-2, -1, 0, 1, and 2 from furthest to nearest the shoal) and then summing the products of the compartment number and the time in each compartment (in seconds). These two measurements were used to assess interaction with shoals and to examine active choice to use the space within the arena. Finally, we measured 4a) ***activity***, as described above, for the 5 min immediately after the predator cue was introduced into the arena. These values were normalized to baseline activity levels measured in the boldness and exploratory assays to determine 4b) the ***change in activity*** in response to predator cue, and to thus examine how long fish took to resume baseline levels of activity**.**

Respirometry

We used stop-flow respirometry in clean water to measure standard metabolic rate (SMR) and maximum metabolic rate (MMR). Fish were fasted for the last 48 h of chronic exposures and were then transferred at ~1700-1900 local time to 90 ml cylindrical acrylic chambers containing normoxic water, which were covered in dark plastic to minimize visual disturbance. These respirometry chambers were connected to two water circulation circuits: a ‘flushing circuit’ that used a pump to flush the respirometry chamber with water

from a surrounding buffer tank (which was bubbled with air to maintain normoxic O_2 levels); and a 'measurement circuit' that continuously pumped water from the respirometry chamber across a fibre-optic O_2 sensor (FireSting FSO2-4, PyroScience GmbH) in a closed loop. Measurements of O_2 consumption rate (MO_2) were made overnight in normoxia using an automated respirometry system (AutoResp, Loligo Systems, Viborg, Denmark) by alternating between 4.5 min flush periods (when both flushing and measurement circuits were active) and 4 min measurement periods (when only the measurement circuit was active such that the respirometry chamber acted as a closed system), during which MO_2 was determined from the depletion of water O_2 content over time (based on measurements of water O_2 content taken every 1 s), and is expressed relative to body mass ($\mu mol\ g^{-1}\ h^{-1}$). Standard metabolic rate (SMR) was calculated as the average of the lowest 10 MO_2 measurements that were made overnight. Starting at ~1100-1200 the following day, maximum metabolic rate (MMR) was measured in the same individuals. Fish were removed from the respirometers, placed in a bucket (30 cm in diameter) with clean water, chased using a dip net for 4 min, subjected to air exposure for 1 min, and then returned to the respirometer. MMR was determined as the MO_2 measured over the first 85 min after the fish was returned to the respirometer.

Statistical analysis

One series of statistical tests focussed on examining the effects of chronic hypoxia in clean water, because this subset of experiments will form the basis for a future publication. One-way ANOVA was used to test for main effect of chronic hypoxia on most metabolic and behavioural measurements, followed by Dunnett's post-hoc tests to identify pairwise differences between hypoxic treatment groups and the normoxic controls. The only exception was the response to predator cue, for which a two-way ANOVA was used to examine the effects of chronic hypoxia and time (repeated measure) on activity. Sidak's post-hoc tests identified pairwise differences between the activity at each time point during recovery compared to baseline activity levels within each treatment group. Pearson correlations were calculated between metabolic and behavioural parameters. I also

conducted a series of statistical tests examining the full set of data in both clean water and wastewater, in which two-water ANOVA was used to test for the main effects of chronic hypoxia and wastewater along with their interaction. All statistical analyses were performed using GraphPad Prism (version 8.4.2, San Diego, California USA). Data are presented as means ± SEM, and $p < 0.05$ was considered significant.

A.2 Results

Effects of hypoxia on metabolic rate and aerobic scope

Chronic exposure to hypoxia reduced standard metabolic rate (main effect of hypoxia in one-way ANOVA, $p = 0.0039$) and maximum metabolic rate (main effect of hypoxia, $p = 0.0067$; Fig. A-1A-B). There was a 25% decrease in SMR and 26% decrease in MMR in moderate hypoxia. Additionally, in severe hypoxia, SMR and MMR decreased 0.17 and 0.19-fold, respectively. As a result of the reductions in both SMR and MMR, chronic hypoxia had no significant effects on absolute aerobic scope (main effect of hypoxia, $p = 0.1974$; Fig. A-1C) or factorial aerobic scope (main effect of hypoxia, $p = 0.2356$; Fig. A-1D).

