Impacts of acute and anthropogenic stress on fish microRNA

by

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A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Doctor of Philosophy
in
Biology

Waterloo, Ontario, Canada, 2021

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Author's Declaration

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

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Statement of Contributions

Heather Ikert was the sole author for Chapters 1 and 6 which were written under the supervision of Dr. Paul Craig and Dr. Barb Katzenback and were not written for publication.

This thesis consists in part of four manuscripts written for publication. Exceptions to sole authorship of material are as follows:

Research presented in Chapter 2:

Dr. Paul Craig was the primary investigator on the Natural Sciences and Engineering Research Council (NSERC) and Canadian Foundation for Innovation (CFI) grants which supported conducting this work. This research was conducted at the University of Waterloo by Heather Ikert under the supervision of Dr. Paul Craig. Dr. Craig provided input on study design, data interpretation, and edited the manuscript. Heather Ikert conducted the study, collecting and analyzing the data and writing the manuscript. This research is published.

Ikert, H., Craig, P.M., 2020. Chronic exposure to venlafaxine and increased water temperature reversibly alters microRNA in zebrafish gonads (*Danio rerio*). Comparative Biochemistry and Physiology Part D: Genomics and Proteomics 33, 100634. https://doi.org/10.1016/j.cbd.2019.100634

Research presented in Chapter 3:

Dr. Paul Craig was the primary investigator on the Natural Sciences and Engineering Research Council (NSERC) and Canadian Foundation for Innovation (CFI) grants which supported this work. Heather Ikert was supported by an NSERC Postgraduate Scholarship - Doctoral (PGS D) grant. This research was conducted at the University of Waterloo by Heather Ikert under the supervision of Dr. Paul Craig and Dr. Barb Katzenback. Heather Ikert conducted the experimental design, sample collection and analysis, data analysis, and manuscript writing. Mark Lubberts assisted with proteomics experimental design, sample and data analysis. Dr. Brendan McConkey assisted with proteomic experimental design and provided funding. Dr. Barb Katzenback assisted with data interpretation and manuscript editing. Dr. Paul M. Craig assisted with experimental design, manuscript edits and provided funding. This research is formatted for publication.

Ikert, H., Lubberts, M., McConkey, B., Katzenback, B.A., Craig, P.M. Acute and chronic exposure to multiple anthropogenic stressors alters microRNA, mRNA, and proteins in zebrafish (*Danio rerio*) liver and muscle. This chapter will be submitted to Aquatic Toxicology.

Research presented in Chapter 4:

Dr. Paul Craig was the primary investigator on the Natural Sciences and Engineering Research Council (NSERC) and Canadian Foundation for Innovation (CFI) grants which supported conducting this work. Heather Ikert was supported by an NSERC PGS D grant. Slava Osokin was funded through the University of Waterloo Science Research Internship program and the Government of Canada's Student Work Placement Program. This research was conducted at the University of Waterloo by Heather Ikert under the supervision of Dr. Paul Craig and Dr. Barb Katzenback. Heather Ikert designed the experiment, conducted experimental and data analysis, and wrote the manuscript. Slava Osokin conducted the RNA and enzyme analysis. Joshua Raiden Saito conducted the enzyme analysis. Dr. Paul M. Craig assisted with the experimental design and editing the manuscript. This research is published.

Ikert, H., Osokin, S., Saito, J.R., Craig, P.M., 2021. Responses of microRNA and predicted mRNA and enzymatic targets in liver of two salmonids (*Oncorhynchus mykiss* and *Salvelinus fontinalis*) following air exposure. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 256, 110646. https://doi.org/10.1016/j.cbpb.2021.110646

Research presented in Chapter 5:

This research was funded through the Global Water Futures Grant #419205. Heather Ikert was supported by an NSERC PGS D. This research was conducted at the University of Waterloo by Heather Ikert under the supervision of Dr. Paul Craig and Dr. Barb Katzenback. Heather Ikert conducted the experimental design, collected and analyzed data, and wrote the manuscript. Dr. Michael Lynch assisted in bioinformatic data analysis and edited the manuscript. Dr. Mark Servos and Dr. Andrew Doxey acquired grant funding. Dr. John Giesy acquired funding and provided minor edits. Dr. Barb Katzenback acquired funding and edited the manuscript. Dr. Paul Craig acquired funding, assisted in experimental design and manuscript writing, and edited the manuscript. This research is published.

Ikert, H., Lynch, M.D.J., Doxey, A.C., Giesy, J.P., Servos, M.R., Katzenback, B.A., Craig, P.M., 2021. High Throughput Sequencing of MicroRNA in Rainbow Trout Plasma, Mucus, and Surrounding Water Following Acute Stress. Front. Physiol. 11. https://doi.org/10.3389/fphys.2020.588313

Abstract

Fishes play crucial roles in the ecology of aquatic environments and contribute to the multi-billion-dollar fisheries industry. The integrity of their populations and health needs to be maintained for future generations and research on the biology and effects of stress on fish can contribute to this cause. While much is understood about the adrenergic response to stress which results in the secretion of catecholamines and cortisol, there is much to be understood about molecular mechanisms of stress, such as the role of microRNAs.

MicroRNAs (miRNAs) regulate post-transcriptional molecular responses by binding to mRNA and labelling them for degradation or blocking translation, effectively decreasing target protein translation levels. The response of miRNA transcript levels to environmental stressors, such as increased water temperatures, have been measured in fish since 2009. However, there is still much that is poorly understood about the effects of fish stress on miRNA levels, such as how time sensitive the response is, whether the response is tissue specific, and whether it is possible to measure miRNAs in non-lethal or non-invasive samples, such as mucus or the water surrounding fish. Furthermore, there are many gaps in understanding of how miRNA levels are altered in non-model species, such as salmonids. Most studies of fish stressors often focus on single stressor studies to elucidate the molecular mechanisms however, fish are not exposed to stressors individually in the aquatic environment. Therefore, it is important to study the simultaneous effects of multiple, emerging anthropogenic stressors of concern (e.g., increased water temperature, decreased dissolved oxygen, and pharmaceutical contaminants), on fish and their miRNA levels.

The overall goal of my thesis is to determine how transcript levels of miRNAs are regulated when fishes are exposed to stress. More specifically, I wanted to further characterize the miRNA response in different tissues and at different timepoints, in both model and non-model fish species, to determine the conservation or specificity of the miRNA response. I also aimed to determine if it was possible to sample miRNAs in non-lethally collected samples as a novel method of measuring stress in fish. Furthermore, I measured predicted downstream responses (mRNA transcript levels, protein abundances, and enzyme activities) to understand the functional implications of changes to miRNA transcript levels and to describe the molecular response to acute and anthropogenic stressors.

In studying the effects of chronic exposure to anthropogenic stressors on zebrafish gonads, I found that the miRNA response was reversible and associated with adverse reproductive impacts (Chapter

2). In studying the effects of different lengths of exposure to anthropogenic stressors on zebrafish liver and muscle tissues, I determined that the miRNA response was specific to length of exposure, tissue type, as well as the sex of the fish, and that fish were activating cell stress, decontamination, metabolic, and reproductive responses (Chapter 3). In studying the effects of acute stress on rainbow and brook trout liver tissues, I found that miRNA transcript levels, mRNA transcript levels, and metabolic enzyme activities were altered in a time-dependent manner post-stress and that there was much intra-species and inter-species variability (Chapter 4). In studying the effects of acute stress on rainbow trout blood plasma, mucus, and the surrounding water, I found that miRNAs were able to be measured and transcript levels were altered following stress in all three non-lethal sampling locations (Chapter 5).

Altogether, I have contributed further to identifying specific transcript levels of miRNA that respond to acute and anthropogenic stressors in multiple fish species. I have also characterized how the miRNA response is associated with the presence of the stressor, the length of exposure to the stressor, and the length of time following exposure to the stressor. These data are helpful in understanding the molecular regulation and response to stress and broadly contribute to understanding how miRNAs play a role in how organisms can adapt to transient or ongoing stressors. In addition, the downstream molecular responses associated with changes in miRNA transcript levels were also measured in response to these stressors and highlight the metabolic, reproductive, and cellular stress responses that the fish were activating when exposed to anthropogenic stressors, as well as filling in gaps of metabolic enzymes that are part of the acute stress response. My research also highlights the complex role that miRNAs play in finetuning the molecular response to stress, as there are still many gaps in understanding what the altered miRNA transcript levels are targeting and post transcriptionally regulating. In the future, instead of focusing on identifying miRNAs that are regulating a particular transcript or pathway of interest, priority can be given to identifying miRNAs that are crucial in driving the stress response or in allowing a particular individual or species to adapt to stress.

Acknowledgements

A thesis cannot be completed without the assistance and support of many people. I would first like to thank my supervisor, Dr. Paul Craig. You have been a wonderful source of direction and support from the very start of my tenure in your lab as an undergraduate in 2015. The direction you provided while simultaneously giving me lots of room to try things out and learn independently has been hugely helpful in my growth and learning journey as a graduate student. Thank you too for the many opportunities to share my research and meet other scientists at conferences over the years. I also really appreciate the passion for science that you have demonstrated while prioritizing life and health for yourself and your students. I would also like to thank my co-supervisor Dr. Barb Katzenback. You have been a source of immensely helpful feedback and your level of investment in my learning and success is greatly appreciated. You have contributed greatly to helping me refine my critical thinking and communication skills. I would also like to thank my committee members, Dr. Brian Dixon and Dr. Michael Wilkie. You have provided valuable feedback and support over the years that has improved my study design and interpretation of my findings.

This thesis would also not have been completed without the support and commiseration of many people that have been a part of the Craig lab and Biology Department at the University of Waterloo. I would like to thank Hossein Mehdi for putting up with my constant teasing about his love of birds and for always being ready to discuss and hash out our findings. I would also like to thank Chris Kuc for his support, his wonderful British accent, and making the lab a fun place to be. I would like to thank Ivan Cadonic, firstly for his last-minute help in editing this thesis (since it is so fresh in my mind). I am so glad that have gotten to share the highs and lows of research with you over the years, even if it seems like the lows predominate sometimes (ex. fish surgeries and bioinformatic issues). I want to thank Nathan Bennoit for many fun moments in the lab, from scaring Ivan while he performed fish surgeries to having an hours-long debate about the method of iron absorption in the gut. I also want to thank Rhiannon Hodgson for being a part of general lab shenanigans, including a random, mid-week trip to Canada's Wonderland. Thanks to Louis Pfeifer for placing the knowledge in my head that brown isn't a colour… I still don't accept that it's only dark orange. Nathanael Harper, thanks for the fun and productive days in the field and for allowing me to hear the story about you accidentally bear spraying yourself multiple times. Thanks to the many, many wonderful undergraduate and graduate students that I have had the opportunity of working with over the years.

Thank you to Quinn Abram, Éric Le Dreff-Kerwin, and Ben Morrow for many fun evenings at the grad house and playing board games. Steph Slowinski, thank you for your friendship and support through the years of graduate school.

Outside the sphere of academia, I have had the support of so many people. James and Karen and Judah and Anna Kelly – thank you so much for welcoming me into your home and hearts over these past five years. Your love and support mean so much to me – and the numerous fish drawings created by Judah and Anna that I now own will always be cherished.

Laura, I could have never imagined the support and kindness that you have shown me over the past two years. Our daily walks to get some exercise and human interaction early in the pandemic were life-giving and your friendship has been (and continues to be) a treasure.

Joseph, I don't know how to put into words what your support has meant to me over these past months. All I can say is that I am so appreciative of all the fish puns and the encouraging/ridiculous gifs, and I am very grateful that you are in my life. I love you bunches and bunches.

Thank you to my wonderfully supportive family: Mom and Dad, thank you for being constant sources of love and helpful advice. Jenn & Josiah & Peter & Eileen & Malachi, Robyn & Graham, Angela & Stephen, thank you for your love and encouragement.

Michelle Koop, Emily Robinson, Miraya Groot, Jani Harris, Ashley Ross and many other friends who have listened to my nerdy excitement about fish stress and have encouraged me throughout this journey – thank you, thank you, thank you.

And of course, shoutout to my best friend (not sure what I ever did to deserve that distinction and your love), Addie Malecki.

And I'm sure that I have forgotten someone… please know that I am grateful for your support!

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I would also like to do the opposite of thanking the COVID-19 pandemic and would like to cordially invite several hundred mantis shrimps to punch the virus in the face repeatedly.