Effects of hypoxia on behaviour

Chronic exposure to hypoxia increased latency to exit the refuge, reflecting a decrease in boldness to explore a novel environment (main effect of hypoxia, $p = 0.0057$; Fig. A-2B). Chronic hypoxia also decreased the proportion of time active during the boldness and exploration assays (main effect of hypoxia, $p = 0.0002$; Fig. A-2D). The measures of sociality used here demonstrated that killifish discerned and responded to the visual cues from a shoal of conspecifics during the sociality assay. In general, killifish preferentially spent time on the side of the tank nearest the shoal, as reflected by positive sociality index scores. The possible range of sociality index scores was from -12000 to +12000, reflecting the extreme conditions in which fish spent all their time in the locations of the tank that were furthest and nearest to the visible shoal, respectively. Killifish also carried out a characteristic behaviour in which they would ‘nose’ the glass separating the shoal from the behavioural arena. However, neither sociality index nor time nosing with

shoals (another measured sociality behaviour) were affected by chronic exposure to hypoxia (main effect of hypoxia, $p = 0.5337$; Fig. A-3BC). Killifish responded strongly to a model predator cue, with pronounced reductions in activity, but the temporal pattern of this response was affected by chronic hypoxia (main effect of hypoxia, $p = 0.015$). Specifically, killifish exposed to chronic hypoxia exhibited a much shorter response, recovering and/or exceeding baseline activity levels within 5 min (Fig. A-4B).

What is the relationship between metabolism and behaviour?

There were strong correlations between metabolism and several behavioural traits across all treatment groups (Fig. A-5A). Standard metabolic rate correlated positively with maximum metabolic rate (Pearson's r = 0.86, $p < 0.001$) and activity (Pearson's r = 0.44, $p = 0.02$). Both standard and maximum metabolic rates correlated negatively with the boldness metric, latency to exit refuge (SMR and Latency, Pearson's r = -0.40, $p = 0.03$; MMR and Latency, Pearson's r = -0.61, $p = 0.02$). Latency to exit refuge negatively correlated with activity (Pearson's r = -0.57, $p < 0.001$) and positively correlated with relative activity after predator cue (Pearson's r = 0.43, $p = 0.02$). Activity was negatively correlated with predator response (Pearson's r = -0.45, $p < 0.01$).

In comparison to correlations across all treatment groups, only some relationships persisted in the within-group correlations (Fig. A-5B-D). A positive correlation persisted between standard metabolic rate and activity in normoxia (Pearson's r = 0.71, $p = 0.049$). Contrastingly, the relationship between activity and predator response was positive in normoxia (Pearson's r = 0.82, $p < 0.01$). An additional negative correlation between sociality index and predator response was identified in moderate hypoxia (Pearson's r = -0.06, $p = 0.02$). The negative correlation between latency to exit refuge and activity persisted in both hypoxia groups (moderate hypoxia, Pearson's r = -0.75, $p < 0.01$; severe hypoxia, Pearson's r = -0.49, $p = 0.03$). The positive correlation between standard metabolic rate and maximum metabolic rate (Pearson's r = 0.98, $p = 0.02$) remained in severe hypoxia.

Modest effects of combined exposure to wastewater and hypoxia on behaviour

Many of the effects of chronic hypoxia on behaviour were unaltered by combined exposure to wastewater. The results for all wastewater groups are shown in Table A-1. There were two notable effects of exposure to wastewater on behaviour in normoxia or hypoxia. Firstly, whereas moderate hypoxia in clean water decreased boldness (Fig. A-2B), combined exposure with wastewater eliminated this effect on the latency time to exit the refuge (main effect of hypoxia, $p = 0.049$). Wastewater exposure in normoxia shortened the response to predator cue, where fish returned to ~80% of baseline activity by 5 min after a predator cue. Like the results in clean water, fish exposed to moderate or severe hypoxia in wastewater recovered and exceeded baseline activity within 5 min of the predator cue.

A.3 Figures and Tables

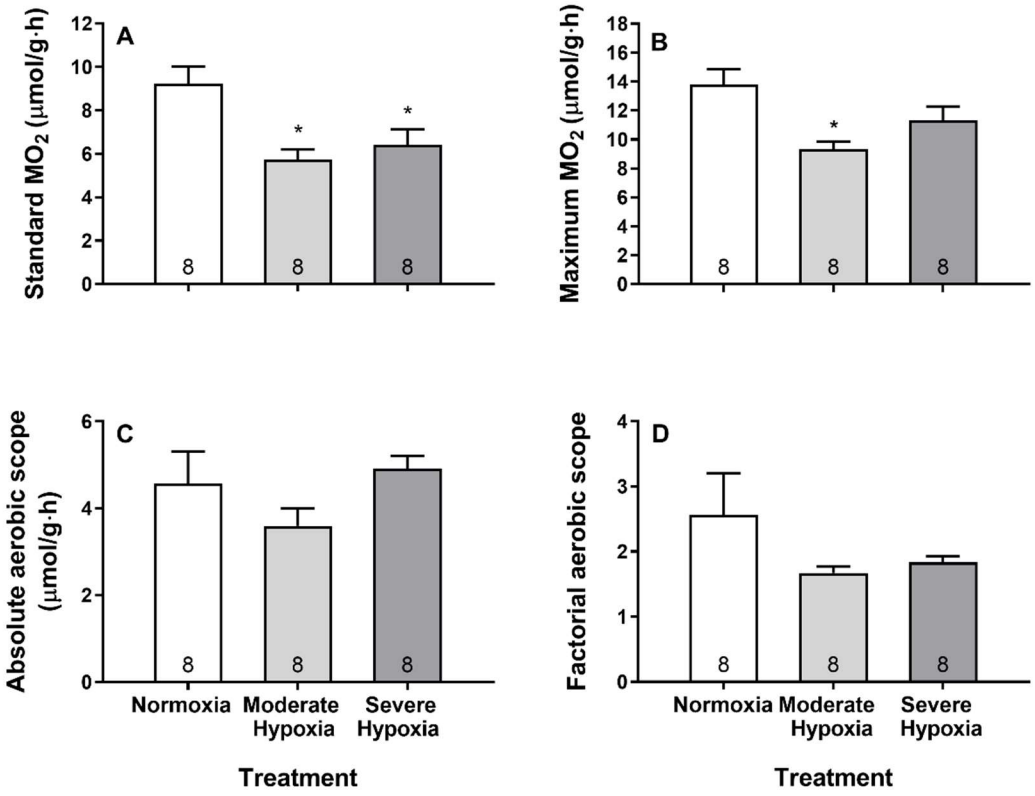


Figure A-1: Chronic exposure to hypoxia reduced (A) standard metabolic rate (MO_2) and (B) maximum MO_2 but had no effects on absolute (C) or factorial aerobic scope (D). *Significant pairwise difference from normoxic controls ($p<0.05$). Data are presented as means ± SEM, with sample sizes indicated within each bar.