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List of abbreviations

ABC ATP-binding cassette
ADP adenosine diphosphate
AGC automatic gain control
AMPK AMP-activated protein kinase
ANOVA analysis of variance
ATP adenosine triphosphate
AUPP animal utilization project protocol
BCA bicinchoninic acid
BLAST basic local alignment search tool
CAT catalase
CCO cytochrome c oxidase
CCO5B cytochrome c oxidase subunit 5B
cDNA complementary DNA
CPM counts-per-million
CS citrate synthase
Ct or Cq Cycle threshold
CYP1A cytochrome P450, family 1, subfamily A
CYP3A65 cytochrome P450, family 3, subfamily A, polypeptide 65
DAVID Database for Annotation, Visualization, and Integrated Discovery
DFO Fisheries and Oceans Canada
DNA deoxyribonucleic acid
DTNB 5,5'-dithio-bis-(2-nitrobenzoic acid)
DTT dithiothreitol
eDNA environmental DNA
EDTA ethylenediaminetetraacetic acid
ELISA enzyme-linked immunosorbent assay
e-miRNA environmental microRNA
ES enrichment score
FAO Food and Agriculture Organization of the United Nations
FDR false discovery rate
FLX fluoxetine
GEO Gene Expression Omnibus
GK glucokinase
GSEA gene set enrichment analysis
HCD higher-energy collisional dissociation
HCl hydrochloric acid
HPI hypothalamus-pituitary-interrenal
HSC hypothalamic-sympathetic-chromaffin
HSP70 heat shock protein of 70 kDa
HSP90 heat shock protein of 90 kDa
IGFBP1A insulin-like growth factor binding protein 1A
IT injection time
KEGG Kyoto Encyclopedia of Genes and Genomes
LC-MS/MS liquid chromatography–mass spectrometry
LDH lactate dehydrogenase

MDL method detection limit
MDS multidimensional scaling
miARma-Seq miRNA-Seq And RNA-Seq Multiprocess Analysis
miRNA microRNA
mRNA messenger RNA
MS mass spectrometry
MS-222 tricaine mesylate
MTBE methyl tert-butyl ether
NAD$^+$ nicotinamide adenine dinucleotide
NADP nicotinamide adenine dinucleotide phosphate
NCBI National Center for Biotechnology Information
NCE normalized collisional energy
NES normalized enrichment score
PCA perchloric acid
PCR polymerase chain reaction
PGC1A PPARG coactivator 1 alpha
pH power of hydrogen
PK pyruvate kinase
PSM peptide spectral match
RISC RNA-induced silencing complex
RNA ribonucleic acid
RNA-Seq RNA sequencing
ROUT Robust regression and outlier removal
ROS reactive oxygen species
RPM rotations per minute
RT reverse transcriptase
RT-qPCR reverse transcription quantitative PCR
SDS sodium dodecyl sulfate
SEM standard error of the mean
snRNA small nuclear RNA
SPE solid-phase extraction
SPS synchronous precursor selection
TEAB triethylammonium bicarbonate
TEMP temperature
TMM trimmed mean of m-values
TMT tandem mass tag
TUT terminal uridyl-transferase
UNC45B unc-45 myosin chaperone B
USD United States dollar
UTR untranslated region
VEGF vascular endothelial growth factor
VFX venlafaxine
VTG vitellogenin
WWTP wastewater treatment plant

Judah & Anna Kelly ©2021

Chapter 1

Introduction

1.1 The importance of fishes

In Canada, fishes generate more than CAD$6 billion annually to the economy through fisheries, aquaculture, the aquarium industry, and recreational activities (DFO, 2021; Homlund & Hammer, 1999; FAO, 2020; Lynch et al., 2016). Globally, aquaculture production alone generated a total value of USD$263.6 billion in 2020 (FAO, 2020). Fishes are also an important food source for animal and human consumption with an estimated total of 156 million tonnes of fishes consumed globally by humans in 2020 (Homlund & Hammer, 1999; Lynch et al., 2016; FAO, 2020). Ecologically, fishes contribute to the food web, cycle and balance nutrient levels, regulate sediment conditions, and regulate carbon flux (Homlund & Hammer, 1999; McIntyre et al., 2007). Altogether, the importance of fishes is demonstrated by their enormous economic, nutritional, and ecological impacts on our world. As such, it is essential that researchers, end users, and policy managers monitor fish stress in order to maintain the integrity of fish communities for future generations.

1.2 Fish stress response

The adrenergic stress response in fish includes the activation of the hypothalamic-sympathetic-chromaffin (HSC) cell axis which releases the catecholamines, epinephrine and norepinephrine, as well as the hypothalamus-pituitary-interrenal (HPI) axis which releases cortisol (**Figure 1.1**; Wendelaar Bonga, 1997). Catecholamines and cortisol act on tissues to control osmotic balance, mobilize energy for metabolic needs, alter cardiac output and blood flow, alter immune function, and allocate energy away from reproduction and growth (Wendelaar Bonga, 1997). In more recent years, the molecular mechanisms underpinning the adrenergic stress response have been studied in fish (López-Patiño et al., 2014; Wiseman et al., 2007). Though much has been characterized about the adrenergic stress response, there are still critical molecular aspects that are unknown, especially in response to particular stressors and mixtures of stressors.

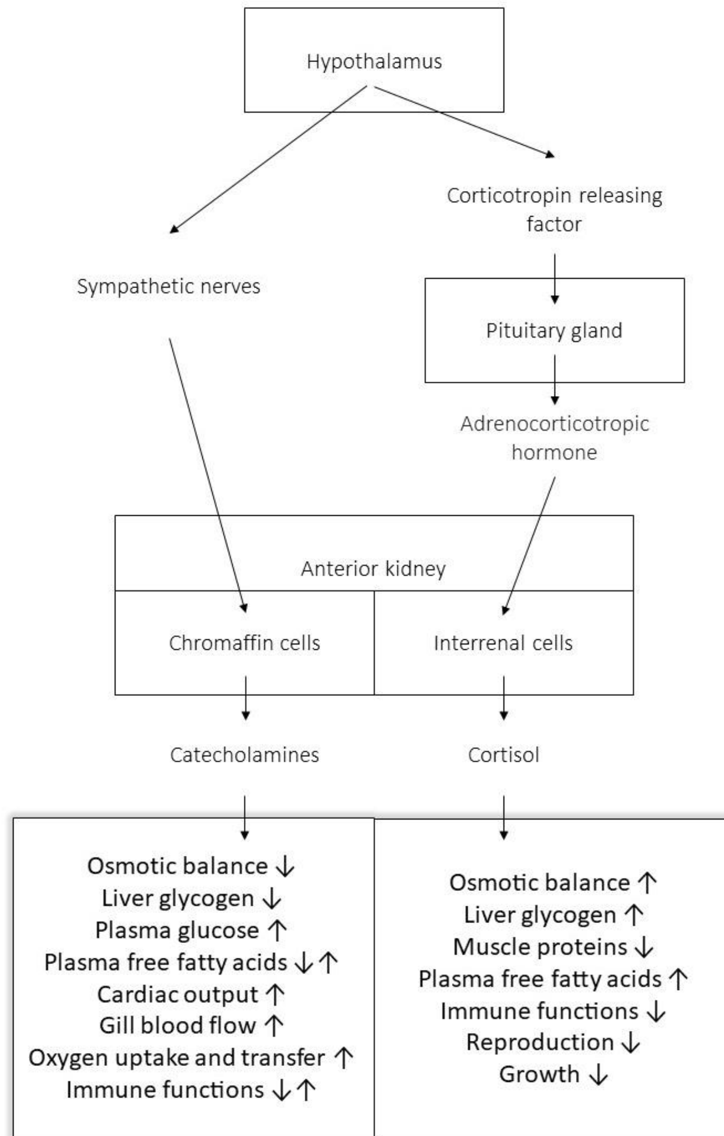

Figure 1.1 Acute stress response in teleosts.
The adrenergic stress response in teleosts is depicted beginning from the hypothalamus and ending in the physiological effects experienced. The stimulation (↑) or inhibition (↓) of processes due to release of catecholamines and cortisol are indicated. Figure modified from Wendelaar Bonga (1997).

1.2.1 Environmental stressors and how fish respond

Climate change stressors, wastewater input, and urbanization are causing increased water temperatures and decreased dissolved oxygen levels which negatively impact fish (Holeton et al., 2011; Jenny et al., 2016; Kinouchi et al., 2007; Paul and Meyer, 2001; Poesch et al., 2016).

Wastewater treatment plants (WWTPs) also introduce contaminants, such as pharmaceuticals, into surface waters (Holeton et al., 2011; Metcalfe et al., 2010). One pharmaceutical of interest due its consumption worldwide, persistence in surface waters, and biological activity is venlafaxine (VFX; Cornwall et al., 2006; Harvey et al., 2000; O'Flynn et al., 2021; Appendix A). While the response to individual environmental stressors has been characterized at the whole organism level, the effects at the molecular level are still poorly understood. For example, exposure to increased water temperature is known to increase metabolic rate and oxygen consumption of zebrafish (López-Olmeda and Sánchez-Vázquez, 2011; Mottola et al., 2020; Scott and Johnston, 2012). Also, decreased dissolved oxygen (mild hypoxia) impairs the HPI axis response in lake whitefish (*Coregonus clupeaformis*) and also impairs reproductive success in zebrafish and marine medaka (*Oryzias melastigma*; Ho and Burggren, 2012; Lai et al., 2019; Whitehouse et al., 2020). Exposure to VFX alters brain function in rainbow trout and European seabass, and negatively impacts the behaviour, visual motor response, and cortisol response in zebrafish (Costa et al., 2021; Huang et al., 2019; Melnyk-Lamont et al., 2014; Tang et al., 2021; Thompson and Vijayan, 2021a; 2021b). While these single stressor studies exemplify the effects that each stressor has on fish, there are limited studies that examine the molecular initiating events that drive the adverse phenotypic responses and even fewer that examine the impacts of these stressors in combination (Chapter 2 & 3).

1.2.2 Multiple stressors

Environmental stressors can exacerbate each other during simultaneous exposure, which commonly occurs in natural environments. Adverse metabolic impacts caused by exposure to increased water temperature can be exacerbated by concurrent exposure to decreased dissolved oxygen. This is experienced due to the simultaneous increase in oxygen demand and decrease in oxygen availability in warm, hypoxic conditions (Barrionuevo & Burggren, 1999; Ficke et al., 2007; Pan & Herbing, 2017). Toxicity of contaminants can also be altered with simultaneous exposure to increased water temperature or decreased dissolved oxygen (Kennedy & Ross, 2012; Osterauer & Köhler, 2008; Dasgupta et al., 2016; Dolci et al., 2013; McElroy et al., 2012). In fact, warmer water temperatures increase the toxicity of VFX in fish due to increased absorption across the gills (Maulvault, Santos, Camacho, et al., 2018; Maulvault, Santos, Paula, et al., 2018; Mehdi et al., 2019). While adverse phenotypic outcomes of these combined stressor exposures have been identified, the molecular initiating events have been poorly explored. Recent studies (Best et al., 2018; Navarro-Martin et al.,

2020) have suggested that epigenetics and epigenetic modulators, such as microRNAs, may play a role in driving phenotypic responses at the molecular level (Chapters 2 & 3).

1.3 MicroRNA

1.3.1 MicroRNA function

MicroRNAs (miRNAs) are a well-conserved facet of post-transcriptional regulation and are small, non-coding RNA (Bartel, 2004; Lee et al., 1993; Ruvkun et al., 1993). MiRNAs can be intergenic, intronic, within repetitive elements, or on their own, and are primarily transcribed in the nucleus (pri-miRNAs), processed by DROSHA and DGCR8 (pre-miRNAs), and exported from the nucleus by exportin-5 where they are further processed by DICER to produce mature miRNAs (Bartel, 2009, 2004; Bizuayehu and Babiak, 2014). Mature miRNAs are loaded into the RNA-induced silencing complex (RISC) which primarily functions by binding to the 3′ untranslated region (UTR) of mRNA to repress translation of the mRNA or to target the transcript for degradation, ultimately decreasing the abundance of protein translated (**Figure 1.2**; Bartel, 2009, 2004; Bizuayehu and Babiak, 2014). These relationships between miRNAs and target mRNA can be computationally predicted based on sequence complementarity and RNA stability (Bartel, 2009; Garcia et al., 2011; Grimson et al., 2007; Lewis et al., 2005). Though the binding of miRNAs to the 3′ UTR is the primary method of post-transcriptional regulation by miRNAs, they can also bind to the 5′ end of mRNA, exons, pre-mRNA, or even DNA, occasionally resulting in increases in translation instead of the typical decreases in translation (Bizuayehu and Babiak, 2014). To further highlight the complexity of the regulation via miRNAs, each miRNA can bind to several mRNA and each mRNA can be regulated by several miRNAs (Bartel, 2009). Therefore, miRNAs are involved in finetuning molecular responses and though it is known that they play a role in most biological processes in organisms, including fish, there is much that is not understood about their regulation.

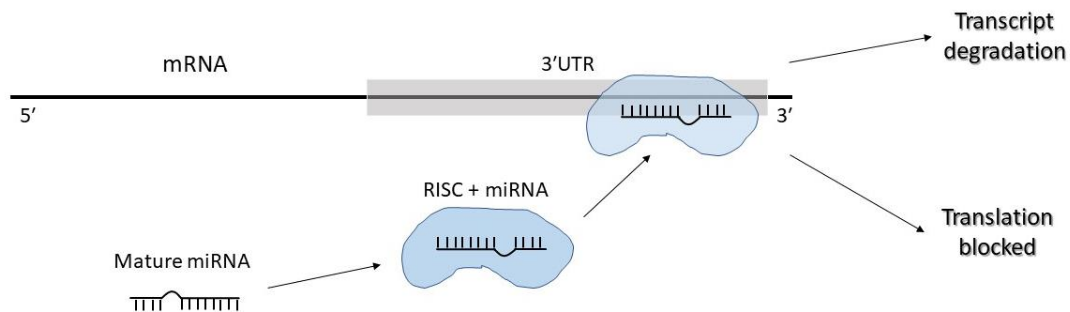

Figure 1.2 The primary methods of post-transcriptional regulation via microRNA. Mature microRNAs (miRNAs) primarily function by being loaded into the RNA-induced silencing complex (RISC) and binding to the 3′ untranslated region (UTR) of mRNA. This leads to mRNA transcript degradation or translation of proteins being blocked.

1.3.2 MicroRNA in fish

To date, miRNAs have only been characterized in 16 fish species in miRBase.org (the microRNA database) although miRNAs have been characterized in some other fish species (e.g. rainbow trout) which are not yet present in the miRNA database (Griffiths-Jones, 2006, 2004; Griffiths-Jones et al., 2007; Juanchich et al., 2016; Kozomara et al., 2019; Kozomara and Griffiths-Jones, 2014, 2011). Fish miRNAs, like all other miRNAs, are named first by a three-letter code comprised of the first letter of the species name and the first two letters of the genus name (ex. *Danio rerio* = dre), followed by miR, to indicate that it is a miRNA, and then by a number to identify which miRNA family it belongs to (ex. dre-miR-1 would have the same/similar seed sequence as hsa-miR-1 and is therefore part of the same miRNA family). If both the 5′ and 3′ ends of the pre-miRNA hairpin loop form mature miRNA, then the particular end of the miRNA is indicated by 5p or 3p notation (ex. dre-miR-1-5p indicates that the miRNA originates from the 5′ end of the hairpin loop of the pre-miRNA). Therefore, the study of the presence and role of miRNAs in fish have begun to be studied but there are still many gaps in understanding the regulation of miRNAs in certain condition and in different fish species.

The impacts of environmental stressors on fish miRNAs have only been measured since 2009 and since then, fish miRNA levels have also been measured in response to hypoxia, cold, heat, toxicants, salinity, infection, and radiation (Flynt et al., 2009; Johnston et al., 2009; Best et al., 2018). Changes in fish miRNA transcript levels have even been measured in response to emerging anthropogenic stressors of interest, including in response to venlafaxine exposure). Specifically, VFX exposure decreased hepatic transcript levels of miR-129 and miR-142 in zebrafish (Luu et al., 2021).

Therefore, it is well established that exposure to stressors, including emerging anthropogenic stressors, alters transcript levels of miRNA. However, there are still critical gaps in our knowledge regarding the effects of multiple stressor exposure, varying lengths of stressor exposure, tissue-specific responses, and species-specific responses (Chapters 2 – 5).

1.3.3 Environmental microRNAs

Since 2011, the presence of fish species in a variety of environments has been measured by extracting and characterizing DNA from water samples, termed environmental DNA or eDNA (Jerde et al., 2011). Fish eDNA detection has become a useful biomonitoring method, however, current eDNA techniques can only inform on the presence or absence of fish in an aquatic environment and it would be useful to have a non-invasive biomarker of fish stress levels (Thomsen and Willerslev, 2015). Therefore, knowing that miRNA transcript levels are regulated by exposure to stress and are part of the post-transcriptional regulation of a fish's phenotype, I wondered if miRNAs could be measured from the water column, much like eDNA and also be altered following exposure to stress, much like internally measured miRNAs. Since eDNA originates from skin cells, blood, urine, feces, or gametes, and these tissues and sample types all contain miRNAs, it is likely that miRNAs are present in the water column (Ficetola et al., 2008; Höss et al., 1992; Jerde et al., 2011; Valiere and Taberlet, 2000). Additionally, miRNAs are known to be more stable than mRNA with a measured half-life of 5 days in cell culture as compared to mRNA with an average half-life of 5 min, providing more evidence of their potential to be measured in water samples (Gantier et al., 2011; Moran et al., 2013). Therefore, though miRNAs have never been measured in the water column and may not be associated with the internal miRNA transcript level changes following exposure to stress, there is potential for their (environmental miRNA or e-miRNA) measurement and potential for the levels of e-miRNA to be correlated to the fish's internal response to stress.

1.4 Which fish species were studied?

There are over 35,000 fish species in the world which live in a variety of habitats and have a variety of tolerances to environmental perturbations, highlighting the importance of considering multiple species when studying the effects of stress on fish (Bordin and Freire, 2021; Froese and Pauly, 2021). However, there are limitations on which fish species can be used due to availability, ability to house in a laboratory, and whether genetic information is available. For this reason, I began my research with zebrafish (*Danio rerio*), due to its accessibility, ease of husbandry, and the wealth of genetic

information available (Chapters 2 &3; Ahkin Chin Tai and Freeman, 2020; Grunwald and Eisen, 2002; Hill, 2005; Howe et al., 2013; Laale, 1977; Lawrence, 2007; Metscher and Ahlberg, 1999; Nüsslein-Volhard and Dahm, 2002). However, zebrafish are a tropical species and tolerant to many stressors, especially fluctuations in temperature (Engeszer et al., 2007; Arunachalam et al., 2013; Morgan et al., 2019). Therefore, it is important to also study temperate, endemic species due to the presence of stressors in their environment and lower tolerance to stressors. For this reason, I also studied rainbow trout (*Oncorhynchus mykiss*), as it is a local species that is readily available and relatively easy to culture (Chapters 4 & 5; Tort, 2013; Woodward and Strange, 1987). Furthermore, it is both a model of fish physiology and a model for miRNA within salmonids, with a sequenced genome and miRNAome (Zardoya et al., 1995; Salem et al., 2010; Tort, 2013; Berthelot et al., 2014; Juanchich et al., 2016; Mennigen and Zhang, Pasquier et al., 2016; 2016; Gao et al., 2021). However, it is important to extend research to less studied species such as brook trout (*Salvelinus fontinalis*). Brook trout are a local species, currently endangered in southwestern Ontario due to anthropogenic influences, and there is minimal study of fish stress in this species (Chapter 4; Biron and Benfey, 1994; Stranko et al., 2008). To date, only the mitochondrial genome has been sequenced and no miRNAs have been measured in brook trout (Doiron et al., 2002). Therefore, by using zebrafish, rainbow trout and brook trout to study miRNA in response to fish stress, I can leverage available knowledge and extend it to less studied species.

1.5 Objectives

The overall goal of my thesis was to determine how transcript levels of miRNAs are regulated when fishes are exposed to stress (**Figure 1.3**). Specifically, I wanted to understand how time sensitive this response is, what factors (such as tissue type) affected this response, and if this response could be measured externally to the fish. The stressors I studied were emerging anthropogenic stressors in combination (increased water temperature, decreased dissolved oxygen, and venlafaxine) and also acute stress induced by air exposure. Furthermore, I aimed to understand the implications of the miRNA response following fish stress by measuring mRNA transcript levels, protein abundances, and enzyme activities that they are predicted to regulate.

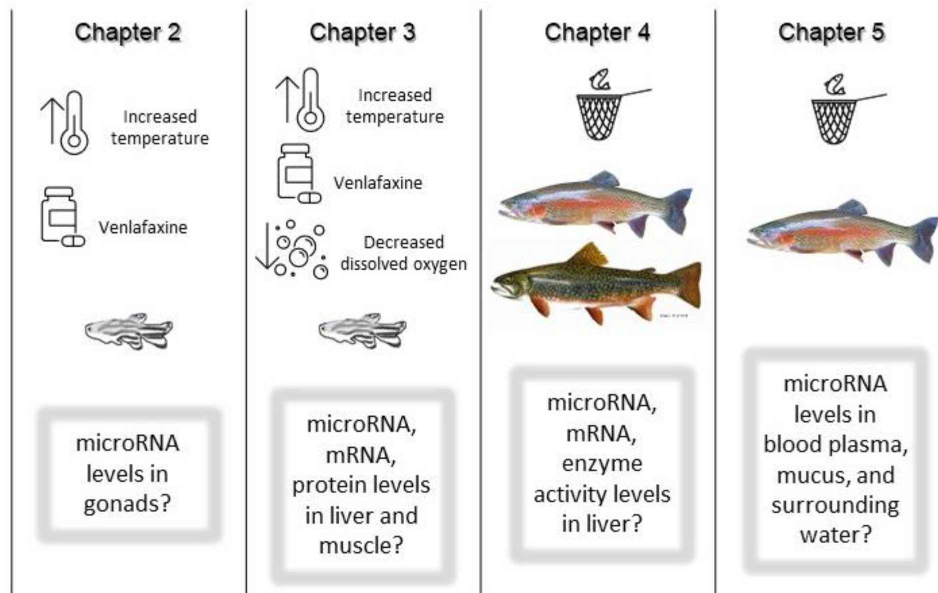

Figure 1.3 Graphical summary of thesis research objectives.
The stressors, fish species, and endpoints measured are depicted under their respective chapter headings. In Chapters 2 and 3, endpoints were measured in zebrafish following anthropogenic stressors and in Chapters 4 and 5, endpoints were measured in salmonids (Chapter 4: rainbow and brook trout; Chapter 5: rainbow trout) following acute air exposure. Gonad tissue was sampled in Chapter 2, muscle tissue was sampled in Chapter 3, liver tissue was sampled in Chapters 3 and 4, and blood, mucus, and water were sampled in Chapter 5.

1.5.1 Specific chapter objectives

The objective of **Chapter 2** was to determine the effects of chronic exposure to and depuration from VFX and/or increased water temperature on miRNAs in the gonad tissue of zebrafish. This chapter provides data on the plasticity of the miRNA response and on reproductive impacts of anthropogenic stressors. Furthermore, the individual and combined impacts of the stressors were investigated. Gaps in knowledge were filled on the impacts of multiple, emerging anthropogenic stressors on miRNA levels in a model fish species and how these impacts respond over time.

The objective of **Chapter 3** was to determine the effects of acute and chronic exposure to VFX, increased water temperature, and decreased dissolved oxygen on miRNAs, mRNA and proteins in liver and muscle tissue of zebrafish. This chapter provides data on the combined impacts of the stressors in multiple tissues and on downstream phenotypic responses. Gaps in knowledge were filled on the impacts of multiple, emerging anthropogenic stressors on miRNA levels, and other molecular

endpoints in a model fish species. This chapter also filled in gaps about the impact of exposure length and specific tissues on the miRNA response.

The objective of **Chapter 4** was to determine the effects of acute stress via air exposure on miRNAs, mRNA, and enzyme activity in liver tissue of rainbow and brook trout. This chapter provides data on the miRNA response in understudied fish species as well as downstream phenotypic responses. Gaps in knowledge were filled on the impacts of acute stress on miRNA levels, and other molecular endpoints in non-model fish species. This chapter also filled in gaps about the miRNA response during recovery following stress and further characterized the physiological response to acute stress in liver.

The objective of **Chapter 5** was to determine the effects of acute stress via air exposure on miRNA profiles in non-lethal samples (plasma, mucus, and surrounding water) of rainbow trout. This chapter provides data on the feasibility of sampling miRNAs from non-lethal and non-invasive samples and determine if there is potential for the use of miRNAs as non-lethal biomarkers. Gaps in knowledge were filled on the impacts of acute stress on miRNA levels in a salmonid.

1.6 Significance

The research I undertook in my thesis is significant both in further understanding how miRNA transcript levels are regulated in response to stress and in further understanding the impacts of our actions on the aquatic environment. The study of miRNAs and how they are regulated in different conditions and how they correlate to downstream phenotypes allows us to understand how fish adapt to stressful situations. Also, due to the conservation of miRNAs and their function, this research can be added to our overall understanding of the properties of the miRNA response in other organisms, including humans. By characterizing how the miRNA response is associated with the presence of the stressor, the length of exposure to the stressor, and the length of time following exposure to the stressor allows for further understanding of how miRNAs play a role in the adaptive response of organisms to different environmental conditions. Furthermore, by studying the effects of emerging, anthropogenic stressors on miRNA and other molecular endpoints, I have added to our understanding of how we are negatively impacting fish. This improved understanding will allow us to not only know the molecular mechanisms related to adverse outcomes resulting from anthropogenic stress but will also provide us with the tools to continue to monitor the impacts of our actions.

Chapter 2

Chronic exposure to venlafaxine and increased water temperature reversibly alters microRNA in zebrafish gonads (*Danio rerio*)

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***Comparative Biochemistry and Physiology Part D: Genomics and Proteomics,* 33, pp. 100634. 2020.**

2.1 Summary

MicroRNA (miRNA) are short, non-coding RNA that act by downregulating targeted mRNA transcripts. Only recently have they been used as endpoints in studies of aquatic toxicology. The purpose of this study was to determine the effect of an antidepressant contaminant, venlafaxine (VFX), and increased temperature on specific microRNA levels in zebrafish (*Danio rerio*) reproductive tissue. Adult zebrafish were exposed to one of four conditions: control, 1 µg/L VFX (VFX), 32 °C (Temp), or 1 µg/L VFX + 32 °C (VFX & Temp) for 21 days. Half of the fish were returned to control conditions for a 21-day recovery period. RT-qPCR was performed to measure relative abundances of several miRNAs known to respond to antidepressant exposure: dre-miR-22b-3p, dre-miR-301a, dre-miR-140-5p, dre-let-7d-5p, dre-miR-210-5p, and dre-miR-457b-5p. After the exposure period, dre-miR-22b-3p and dre-miR-301a showed a significant downregulation in response to all treatments. In contrast, after the recovery period, there were no significant differences in microRNA abundance. These altered microRNA are predicted to target several genes, including phosphofructokinase, and are associated with ovarian pathologies. Combined, I have shown that VFX and increased water temperature alter miRNA abundances in zebrafish reproductive tissue, an effect correlated with a functional stress response and cell cycle dysregulation.