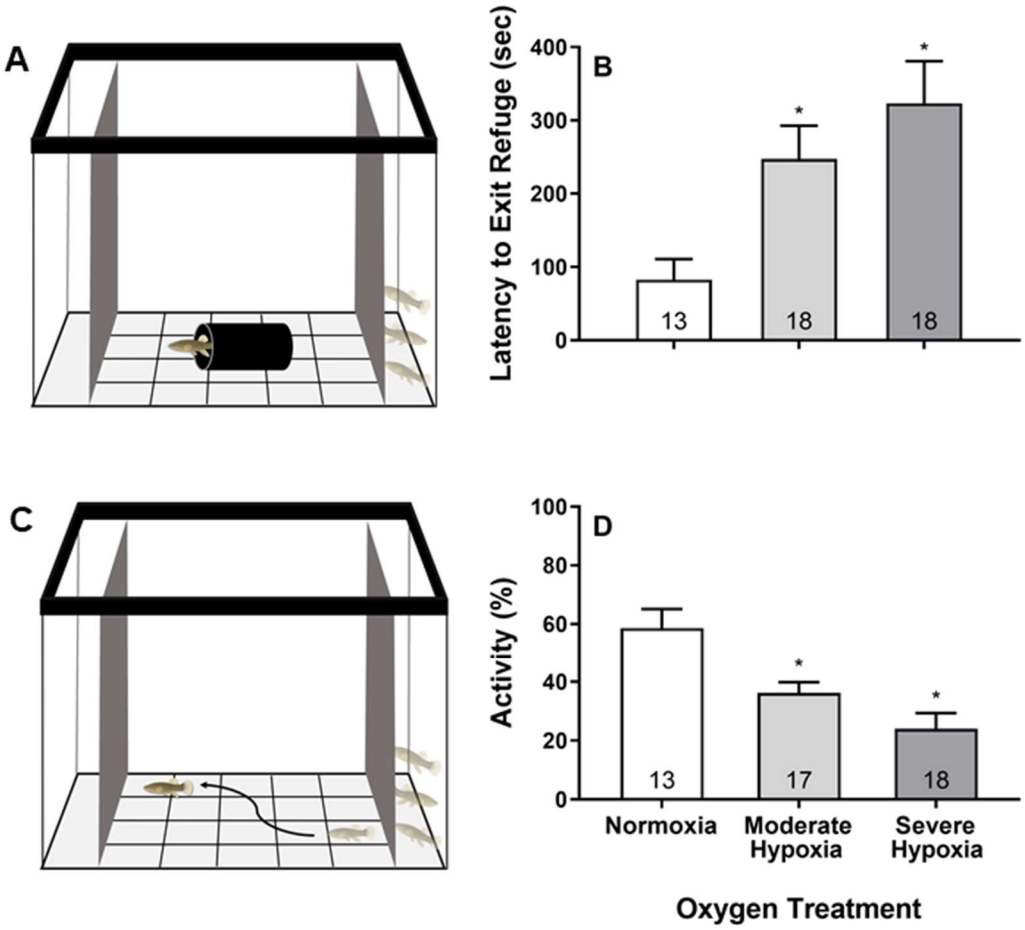

A
B
Latency to Exit Refuge (sec)
400
300
200
100
0
13
18
18
*
*
C
D
Activity (%)
100
80
60
40
20
0
13
17
18
*
*
Normoxia
Moderate Hypoxia
Severe Hypoxia
Oxygen Treatment

Figure A-2: Chronic hypoxia exposure increased latency to exit the refuge (B) and decreased activity (percentage of time spent moving at least half a body length per second) (D). Diagrams of the experimental tank setup for boldness and exploration assays that were used are shown in A and C. *Significant pairwise difference from normoxic controls ($p<0.05$). Data are presented as means ± SEM, with sample sizes indicated within each bar.

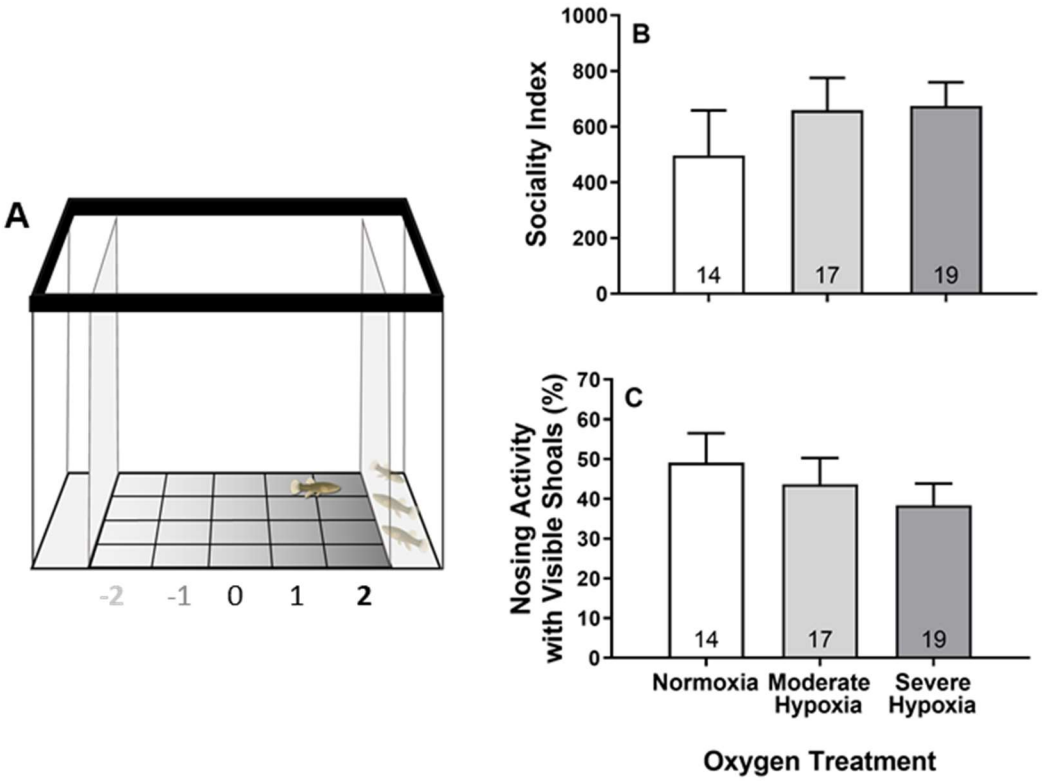

A
-2
-1
0
1
2
B
Sociality Index
1000
800
600
400
200
0
14
17
19
C
Nosing Activity with Visible Shoals (%)
70
60
50
40
30
20
10
0
14
17
19
Normoxia
Moderate Hypoxia
Severe Hypoxia
Oxygen Treatment

Figure A-3: Chronic hypoxia exposure did not affect sociality index (B) nor nosing activity with visible shoals (C). Diagram of the experimental tank setup for sociality assays is shown in A. The possible range of sociality index scores was from -12000 to +12000, obtained by summing the products of the compartment number (shown under each vertical compartment of the tank in A) and the time in each compartment (in seconds), such that a positive index score indicates that fish spent more time in the two columns nearest the shoal. Data are presented as means ± SEM, with sample sizes indicated within each bar.