2.2 Introduction

With an ever-growing human population, increasing anthropogenic stress is placed on the aquatic environment, impacting biodiversity (Johnson et al., 2017). This highlights the need to measure the effects of stressors on aquatic organisms in order to communicate to policy makers that more stringent regulation is required before irreversible impacts to aquatic communities occur. While there are a variety of endpoints that can be used across different levels of biological organization, a molecular approach can indicate adverse effects before lethality occurs. This can be particularly useful since it allows for more time within the aquatic toxicology framework for remediation. Therefore, by studying the effects of aquatic contaminants and stressors in our environment and testing emerging biomarkers, such as microRNA (miRNA), valuable knowledge can be gained to further inform regulators on the effects of our actions.

2.2.1 Venlafaxine

Venlafaxine is a selective serotonin and norepinephrine reuptake inhibitor prescribed for anxiety, lack of energy, pain, psychomotor problems and other symptoms of major depressive disorders (Silvershein and Saltiel, 2015; Appendix A). It preferentially binds to serotonin receptors, meaning at lower doses, it is functionally a serotonin reuptake inhibitor, similar to fluoxetine (FLX), also known as Prozac® (Harvey et al., 2000; Zerjav et al., 2009). VFX appears in the aquatic environment downstream of wastewater treatment plants (WWTPs) as 5% of the ingested dose is recovered in urine as the non-metabolized, active form of the drug (Pubchem, n.d.). VFX has been found at 2.19 μg/L downstream of a WWTP in St. Paul, Minnesota (Schultz et al., 2010). It has also been measured downstream of a WWTP in the Grand River (Waterloo, ON) at approximately 1 μg/L (Metcalfe et al., 2010). Therefore, venlafaxine is a quantifiable, widespread contaminant in freshwater environments. VFX is water-soluble and will be taken up over the gills of fish and circulated throughout the organism. VFX is bio-accumulated in fish in the following tissues, in order from most to least: liver, brain, plasma, and least in muscle (Metcalfe et al., 2010; Schultz et al., 2010; Lajeunesse et al., 2011; Schoenfuss et al., 2011; Grabicova et al., 2014; Arnnok et al., 2017; David et al., 2018; Huerta et al., 2018; Maulvault et al., 2018).

Fish and their tissues are being exposed directly to VFX, and there are a variety of non-target effects on phenotype, behaviour, and physiology of fish (Schultz et al., 2010; Ings et al., 2012; Galus et al., 2013a, 2013b; Best et al., 2014; Bisesi et al., 2014, 2016; Melnyk-Lamont et al., 2014; Mehdi

et al., 2017; Melvin, 2017; Parrott and Metcalfe, 2017, 2018; Simmons et al., 2017; Thompson et al., 2017). However, there is a need to continue research into the effects on emerging biomarkers that signal change at an earlier point and can be more readily measured. Moreover, pharmaceutical exposure of VFX in combination with increased water temperature resulting from climate change is a unique and important system to study as our understanding of the anthropogenic effects on the environment continues.

2.2.2 Increasing water temperature

In Canada, surface air temperatures are conservatively predicted to increase by five degrees by 2050 (Poesch et al., 2016), which will increase surface water temperatures. Water temperatures around the world are increasing as well, with quantifiable increases in Lake Tanganyika (O'Reilly et al., 2003), Lake Baikal (Shimaraev and Domysheva, 2012), and the Yangtze River (Gemmer et al., 2012). The effects of increased water temperature are critical for study as elevated temperature increases metabolic rate in ectothermic poikilotherms, which can exacerbate contaminant uptake and harmful impacts (ex. Maulvault et al., 2018). There is some variation of venlafaxine uptake due to environmental conditions. Specifically, warming and acidification increases uptake of VFX in the brain while decreasing both uptake and elimination in the liver, showing that exposure of fish to VFX will be altered with different environmental conditions (Arnnok et al., 2017; Maulvault et al., 2018). Therefore, there is an inherent need to study the effects of increased water temperature in combination with emerging contaminants in our aquatic environment.

2.2.3 MicroRNA

MicroRNA (miRNA) are conserved, small (22-nucleotide) non-coding RNA which act by decreasing mRNA translation and stability. They function by binding to the 3′ untranslated region of mRNA, activating Slicer (Argonaut 2 endonuclease), which cleaves the mRNA, resulting in reduced mRNA transcript abundance, translational potential, and impacts the functional responses of downstream targets (Best et al., 2017). The miRNA response to stressors has been measured in fish since 2009 and been shown to be mostly conserved across fish species (Johnston et al., 2009; Best et al., 2017). MiRNA are a sensitive biomarker because they respond to both general and specific stressors. MiRNA expression changes in response to altered water temperatures (Johnston et al., 2009; Yang et al., 2011; Campos et al., 2014; Bizuayehu et al., 2015; Hung et al., 2016; Qiang et al., 2017). Additionally, the concentrations of specific miRNA (let-7d-5p, miR-140-5p, miR-210-5p, mir-22b-

3p, miR-301a, miR-457b-5p) are increased in response to fluoxetine exposure in zebrafish (Craig et al., 2014). In this study, the miRNA listed above increased 3–18-fold after exposure to 540 ng/L fluoxetine. Since fluoxetine and venlafaxine both inhibit serotonin reuptake, I want to measure if the same miRNA response will occur and if this response is modified with exposure to warmer temperatures.

Furthermore, in order to develop a useful biomarker of environmental stress it is crucial that the marker be altered in the presence of the stressor but return to normal levels following removal of the stressor. This will allow for time-sensitive measurement of the stress exposure and the organism's physiological state.

2.2.4 Objectives

In this study, I investigated the effects of VFX and increased water temperature on adult zebrafish (*Danio rerio*). This study examined the effects of individual and combined stressors, on miRNA in reproductive tissues of exposed fish. This is a sublethal exposure on zebrafish, a genetic model. Six miRNAs were measured in zebrafish ovaries and testes, previously found by Craig et al. (2014) to be altered following exposure to fluoxetine. I hypothesized that the exposure to stressors would alter the relative abundance of specific miRNA in zebrafish gonads, and that the response would be affected by the individual and combined stressors uniquely. I additionally hypothesized that miRNA levels would no longer be altered after removal of stressors, due to their plasticity in responding to environmental conditions. Altogether, this study aims to demonstrate the use of miRNA as biomarkers of environmental exposure.

2.3 Materials and methods

2.3.1 Zebrafish husbandry

Adult zebrafish were housed in an Aquatic Habitats® Z-Hab Mini System (Pentair, USA) with water quality set points at 28 °C, 675 µS and pH 7.3. They were exposed to a 12 h:12 h light-dark cycle. Fish were fed ground TetraMin™ (Tropical Flakes, Blacksburg, VA, USA), twice daily, until satiety. Zebrafish used in the study had a mean fresh body weight of 0.39 ± 0.12 g. All experimental procedures were approved and carried out in accordance with the University of Waterloo animal care guidelines (AUPP # 30005).

2.3.2 Exposure

Zebrafish were exposed to combinations of increased water temperature and venlafaxine (VFX) for 21 days in a chronic, static exposure. The four treatments are referred to as Control (0 µg/L VFX, 27 °C), VFX (1 µg/L VFX, 27 °C), Temp (0 µg/L VFX, 32 °C), and VFX & Temp (1 µg/L VFX, 32 °C). Each treatment contained 3 tank replicates with 5 male and 5 female zebrafish in each of the 20 L glass aquaria. Prior to exposure, zebrafish were acclimated to their tanks for one week. This included increasing the temperature by one degree per day in the Temp and VFX & Temp treatment tanks. Fish were fed 1% of their average body weight daily. The tanks were not filtered during exposure so that no VFX was removed. Therefore, 50 % static water changes were performed daily with the preheated water from header tanks. The tanks exposed to VFX (Sigma-Aldrich, Oakville, ON, Canada) were spiked with concentrated VFX dissolved in water, following the daily water exchange, to ensure levels were maintained at 1 µg/L. Water samples were collected weekly to ensure there was no VFX contamination in the control tanks and that the venlafaxine treatments were exposed to 1 µg/L consistently. These water samples were collected one-hour post-VFX addition to ensure even mixing throughout the tank. Five fish from each tank or fifteen fish per treatment were sampled at the end of the exposure period. Ovaries and testes were collected and frozen at −80 °C until analysis was performed. Tissue analysis was performed on testes and ovaries separately.

2.3.3 Recovery

Fifteen fish from each treatment were returned to control conditions (0 µg/L VFX, 27 °C) for 21 days post-exposure. They were housed in four tanks within the Aquatic Habitat system, with fish of the same treatment. At the end of the 21-day post exposure period ovaries and testes were collected and

frozen at −80 °C until analysis was performed. Tissue analysis was performed on testes and ovaries separately.

2.3.4 Water quality analysis

Water temperature was recorded daily. The pH, ammonia, nitrate, and nitrite content of the water was measured weekly. Water samples were taken weekly during the acclimation and exposure period from the Control, VFX, Temp, and Temp & VFX tanks to measure venlafaxine levels. Briefly, samples were preserved with 625 µL of 200 g/L sodium azide (Sigma-Aldrich) and 312.5 µL of 20 g/L ascorbic acid (Sigma-Aldrich). They were stored at 4 °C for up to one week. Extraction and quantification of VFX occurred using 100 mL of the water sample. Samples were spiked with 100 µL [100 µg/L] deuterated VFX (Couperus et al., 2016). VFX was extracted from the water samples using solid-phase extraction (SPE) in Oasis HLB cartridges (6 cc, 500 mg, Waters Corporation, Milliford, MA, USA; Rahman et al., 2010; Couperus et al., 2016). The eluents were collected, evaporated, and then reconstituted with 500 µL of methanol and stored at −20 °C until analysis (Rahman et al., 2010; Couperus et al., 2016). VFX was quantified using an API 3200 QTRAP LC-MS/MS system (SCIEX, Concord, Ontario, Canada; Couperus et al., 2016). The method detection limit (MDL) was 5 ng/L. Further details about cartridge conditioning, elution, and LC-MS/MS parameters can be found in Suppl File 1 (Appendix B).

2.3.5 Tissue analysis

After the exposure and recovery periods, fish were euthanized with buffered MS-222; (Sigma-Aldrich), and their testes or ovaries were collected and snap frozen on dry ice. MiRNA was extracted from tissue using the Qiagen miRNeasy Kit protocol (Qiagen, Missisauga, ON, Canada). To homogenize the tissue, an Omni Tip™ tissue homogenizer (OMNI International, Kennesaw, Georgia, USA) was used at 35,000 rpm for 15 s on ice. Samples were centrifuged at 12,000 × *g*, whenever the speed wasn't specified. In the final step, the eluate was run through the column for a second time to ensure all miRNA were collected. Relative abundance of specific miRNA, let-7d-5p, miR-140-5p, miR-210-5p, miR-22b-3p, miR-301a, and miR-457b-5p, was analyzed by synthesizing cDNA using the Qiagen miScript II RT kit using the HiSpec buffer as recommended (Qiagen). Next, RT-qPCR was run using the Qiagen miScript PCR kit. The protocol was followed, using 3 µM primer (Sigma-Aldrich; **Table 2.1**). The relative abundance of small nuclear RNA (snRNA) 7SK was measured as a reference transcript to normalize the miRNA levels. Thermocycler settings were as follows:

incubation at 95 °C for 15 min to activate HotStarTaq DNA polymerase, denaturation for 15 s at 95 °C, annealing for 30 s at 55 °C, and extension for 30 s at 72 °C and with fluorescence capture. Cycles were repeated 40 times. Analysis was performed using a Bio Rad Touch CFX96 RT-qPCR Detection System (Bio Rad, CA, USA), which required the ramp rate to be adjusted to a rate of 1 °C per second. Melt curve analysis was performed using the following settings: sample heated from 65 °C to 95 °C at a rate of 0.5 °C per second, capturing fluorescence data every 5 s. Primers were validated by standard curve analysis, the use of non-template controls, and melt curve analysis, to ensure that efficiency was between 90 and 110 %, that there was no contamination or primer-dimerization, and that samples would be diluted into the linear dynamic range where no inhibition was present.

Table 2.1 Primer sequences used for RT-qPCR measurement of the targets.
The Qiagen Universal Reverse Primer was used for all miRNA. The sequence of the Universal Reverse Primer is unknown due to the proprietary nature.

Oligo name	Accession #	Forward	Reverse
let-7d-5p	**MIMAT0001762**	TGAGGTAGTTGGTTGTATGGTT	Qiagen Universal Reverse Primer
miR-140-5p	**MIMAT0001836**	CAGTGGTTTTACCCTATGGTAG	
miR-210-5p	**MIMA0003392**	AGCCACTGACTAACGCACATTG	
miR-22b-3p	**MIMAT0001789**	CGTTCTTCACTGGCTAGCTTTA	
miR-301a	**MIMAT0001870**	CAGTGCAATAGTATTGTCAAAG	
miR-457b-5p	**MIMAT0001884**	AAGCAGCACATAAATACTGGAG	
7SK-snRNA	**NR_029421.1**	ACGAGCATCGCTGGTATAGAA	GCCTCATTTGGAT GTGTCTGA

2.3.6 *In silico* microRNA target and pathway analysis

Target and functional prediction were performed *in silico* for miRNA that were significantly altered by the treatments. This was performed using the TargetScanFish 6.2 (targetscan.org) database to identify target gene transcripts (Lewis et al., 2005; Grimson et al., 2007; Garcia et al., 2011; Ulitsky et al., 2012). Once a list of target transcripts was compiled for altered miRNA, only genes with a context score of −0.3 or less were used to minimize unlikely gene targets in downstream analysis (Garcia et al., 2011; Lu and Clark, 2012). The list of genes was uploaded to the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.8 tool and underwent Functional Annotation Clustering (Huang et al., 2009a, 2009b). This tool identifies overrepresented groups of genes or functions in a gene list. Enriched gene clusters identified using DAVID were filtered using the highest stringency possible so to remove less definitive results and to avoid over-extrapolating miRNA target results. Enriched groups were cut off at an enrichment score of 1.3 (equivalent of p =

0.05) (Huang et al., 2007). The gene lists were also uploaded to the zebrafish specific KEGG Search & Colour pathway tool to identify pathways that could be affected by the altered miRNA (Kanehisa and Goto, 2000; Kanehisa et al., 2017, 2019). The number of genes targeted in each pathway was compared to the total number of genes in the pathway to identify highly enriched or targeted KEGG pathways.

2.3.7 Statistical analysis

To identify the differences in relative abundance of the miRNA, the $2^{-\Delta\Delta Ct}$ values were calculated in order to compare individuals and treatments (Livak and Schmittgen, 2001). The small RNA 7SK was used as a reference gene. Statistical analysis was performed on GraphPad Prism 8.1.2 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). Outliers were identified and removed using the ROUT method and normality was tested using the Shapiro-Wilk method. Data was analyzed using parametric two-way ANOVAs (Factors: Treatment & Sex). If a significant difference was found at $p < 0.05$, a Holm-Sidak multiple comparison test was used to compare treatments to the control.

2.4 Results

2.4.1 Water quality analysis

The pH, ammonia, nitrate, and nitrate levels of the tanks were measured periodically throughout the acclimation, exposure and recovery period and were always maintained within acceptable levels and treatment tanks did not differ from control tanks. No mortality or morbidity occurred during the experiment. Control and VFX treatments were consistently maintained at 27 °C, while the Temp and VFX & Temp treatments were maintained at 32 °C, during the exposure period (**Figure 2.1A**). During the recovery period, when fish were in the aquatic system, the temperature was maintained at 27.7 ± 0.2 °C. The levels in the VFX and VFX & Temp treatments stayed within 1.01–1.35 μg/L, while levels of VFX in the Control and Temp tanks stayed below the detectable limit of 5 ng/L (**Figure 2.1B**; Suppl File 1).

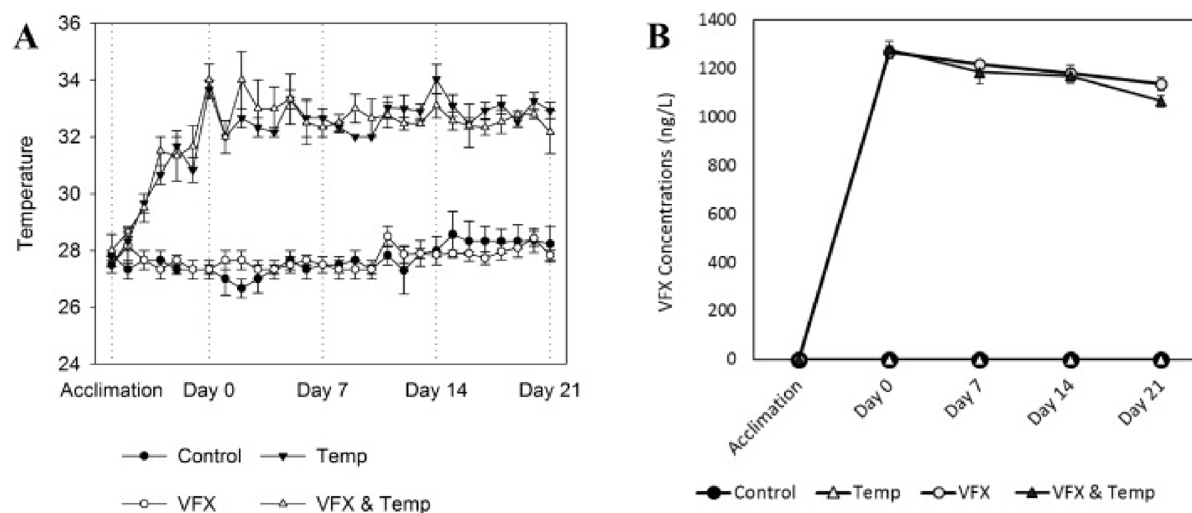

Figure 2.1 Temperature and venlafaxine levels during exposure.
Mean ± SEM **A**. Average daily tank temperature and **B**. venlafaxine concentration in the tanks during the exposure (n = 3). Treatments are indicated by the shape and shading of the symbols.

2.4.2 Tissue analysis

Zebrafish were sampled at the end of the exposure period (Day 21) and the end of the recovery period (Day 42). The sex, mass of fish, mass of reproductive tissue was recorded (no differences between treatments). The relative abundance of miR-22b-3p decreased to 20–30 % of the control abundance when exposed to Temp, VFX, and VFX & Temp (**Figure 2.2D**). This effect is no longer observed after the recovery period (**Figure 2.3D**). The relative abundance of miR-301a decreased to 20–30 % of the control when exposed to any of the treatments, Temp, VFX, and VFX & Temp (**Figure 2.2E**). This effect is no longer observed after the recovery period (**Figure 2.3E**). There were no changes in relative abundance of let-7d-5p, miR-140-5p, miR-210-5p, or miR-457b between treatments at Day 21 (**Figure 2.2A—C**, & **F**) or Day 42 (**Figure 2.3A—C, & F**).

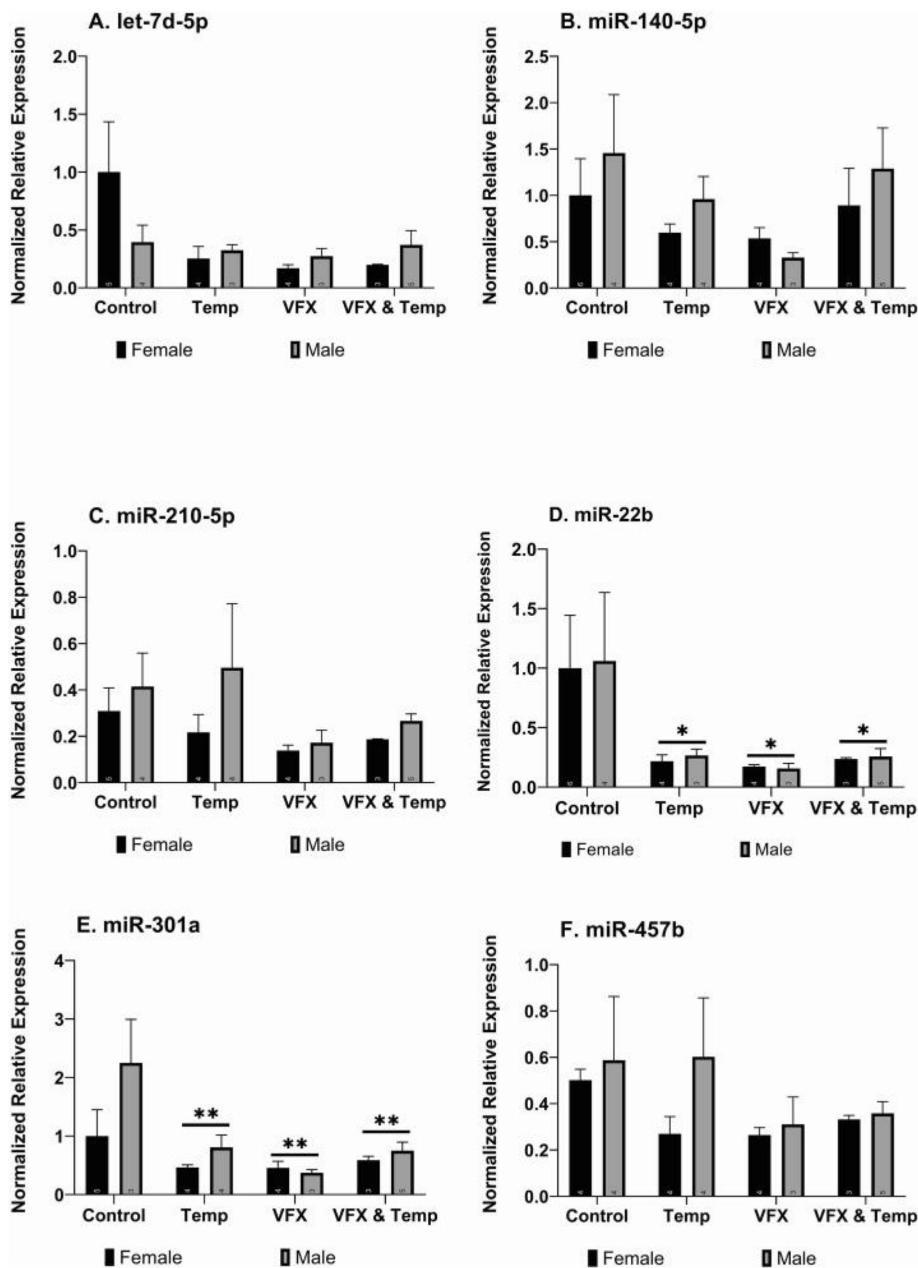

Figure 2.2 MicroRNA expression fold changes relative to the control after the exposure period (Day 21) in zebrafish testes and ovaries.

Data were analyzed using a parametric two-way ANOVA (p = 0.05) where all the treatments were compared to the control using a Holm-Sidak multiple comparison test (p = 0.05) Error bars represent positive standard error. Female fish are indicated by black bars and male fish are indicated by grey bars. N numbers analyzed are indicated within the bars. A single asterisk denotes a p-value between 0.05 and 0.01, a double asterisk denotes a p-value between 0.01 and 0.001. **A**. let-7d-5p. **B**. miR-140-5p.). **C**. miR-210-5p. **D**. miR-22b-3p. **E**. miR-301a. **F**. miR-457b-5p.

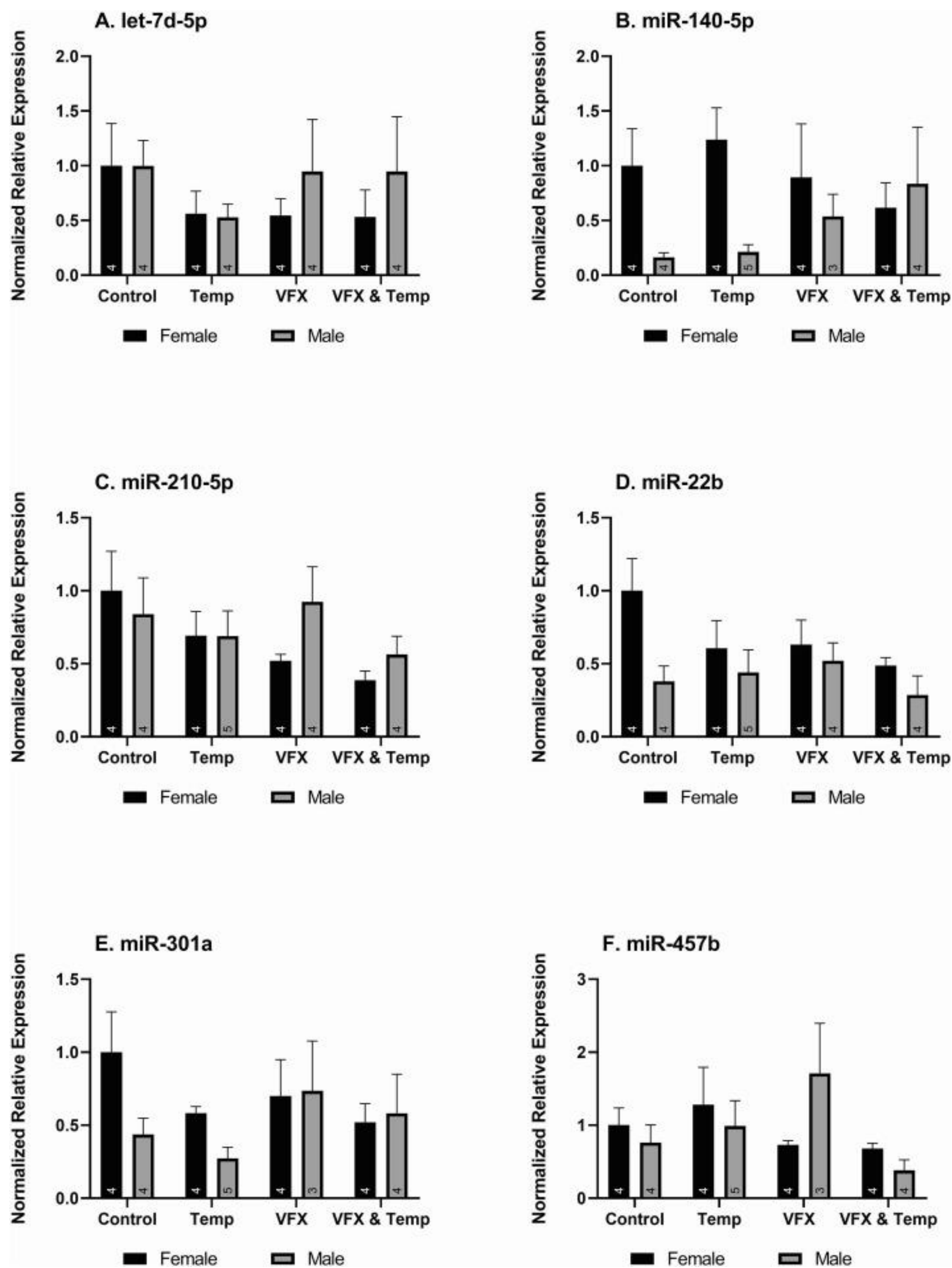

Figure 2.3 MicroRNA expression fold changes relative to the control after the exposure period and the recovery period (Day 42) in zebrafish testes and ovaries.
Error bars represent positive standard error. Female fish are indicated by black bars and male fish are indicated by grey bars. N numbers analyzed are indicated within the bars. A single asterisk denotes a p-value between 0.05 and 0.01, a double asterisk denotes a p-value between 0.01 and 0.001. Data were analyzed using a parametric two-way ANOVA ($p = 0.05$) where all the treatments were compared to the control using a Holm-Sidak multiple comparison test ($p = 0.05$). **A**. let-7d-5p. **B**. miR-140-5p. **C**. miR-210-5p. **D**. miR-22b-3p. **E**. miR-301a. **F**. miR-457b-5p.