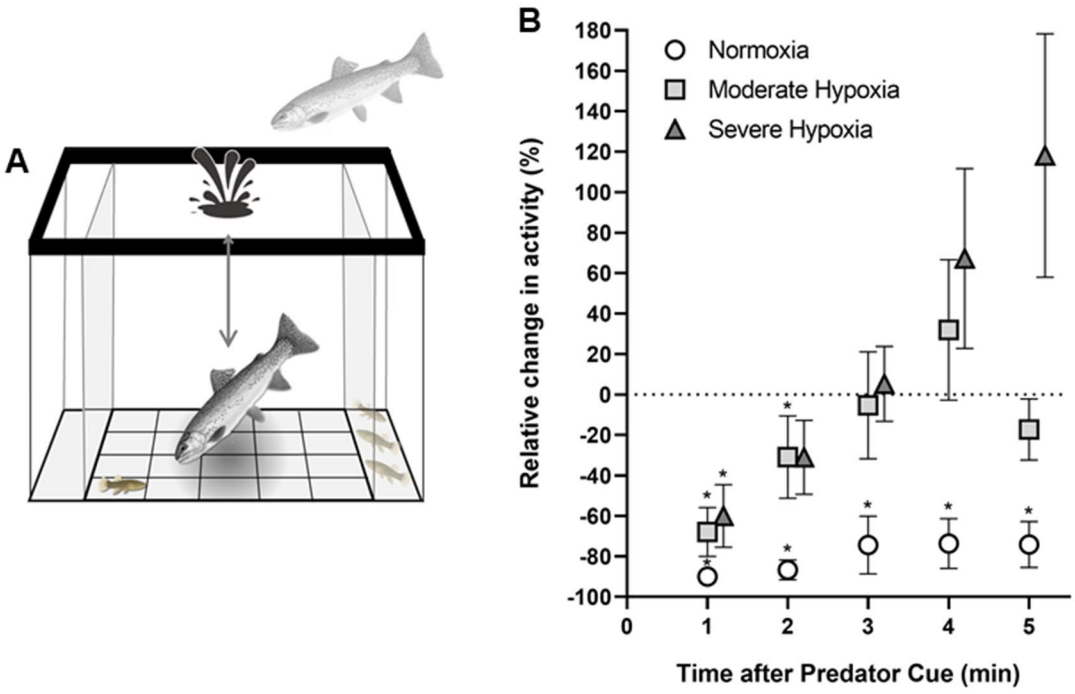

A
B
Normoxia
Moderate Hypoxia
Severe Hypoxia
Relative change in activity (%)
Time after Predator Cue (min)
180
160
140
120
100
80
60
40
20
0
-20
-40
-60
-80
-100
0
1
2
3
4
5

Figure A-4: Hypoxia shortened the response to predator cue, such that fish returned to and exceed baseline activity levels within 5 min after the cue was introduced (B). Graph shows change in the proportion of time active relative to baseline activity (%) after the predator cue. Diagram of the experimental tank setup for the predator cue response assay is shown in A. *Significant pairwise difference in activity compared to baseline within a treatment group ($p<0.05$). Data are presented as means ± SEM (n = 13-18). Symbols representing data points from the Severe Hypoxia treatment group are shifted right for clarity.

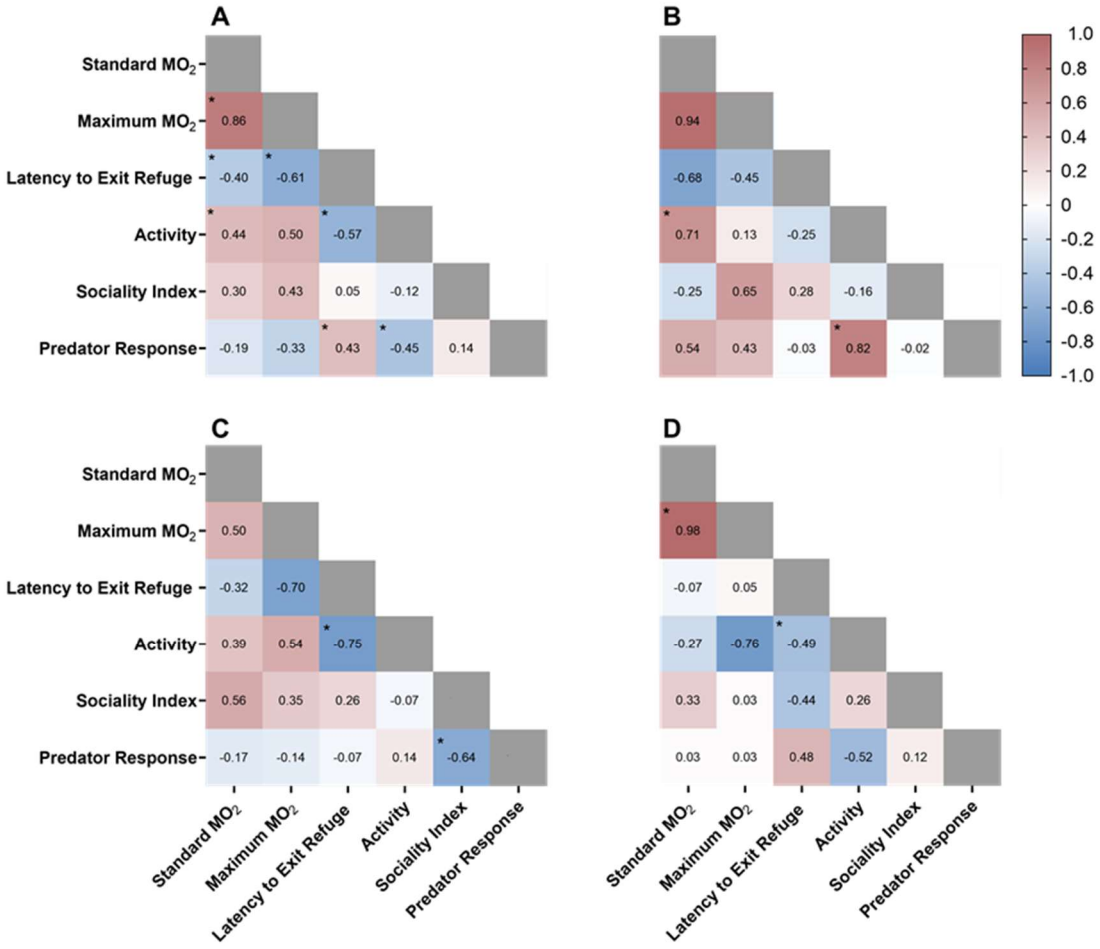

A
B
C
D
Standard MO_2
Maximum MO_2
Latency to Exit Refuge
Activity
Sociality Index
Predator Response
1.0
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1.0

Figure A-5: Correlation matrices of metabolic rates (MO_2) and behavioural parameters assessed in killifish: (A) All treatments combined, or within each treatment group of (B) normoxic controls, (C) chronic moderate hypoxia, and (D) chronic severe hypoxia. The colours and numbers indicate the strength and direction of the Pearson's r correlation. *Significant correlations between respective parameters (p<0.05).

Table A-1: Metabolic and behavioural parameters measured in killifish exposed to wastewater effluent under three oxygen treatments.

Variable	Normoxia (20 kPa)	Moderate Hypoxia (5 kPa)	Severe Hypoxia (2 kPa)
SMR (μmol/g·h)	6.00 ± 0.91 (7)	4.50 ± 0.42 (12)	4.56 ± 0.25 (12)
MMR (μmol/g·h)	10.68 ± 1.28 (7)	8.86 ± 0.88 (12)	8.64 ± 0.78 (12)
Absolute AS (μmol/g·h)	4.67 ± 0.57 (7)	4.37 ± 0.60 (12)	4.08 ± 0.59 (12)
Factorial AS	1.83 ± 0.11 (7)	2.05 ± 0.16 (12)	1.87 ± 0.12 (12)
Latency to exit refuge (sec)	73.42 ± 20.90 (17)	81.08 ± 28.83 (20)	*296.675 ± 37.155 (22)
Activity (%)	67.47 ± 4.54 (17)	*48.50 ±5.10 (15)	*24.83 ±3.94 (22)
Sociality index	725.72 ± 60.22 (19)	651.86 ± 108.66 (17)	836.93 ±57.74 (22)
Time nosing with visible shoals (%)	32.98 ± 5.48 (16)	33.33 ± 4.96 (16)	50.09 ± 4.41 (24)
Relative activity 5 min after predator cue (% change)	-23.79 ± 10.78 (18)	35.85 ± 21.04 (17)	83.30 ± 27.60 (22)

Values are means ± SEM (*N*). *Significant differences from normoxic controls ($p<0.05$). SMR, standard metabolic rate; MMR, maximum metabolic rate; AS, aerobic scope.

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