2.4.3 *In silico* microRNA target and pathway analysis

In order to more effectively analyze target genes, microRNA results were grouped together to analyze mRNA targets that would be altered in all treatments. Overall, 88 mRNA targets were above the cut-off for miR-22b and 188 mRNA targets were above the cut-off for miR-301a. Since miR-301a and miR-22b were downregulated, any gene functions or clusters and pathways would be upregulated in all treatments.

Functional gene enrichment analysis identified BTB domains as the only cluster above the enrichment threshold (1.3 or p = 0.05). The BTB domain functions in transcriptional regulation, regulation of chromatin structure, cytoskeleton organization, ubiquitination, and in ion channels (Interpro: IPR011333; Stogios et al., 2005). BTB domains are found on proteins in ubiquitin mediated proteolysis and sphingolipid metabolism pathways (SMART: SM00225). The key KEGG pathways that were affected by the targets of miR-22b and miR-301a were metabolic pathways (dre01100), specifically glycolytic enzyme phosphofructokinase, the oocyte meiosis pathway (dre04114), and the drug metabolism pathway (dre00983). Also, highly enriched pathways include several biosynthesis and metabolic pathways (**Table 2.2**). For complete gene lists from TargetScanFish, Functional Annotation Clustering results from DAVID, or complete pathway lists from KEGG, please refer to Suppl File 1.

Table 2.2 Enriched KEGG pathways targeted by miR-22b and miR-301a.
The percentage of KEGG genes targeted by miR-22b or miR-301a when compared to the total number of genes in each KEGG pathway. Listed are the pathways with above average percentages.

KEGG pathways targeted by miR-22b & 301a	Percentage	# of genes targeted	Total # of genes
dre00072 Synthesis and degradation of ketone bodies	11.1	1	9
dre00770 Pantothenate and CoA biosynthesis	7.1	1	14
dre00533 Glycosaminoglycan biosynthesis - keratan sulfate	6.7	1	15
dre00670 One carbon pool by folate	5.6	1	18
dre04744 Phototransduction	5.4	2	37
dre00600 Sphingolipid metabolism	5	3	60
dre00532 Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate	4.8	1	21
dre03018 RNA degradation	4.6	4	87
dre01230 Biosynthesis of amino acids	4.5	4	88
dre00250 Alanine, aspartate and glutamate metabolism	4.5	2	44
dre00900 Terpenoid backbone biosynthesis	4.5	1	22
dre00590 Arachidonic acid metabolism	4.3	2	47
dre00650 Butanoate metabolism	4.2	1	24
dre00270 Cysteine and methionine metabolism	3.8	2	53
dre00520 Amino sugar and nucleotide sugar metabolism	3.4	2	58
dre00514 Other types of O-glycan biosynthesis	3.4	1	29
dre00052 Galactose metabolism	3.0	1	33
dre00030 Pentose phosphate pathway	3.0	1	33
dre03030 DNA replication	2.6	1	38
dre02010 ABC transporters	2.6	1	39
dre00830 Retinol metabolism	2.5	1	40
dre00051 Fructose and mannose metabolism	2.3	1	43
dre00260 Glycine, serine and threonine metabolism	2.2	1	45

2.5 Discussion

After exposure to increased water temperature and venlafaxine, individually and in combination, abundances of miR-22b-3p and miR-301a were downregulated in zebrafish gonads. These effects are no longer measured after 21 days in control conditions, demonstrating a plastic and time-sensitive response. These changes in miRNA are all different than the exposure to FLX, a similar antidepressant (Craig et al., 2014). When following up with functional responses of the altered miRNA, links to metabolic stress responses, drug metabolism, and tissue-specific responses are observed. These results demonstrate the usefulness of miRNA as an early-detection biomarker in aquatic toxicology.

2.5.1 MicroRNA respond to venlafaxine and increased temperature stress in the same manner

Both miR-301a and miR-22b-3p are downregulated in all treatments (**Figure 2.2D** & **E**). Their response does not differ significantly between individual stressors of increased temperature or venlafaxine or when both stressors are combined. Therefore, these miRNAs are likely responding in a general stress pathway and not to a specific contaminant or temperature stress. Additionally, there are no additive or synergistic effect of combining VFX and increased water temperature on miRNA in zebrafish gonads.

2.5.2 Reversible microRNA response to environmental stressors

The relative abundances of the six miRNAs were not significantly deregulated when measured after the recovery period (Day 42; **Figure 2.3**). This indicates a return to non-altered miRNA levels for miR-22b-3p and miR-301a after the stressors were removed. When compared to a human model, researchers found that altered miRNA levels used to identify the presence of prostate tumours returned to normal 10 days after the removal of prostate tumours (Kelly et al., 2015). MiRNA undergo active regulation and turnover by terminal uridyl-transferases (TUTs), therefore any differences in abundance would have to be actively maintained following exposure (Best et al., 2017). This highlights the plasticity of the miRNA response, which is an ideal characteristic of a biomarker, as the effects of current conditions can be identified and would not be confounded by legacy stressors.

2.5.3 Venlafaxine and fluoxetine have unique microRNA signatures

It was predicted that there would be changes observed in these specific miRNA as they changed in response to a similar antidepressant, FLX (Craig et al., 2014). At low doses, VFX is functionally an SRI, which is the mode of action of FLX (Harvey et al., 2000; Zerjav et al., 2009). However, out of the six miRNA that were measured, only two were altered in our VFX exposure and the response was opposite (decrease instead of the increase measured in the FLX exposure). The differences in responses could be due to the tissue differences since the FLX exposure measured miRNA in the liver of zebrafish whereas I measured the same miRNA in response to VFX in gonads. These tissue-specific effects have been measured in fish previously (Bizuayehu et al., 2012; Lau et al., 2014) and reviewed in general by Bartel (2004). However, more experimental comparisons are warranted to define tissue specificity of the miRNA response to environmental stressors.

2.5.4 Conserved and *in silico* miRNA target analysis

Out of the two downregulated miRNA, miR-22b-3p is the only miRNA that has been measured in response to other gonad phenotypes and pathologies (Nagaraja et al., 2010; Dang et al., 2015; Guo et al., 2017). Due to the conserved nature of miRNA, the responses to miRNA in mammals are useful in understanding the phenotypic changes that occur with deregulation of mir-22b-3p in ovaries or testes. Downregulation of miR-22 plays a role in humans with premature ovarian insufficiency/failure (Dang et al., 2015; Guo et al., 2017). It has been found that deregulation of miR-22 affects migration/invasion of human ovarian cancer cells, and that miR-22 was downregulated in ovarian cancer cell lines and tissues (Nagaraja et al., 2010). These phenotypic responses to changes in gonadal miR-22 abundance are linked to ovarian pathologies, especially those involving cell-cycle deregulation. These miRNAs do not have current implications in testicular pathologies. This links the results found in this chronic exposure with adverse long-term effects for zebrafish ovarian health, especially with cell cycle dysregulation.

To understand the functional responses that the alteration of both miRNA in this exposure, I performed *in silico* target analysis to identify mRNA that could be upregulated and use computational tools (DAVID and KEGG) to derive meaning from the large gene lists. While these are not functionally validated, they can be useful to understand the data and to identify future targets of functional studies. Briefly, mRNA targets were combined into a group of 276 genes and then analyzed with DAVID functional annotation clustering and KEGG Search and Colour pathways. In

the list of combined genes, KEGG identified the upregulation of PFK within the metabolic pathways. This increase in glycolytic catabolism is a well-accepted response to stress, which validates this high-throughput pathway analysis (Wendelaar, 1997). Other pathways of interest were the drug metabolism pathway and the oocyte meiosis pathway, due to the exposure and the tissue measured, respectively. The other genes were enriched in BTB protein domains which are a large group of protein domains that function in structure, regulation, and signaling. This enrichment data doesn't highlight a specific functional response but can be used to develop future targets and hypotheses.

2.5.5 Conclusions & future directions

MiRNA are useful sub-lethal biomarkers that can be used within aquatic toxicology to detect current effects that can be linked to downstream phenotypic responses. Therefore, this study contributes to aquatic toxicology by studying the effects of emerging contaminants while using an emerging biomarker. It would be advantageous to repeat this in a local, temperate species, such as rainbow trout. This would validate the conservation of the miRNA response as a biomarker and further investigate the effects of combining temperature and pharmaceutical stress in a more stenothermal species.

2.6 Thank you

The authors would like to thank Ivan Cadonic, Hossein Mehdi, Shahithiya Santoshkumar, and Christopher Kuc for their assistance with the experimental set up, maintenance, takedown, and with tissue processing. The authors would like to thank Leslie Bragg for assisting in venlafaxine extraction and measurement.

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Chapter 3

Acute and chronic exposure to multiple anthropogenic stressors alters microRNAs, mRNA, and proteins in zebrafish (*Danio rerio*) liver and muscle

3.1 Summary

Emerging anthropogenic stressors of concern include increased water temperature, decreased dissolved oxygen, and pharmaceutical contaminants (such as venlafaxine, VFX). Though effects of these stressors on fish have been characterized individually, the impacts of these stressors in combination are largely uncharacterized, especially at the level of molecular regulation. MicroRNAs (miRNAs) are non-coding RNAs that post-transcriptionally mRNA by targeting them for decay or blocking translation. Though, miRNAs have been measured in fish and levels of specific miRNAs are altered in response to environmental stressors, the impact of different lengths of exposures to multiple anthropogenic stressors on different tissues is unknown. Therefore, the objective of this study was to measure the miRNA and associated molecular responses in zebrafish liver and muscle tissue following either acute and chronic exposure to increased water temperature, decreased dissolved oxygen, and venlafaxine. I hypothesized that stress-responsive mRNA (hsp70, igfbp1a, cyp1a, cyp3a65, and pgc1a) and protein levels would be increased in response to the multiple stressor exposure and that the miRNAs predicted to target them (miR-16c-5p, miR-214, miR-142a-3p, miR-181b-5p, and miR-129-5p) would be inversely related, indicating a potential regulatory role. Several miRNAs (miR-142a-3p, miR-16c-5p, miR-214, miR-129-5p), mRNA (hsp70, cyp3a65, cyp1a, pgc1a), and proteins (Vtg2, Vtg3, Si:dkeyp-46h3.3, Unc45b) were altered in liver and/or muscle tissue exposed to multiple stressors. The miRNA and mRNA responses were unique to the length of exposure, tissue, and the sex of the fish. This study is significant as it expands our understanding of the sublethal, molecular mechanisms that are initiated following exposure to multiple, emerging anthropogenic stressors. Specifically, exposure to these multiple stressors initiates transcript and protein levels responses in cell chaperone, decontamination, metabolic, and reproductive pathways which are associated with changes in miRNA transcript levels.

3.2 Introduction

Anthropogenic stressors stemming from urbanization, wastewater inputs, and climate change pose a serious threat to the environment, increasing temperatures, decreasing dissolved oxygen levels, and introducing pharmaceutical contaminants in surface waters. Urbanization-related changes in land use (i.e., increased presence of non-porous surfaces) increases runoff of warm water into streams, resulting in increased stream temperatures (Paul & Meyer, 2001). Increases in water temperature can directly cause decreases in dissolved oxygen due to the inverse relationship between water temperature and oxygen solubility (Colt, 2012). Climate change is also predicted to continue increasing water temperature and reducing dissolved oxygen over the next 25 years (Poesch et al., 2016). Similarly, wastewater effluent increases stream temperatures and nutrient inputs, decreasing dissolved oxygen levels due to increased eutrophication and biological oxygen demand (Jenny et al., 2016). Effluent from wastewater treatment plants (WWTPs) is also the source of pharmaceutical contaminants into surface water as they are not fully removed during treatment (Grabicová et al., 2020). Thus, there are multiple stressors present in surface waters and it is important to understand their impacts on aquatic organisms such as fish.

Exposure to increased water temperature and decreased dissolved oxygen can adversely affect fish physiology. Concurrent exposure to stressors can alter impacts, either exacerbating or minimizing the effects from individual stressors. Increased water temperatures raise rates of development, feeding, metabolism, growth, and oxygen consumption in fish (López-Olmeda & Sánchez-Vázquez, 2011). Lowered dissolved oxygen levels negatively affect fish by reducing reproductive success and HPI axis responsiveness (Ho & Burggren, 2012; Whitehouse et al., 2020). Together, exposure to increased water temperature with decreased dissolved oxygen can exacerbate the metabolic impacts experienced since oxygen concentration decreases at higher temperature but oxygen consumption rates increase (Pan & Herbing, 2017). Therefore, increased water temperature and decreased dissolved oxygen impact fish metabolism and stress response, and these effects can be exacerbated when stressors are combined.

Toxicity of contaminants can also be altered when fish are also exposed to increased water temperature or decreased dissolved oxygen (Kennedy & Ross, 2012; McElroy et al., 2012). Specifically, toxicity of venlafaxine (VFX) is increased in warmer water (Maulvault et al., 2018). VFX is an antidepressant which is found in surface waters worldwide due to human consumption and

subsequent wastewater effluent input (O'Flynn et al., 2021). VFX is taken up by fish, primarily through the gills, and can be quantified in a variety of tissues including the brain, liver, and plasma (Arnnok et al., 2017; Grabicova et al., 2014). Exposure to VFX negatively affects fishes, specifically impacting behavioral responses to stress in zebrafish (Tang et al., 2021). Therefore, VFX is an anthropogenic contaminant of concern, has known adverse effects on fish, and the effects can be exacerbated by simultaneous exposure to other stressors.

Elements of the molecular response to increased water temperature, decreased dissolved oxygen, and/or VFX exposure have been previously characterized, as indicated by changes in specific transcripts or proteins following exposure. Heat shock protein 70 (hsp70) is a protein chaperone which responds to increased temperature and cell stress (Airaksinen et al., 2003). Cytochrome P450, family 3, subfamily A, polypeptide 65 (cyp3a65) is an enzyme which detoxifies contaminants, such as VFX (Saad et al., 2016). Cytochrome P450, family 1, subfamily A (cyp1a) is an enzyme which responds to contaminant and hypoxia exposure (Oris & Roberts, 2007; Saad et al., 2016). Insulin-like growth factor binding protein 1A (igfbp1a) is a protein coding gene which plays a role in the hypoxia response (Maures & Duan, 2002). PPARG coactivator 1 alpha (pgc1a) is a protein which plays a role in mitochondrial biogenesis and can be altered with different energy needs associated with heat or hypoxia stress (H. Liang & Ward, 2006). The increase in these transcripts or proteins in response to anthropogenic stressors has been characterized, however there are other facets of the molecular response which are unknown.

Abundance of mRNA transcripts can be regulated by microRNAs (miRNA). MiRNAs are short, non-coding RNA that primarily function by binding to the 3′ untranslated region (UTR) of specific mRNA and block translation or recruit enzymes to degrade the transcript in a concentration based inverse relationship (O'Brien et al., 2018). MiRNAs are altered in fish in response to increased temperature (J. Sun et al., 2019), decreased dissolved oxygen (Lai et al., 2016), and venlafaxine exposure (individually or in combination) (Luu et al., 2021). Therefore, miRNAs are known to respond to anthropogenic stressors. Additionally, computational relationships between miRNAs and mRNA can be predicted based on the RNA binding stability and sequence complementarity (Grimson et al., 2007). Since specific mRNA transcripts are altered in response to anthropogenic stressors, the miRNAs that are predicted to bind and regulate the mRNA can be bioinformatically identified. Taken together, the molecular response to multiple stressors can be further characterized by

measuring changes in specific mRNA transcript levels, as well as changes in the transcript levels of miRNA that are predicted to target them.

It is known that miRNA and mRNA can be differentially expressed based on the time post-stressor or the length of exposure to stressors (Cadonic et al., 2020; Shaya et al., 2019). However, the acute response (hours) to VFX, increased water temperature, and decreased dissolved oxygen has never been measured alongside the chronic response (days to weeks). Understanding this will add to our comprehension of how fish are able to respond to stressors acutely and cope with long term perturbations. Furthermore different tissues have different physiological roles and therefore it is important to measure how different tissues respond to environmental perturbations. Liver tissue is involved in detoxification of contaminants, making it an important tissue to study when measuring the effects of venlafaxine, a pharmaceutical contaminant (Bruslé & Anadon, 1996). Furthermore, the liver plays a role in energy allocation during the stress response making it an important tissue to study when measuring the effects of increased water temperature and decreased dissolved oxygen, stressors with metabolic impacts (Bruslé & Anadon, 1996). Muscle tissue is physiologically relevant due to its role in movement and aerobic energy usage and is therefore an important tissue to study when studying metabolic stressors. Lastly, it is known that alterations in mRNA expression are not always reflected in changes in protein abundances; therefore, it is valuable to measure the protein response to understand the phenotypic response of the organism to multiple stressors (X. Liang et al., 2020; Mottola et al., 2020). Altogether, it is important to understand how different lengths of exposure to these environmental stressors alter miRNA, mRNA, and proteins in muscle and liver tissues.

The objective of this chapter was to determine the effects of acute and chronic exposure to VFX, increased water temperature, and decreased dissolved oxygen on miRNA, mRNA and corresponding proteins in liver and muscle tissue of zebrafish, a vertebrate miRNA toxicity model (Ahkin Chin Tai & Freeman, 2020). I hypothesized that the stress-responsive mRNA and protein levels will be increased in response to the multiple stressor exposure and that the miRNA predicted to target them will be inversely related, indicating a potential regulatory role. By conducting this study, more can be understood about the molecular response to multiple environmentally relevant stressors.

3.3 Methods

3.3.1 Husbandry Data

Mixed sex adult zebrafish were purchased from PetSmart (Waterloo, Ontario) and housed in an Aquatic Habitats® Z-Hab Mini System (Pentair, USA) with water quality set points at 28 °C, 675 μS and pH 7.3, with a 12 h:12 h light: dark cycle. Fish were fed GEMMA Micro 300 (Skretting, St. Andrews) zebrafish food or ground Tetramin™ fish flakes (Virginia, USA) daily until satiety. No disease, loss of condition, or mortalities occurred throughout the exposures. All experimental procedures were approved and carried out in accordance with the University of Waterloo Animal Care (Animal Utilization Project Protocol #40989) and Canadian Council of Animal Care guidelines.

3.3.2 Exposures & Sampling

Exposures occurred in 40 L aquaria, one tank for each treatment. Individual fish were treated as replicates as there were no tank effects in preliminary experiments, which allowed for a reduction in the number of fish used. The control treatment parameters were 0 μg/L VFX, 27 °C, and 100 % dissolved oxygen (8.5 mg/L) and exposed treatment parameters were 1 μg/L VFX, 32 °C, 50 % dissolved oxygen (4.2 mg/L). These stressors are environmentally relevant as 1 μg/L VFX has been measured in a Canadian river and Canadian surface waters are expected to increase by five degrees by 2050 (Metcalfe et al., 2010; Poesch et al., 2016). The exposure to 50 % dissolved oxygen was used due to its known adverse, but sublethal effects, on zebrafish and is environmentally relevant due to the occurrence of persistent and temporary bouts of hypoxia in freshwater environments (Ho & Burggren, 2012; Jenny et al., 2016). There were two lengths of exposure, acute and chronic. To capture the initial response to the multiple stressors, fish were exposed for 24 h with no acclimation (acute). To capture the long-term response to multiple stressors, fish were exposed for 21 days with a week of acclimation, where temperature was increased, and dissolved oxygen was decreased incrementally (chronic). In the acute exposure, 11 males and 11 females were used in each treatment, and in the chronic exposure, 20 males and 20 females were used in each treatment. The tanks were static with 50% pre-conditioned water renewals every other day which occurred one hour following feeding and was accompanied by re-spiking VFX in the exposed tank. Temperature and dissolved oxygen levels were maintained using a Qubit aquatic control system and recorded at 10 to 30 s intervals using Vernier probes and LoggerPro software. Total ammonia, nitrite, and nitrate were monitored weekly and maintained within healthy limits (NH_3 < 0.1 mg/L, NO_2^- < 0.3 mg/L, NO_3^- <

50 mg/L) and pH levels were monitored weekly and maintained between 7.5 - 8. At the end of the exposure, fish were euthanized in a buffered solution of MS-222 (0.5 mg/L MS-222 with 1.0 mg/L $NaHCO_3$) and weighed. Zebrafish used in the acute study had a mean fresh body weight of 0.66 ± 0.036 g in the control treatment and 0.60 ± 0.038 g in the exposed treatment. Zebrafish used in the chronic study had a mean fresh body weight of 0.55 ± 0.034 g in the control treatment and 0.53 ± 0.011 g in the exposed treatment. Livers and muscles were sampled, placed on dry ice immediately, and stored at -80 °C for miRNA, mRNA, or protein analysis.

3.3.3 Venlafaxine measurement

Water samples were collected once during the acute exposure and weekly during the chronic acclimation and exposure period to measure venlafaxine levels. Briefly, 125 mL samples were frozen at -20 °C on the day they were collected and thawed on the day of extraction. Samples of 100 mL were spiked with 100 µL [100 µg/L] deuterated VFX before extraction using solid-phase extraction in Oasis HLB cartridges (6 cc, 500 mg, Waters Corporation, Milliford, MA, USA; Couperus et al., 2016; M. F. Rahman et al., 2010). Cartridges were conditioned with 5 mL methyl tert-butyl ether (MTBE), 5 mL of methanol, then 5 mL Milli-Q water. Samples were then introduced with Milli-Q water and eluted with 5 mL methanol and 5 mL of 10:90 methanol:MTBE. The eluents were collected, evaporated under nitrogen, and then reconstituted with 500 µL of methanol and stored at −20 °C until analysis (Couperus et al., 2016; M. F. Rahman et al., 2010). VFX was quantified with a method detection limit of 5 ng/L using an API 3200 QTRAP LC-MS/MS system (SCIEX, Concord, Ontario, Canada; (Couperus et al., 2016). Further specifications about cartridge conditioning, elution, and LC-MS/MS parameters can be found in the supplemental files (Appendix C).

3.3.4 RNA target selection

Fold change relative to the control of several mRNA was measured following the exposure. These mRNA were chosen based on expected effects of the multiple stressor exposure to VFX, increased water temperature, and decreased dissolved oxygen in liver and muscle tissue. In the liver tissue, *hsp70*, *cyp1a*, *cyp3a65*, and *igfbp1a* were quantified. These mRNA were measured within the liver tissue as they respond to temperature and cellular stress (*hsp70*), contaminant stress (*cyp1a* & *cyp3a65*), and hypoxia stress (*cyp1a* & *igfbp1a*) (Airaksinen et al., 2003; Goldstone et al., 2010; Saad et al., 2016; Oris & Roberts, 2007; M. S. Rahman & Thomas, 2012; Saad et al., 2016; Duan & Xu, 2005; Kajimura et al., 2006; Maures & Duan, 2002; Naya-Català et al., 2021; C. F. Sun et al., 2011).

In the muscle tissue, *pgc1a* and *hsp70* were quantified, due to their roles in responding to metabolic, temperature, and cellular stress (Airaksinen et al., 2003; LeMoine et al., 2008; Moyes et al., 2010). These mRNA were then input into TargetScanFish, a miRNA target prediction tool to select known zebrafish miRNA to measure alongside the mRNA (Grimson et al., 2007; Lewis et al., 2005). At least one miRNA was chosen for each mRNA (**Table 3.1**). The miRNAs would be expected to be inversely related to the mRNA they are predicted to target, which would provide a preliminary indication of regulation via mRNA transcript decay.

Table 3.1 Binding sites between microRNA and mRNA.
Binding sites identified by TargetScanFish between miRNA (bottom sequence) and the mRNA 3′ untranslated region (top sequence). The position on the 3′UTR begins immediately after the stop codon and ignores any 3’UTR introns. Lower context-plus scores indicate higher likelihood of binding with no specific cutoff to indicate that binding will not occur (Garcia et al., 2011).

<table>
<tr><th>Binding pair</th><th>Binding sites</th><th>Position on 3′UTR</th><th>Context+</th></tr>
<tr><td>hsp70
miR-16c-5p</td><td>5' ...AUUUUUAUACAACACUGCUGCUC...
||| |||||||
3' GAGGUUAUAAAUGU--ACGACGAU</td><td>79-85</td><td>-0.26</td></tr>
<tr><td>hsp70
miR-129-5p</td><td>5' ...AUAUUUUCAUGAAUUCAAAAAAC...
||||||
3' UCGUUCGGGUCUGGCGUUUUUC</td><td>183-189</td><td>-0.02</td></tr>
<tr><td>pgc1a
miR-16c-5p</td><td>5' ...GCUCUUGAUGAACUUUGCUGCUC...
|||||||
3' GAGGUUAUAAAUGUACGACGAU</td><td>3597-3603</td><td>-0.23</td></tr>
<tr><td>pgc1a
miR-129-5p</td><td>5' ...AAACACAAAAACGAACAAAAAAA...
||||||
3' UCGUUCGGGUCUGGCGUUUUUC</td><td>334-340</td><td>> -0.01</td></tr>
<tr><td></td><td>5' ...GGCUAUUUACAACAACAAAAAAA...
||||||
3' UCGUUCGGGUCUGGCGUUUUUC</td><td>557-563</td><td>> -0.01</td></tr>
<tr><td></td><td>5' ...ACGUGUGCGCUUGACGCAAAAAU...
|||||||
3' UCGUUCGGGUCUGGCGUUUUUC</td><td>2669-2675</td><td>> -0.02</td></tr>
<tr><td></td><td>5' ...CGAAAACAAAAACUACAAAAAAG...
||||||
3' UCGUUCGGGUCUGGCGUUUUUC</td><td>2802-2808</td><td>> -0.01</td></tr>
<tr><td></td><td>5' ...AGAGAUAGAACAAGACAAAAAAA...
||||||
3' UCGUUCGGGUCUGGCGUUUUUC</td><td>3547-3553</td><td>> -0.01</td></tr>
<tr><td>cyp3a65
miR142a-3p</td><td>5' ...ACUAUUGGAGUUUAA-ACACUACA...
||| |||||||
3' AGGUAUUUCAUCCUUUGUGAUGU</td><td>205-212</td><td>-0.41</td></tr>
<tr><td>cyp1a
miR-214</td><td>5' ...AUAUCGUGUCUGUGACCUGCUGA...
|||||||| |||||||
3' GACGGACAGACAC-GGACGACA</td><td>799-806</td><td>-0.48</td></tr>
<tr><td>igfbp1a
miR-181c-5p</td><td>5' ...CGUUAUUUAUUUAAAUGAAUGUA...
|||||||||
3' GGGUGGCUGUCGUUACUUACAC</td><td>223-230</td><td>-0.27</td></tr>
</table>

3.3.5 RNA extraction and quantification

A maximum of 50 mg of liver or muscle tissue was used to co-extract miRNA and mRNA using the Qiagen miRNeasy Mini Kit (CAT 217004, Qiagen, Canada). The manufacturer's directions were followed with the following specifications. Tissues were homogenized on ice for 15 s at 35,000 rpm with an Omni Tip™ tissue homogenizer (OMNI International, USA). Samples were centrifuged at 12,000 × *g* when the speed was not specified. Nucleic acids were quantified using a SpectraDrop™ Micro-Volume Microplate and SpectraMax 190 plate reader and A260/A280 ratios were used to assess purity (Molecular Devices, California, USA). The miScript RT II kit (CAT 218161, Qiagen, Canada) was used to reverse transcribe 1 µg RNA using the HiFlex buffer to quantify both mature miRNA and mRNA. Manufacturer's directions for the 20 µL reverse transcription reaction were followed with no deviations and cDNA was stored at -20 °C until qPCR quantification. Primers were validated by standard curves made from pooled cDNA to ensure that primer efficiency was between 90 and 110 % and to identify the dilution of cDNA to use where no amplification inhibition was present (linear dynamic range; **Table 3.2**). Melt curve analysis and non-template controls were used to ensure that no contamination or dimerization of the primers occurred. Samples were diluted 20× to fit within the linear dynamic range of the standard curve and 1 µL was used in a 10 µL PCR reaction (5 µL 2× SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Canada), 1 µL of 5 µM forward primer and 1 µL of 5 µM reverse primer, and 2 µL molecular grade water). The RT-qPCR analysis was conducted with a BioRad CFX96 Touch Thermal Cycler (Bio-Rad Laboratories, Canada), which incubated samples at 95 °C for 30 s, denatured at 95 °C for 10 s, annealed at 60 °C for 20 s, and then detected fluorescence (denaturation, annealing, and fluorescence detection occurred for 40 cycles). Following each run, a melt curve analysis was performed by increasing the temperature from 65 °C to 95 °C in 0.5 °C increments every 5 s, with fluorescence measurements occurring at each temperature increment. Samples were run in duplicate and only samples with a standard deviation < 0.35 between technical replicates were used. Relative quantities of miRNA and mRNA were calculated using Bio-Rad Maestro software and were normalized using the NORMA-Gene algorithm (Heckmann et al., 2011). This algorithm allows for the robust normalization of relative quantities of RNA without housekeeping genes, if more than five RNA targets are measured (Heckmann et al., 2011). This is useful when measuring miRNA levels, as the selection of an appropriate, non-biased reference gene can be challenging.

Table 3.2 Primers used for RT-qPCR quantification of relative miRNA and mRNA expression. The primers used to quantify the relative abundance of miRNA and mRNA are listed below. The NCBI accessions are provided for the mRNA sequences and the miRbase accessions are provided for the miRNA sequences.

mRNA/miRNA	**Accession**	**Forward**	**Reverse**
hsp70	AF210640.1	AAAGCACTGAGGGACGCTAA	TGTTCAGTTCTCTGCCGTTG
igfbp1a	NM_173283.4	AAAGCGAGACAGCACCAGAT	TTGGCCTTCATGCTCTCTTT
cyp1a	AB078927.1	TGGATGAAAAGATCGGGAAG	TGAGGAATGGTGAAGGGAAG
cyp3a65	NM_001037438.1	CGGTGCGTACAGTATGGATG	AGAGAGGGTTCAGCAGGTCA
pgc1a	AY998087.2	TGAGGAAAATGAGGCCAACT	AGCTTCTTCAGCAGGGAAGG
18S	FJ915075.1	ATGGCCGTTCTTAGTTGGTG	GAACGCCACTTGTCCCTCTA
dre-miR-16c-5p	MIMAT0001776	TAGCAGCATGTAAATATTGGAG	GAATCGAGCACCAGTTACGC
dre-miR-214	MIMAT0001283	ACAGCAGGCACAGACAGGCAG	GAATCGAGCACCAGTTACGC
dre-miR-142a-3p	MIMAT0003160	TGTAGTGTTTCCTACTTTATGGA	GAATCGAGCACCAGTTACGC
dre-miR-181b-5p	MIMAT0001270	AACATTCATTGCTGTCGGTGGG	GAATCGAGCACCAGTTACGC
dre-miR-129-5p	MIMAT0001825	CTTTTTGCGGTCTGGGCTTGCT	GAATCGAGCACCAGTTACGC

3.3.6 Protein extraction and quantification

Proteins were extracted from liver and muscle samples using a modified filter-aided sample preparation (Erde et al., 2014). Briefly, a maximum of 10 mg of sample (muscle tissues were ground under liquid nitrogen before weighing out amount for extraction, whereas the entire liver tissue was used) was lysed using a tissue homogenizer (prewashed with methanol) in 4 % (w/v) SDS, 100 mM HEPES/HCl pH 7.6, 100 mM 1,4-dithiothreitol, and 1× MS-SAFE protease inhibitor (Sigma-Aldrich, Canada, CAT MSSAFE-1VL). Samples were incubated for 5 min at 95 °C, then sonicated for 30 s on ice before a second incubation at 95 °C for 5 min before being centrifuged at 12,000 × *g* for 10 min. Fifty microliters of muscle sample and 200 µL of UA buffer (8 M urea in 100 mM HEPES/HCL pH 8.5) or 250 µL liver sample were loaded into a Microcon 10 kDa filter (MilliporeSigma™, CAT MRCPRT010) for digestion. Filters had been washed immediately before with two washes of 500 µL 60 % methanol and spun for 25 min at 14,000 × *g*, followed by two washes with 500 µL ultrapure water and spun for 20 min at 14,000 × *g*, followed by a final two washes with 500 µL UA and spun for 20 and 25 min respectively at 14,000 × *g*. Following addition of samples, filters were spun for 25 min at 14,000 × *g*, followed by a wash with 200 µL UA and spun for 30 min at 14,000 × *g*. Next, 100 µL 50 mM iodoacetamide in UA was added and samples were incubated in the dark for 20 min, then centrifuged for 20 min at 14,000 × *g*. The filter was washed three more times with 200 µL UA, then three times with 200 µL of 100 mM triethylammonium bicarbonate (TEAB) and centrifuged for 25 min at 14,000 × *g* each time. Proteins were digested overnight at 37 °C with 0.2 µg/µL of trypsin/LysC (Promega, Madison, WI, CAT V5073). Peptides were eluted four times using 60 µL of 100 mM TEAB and spinning for 30 min at 14,000 × *g*. Peptide concentrations were measured using the Pierce Quantitative Fluorometric Peptide Assay (ThermoFisher Scientific, CAT 23290). Peptides were stored at -80 °C until ready to thaw and dry using a Speedvac to reconstitute 40 µg peptides in 100 µL of 100 mM TEAB. Tandem mass tag labelling was performed according to kit instructions (TMT10plex™ Isobaric Label Reagent Set, CAT 90110) with the exception that half of the label was added to each sample tube (20 µL instead of 41 µL) with the rest of the reagents added in full. This was done because only 40 µg of peptides were labelled in each sample. Samples were dried using a Speedvac and sent for protein identification and quantification via mass spectrometry at the SPARC BioCentre (The Hospital for Sick Children, Toronto, Ontario, Canada).

Protein purification and quantification was carried out at the SPARC BioCentre, The Hospital for Sick Children, and is described in brief as follows (full details in Supplemental Files; Appendix C).

Peptides were purified by homemade C18 tips, lyophilized, and stored at -20 °C. Samples were analyzed on a Thermo Scientific Orbitrap Fusion Lumos Tribrid mass spectrometer (ThermoFisher, San Jose, CA) outfitted with a nanospray source and EASY-nLC 1000 nano-LC system (ThermoFisher, San Jose, CA). Lyophilized peptide mixtures were dissolved in 0.1% formic acid and loaded onto a 75 µm x 50 cm PepMax RSLC EASY-Spray column filled with 2 µM C18 beads (ThermoFisher San, Jose CA) at a pressure of 900 Bar and a temperature of 60°C. Peptides were eluted over 240 min at a rate of 250 nL/min using a gradient set up with 0.1 % formic acid (Buffer A) and 0.1 % formic acid with 80 % acetonitrile (Buffer B) (**Table 3.3**).

Table 3.3 Gradient elution setup for protein quantification.
Buffer A is 0.1% formic acid and Buffer B is made up of 80% acetonitrile, 0.1% formic Acid, all v/v in HPLC grade water.

Time (min)	Duration (min)	% A	% B
0	1	98	2
1	227	56	44
228	2	0	100
230	10	0	100

Mass spectrometry data was acquired using MultiNotch synchronous precursor selection (SPS) MS3 scanning for TMT tags (McAlister et al., 2012). MS1 acquisition was performed with a scan range of 550 - 1800 m/z with resolution set to 120,000, maximum injection time (IT) of 50 ms and automatic gain control (AGC) target set to 4×10^5. Isolation for MS2 scans was performed in the quadrupole; MS2 scans were performed in the linear ion trap with a scan range of 550 - 1800 m/z, maximum IT of 50 ms, AGC target of 1×10^4, and normalized collisional energy (NCE) of 35 using the turbo scan rate. For MS3 scans, higher-energy collisional dissociation (HCD) activation was employed, with NCE of 65, and scans were measured in the Orbitrap with resolution of 50,000, scan range of 100 – 300 m/z, AGC target of 1×10^5, and maximum IT of 50 ms. MS raw files were analyzed with Proteome Discoverer 2.2 (Thermo Fisher Scientific) and fragment lists searched against the UniProt Zebrafish databases by SEQUEST HT and Amanda 2.0, with parent and fragment mass tolerances set to 10 ppm and 0.6 Da, respectively. Complete tryptic peptides with a maximum of two missed cleavages were accepted. Variable modifications included Met oxidation and N-terminal acetylation; fixed modifications included Cys carbamidomethylation and TMT labeling of peptide N-termini and Lys residues. Search results were filtered through Percolator (Spivak et al., 2009) at the peptide spectral match (PSM) level using a strict false discovery rate (FDR) q-value of 0.01 and

relaxed FDR q-value of 0.05 (Elias & Gygi, 2007). The Proteome Discoverer 2.2 reporter ion quantifier node was used to quantify TMT reporter ions, with an integration tolerance of 20 ppm on the MS3 order.

3.3.7 Statistical Analysis

Normalized relative expression of each miRNA and mRNA target were compared using two-way ANOVAs if assumptions of normality were met, otherwise data for the particular target were compared using Kruskal-Wallis non-parametric tests. The factors compared were Treatment and Sex. There were five males and five females in each treatment. Tukey's test was used as a post-hoc test to identify differences between factors ($p < 0.05$).

Linear regressions were performed using GraphPad (Prism, USA) between normalized levels of miRNA and either normalized levels of predicted mRNA targets or normalized abundance of predicted protein targets. These were performed to determine if inverse relationships existed between the miRNAs and the predicted targets as a preliminary indication of regulation (Luu et al., 2021; Ikert et al., 2021). The correlation coefficient and the measure of whether the slope was significantly non-zero ($p < 0.05$) were calculated.

Proteins were quantified only in female zebrafish due to a lack of sufficient protein in male tissue samples. Furthermore, only two control samples and three exposed samples were analyzed in each tissue (muscle and liver) and length of exposure (acute and chronic). Differential abundance analysis was performed using limma to capture any significantly altered proteins ($p < 0.05$) by combining acute and chronic controls to have sufficient sample size. Results and conclusions from this analysis are presented with caveats indicating the limitations with performing this analysis with limited power. Broad phenotypic changes were identified using gene set enrichment analysis (GSEA; Mootha et al., 2003; Subramanian et al., 2005). GSEA identifies significantly enriched protein pathways and processes between treatments. Protein abundances were compared to zebrafish gene lists and processes that were enriched in either Control or Exposed treatments were identified (FDR = 0.25, GSEA default, 1000 permutations).

R scripts of statistical methods (everything except linear regressions and GSEA were performed in R), other statistical methods (detailed GSEA parameters) and all statistical analysis results (including non-significant results) can be found in the supplemental materials archived on FigShare (Appendix C).

3.4 Results

3.4.1 Venlafaxine concentration, temperature, and oxygen levels

For the acute exposure, the control VFX concentration was below the method detection limit (MDL) of 5 ng/L and the exposed tank had a mean ± standard error (SEM) VFX concentration of 942.5 ± 125 ng/L. During the acute exposure, the temperature and oxygen setpoints (control: 27 °C, 100 % dissolved oxygen; exposed: 32 °C, 50 % dissolved oxygen) were maintained with no deviations. For the chronic exposure, control VFX concentrations were below the MDL, and the exposed tanks had a mean ± SEM VFX concentration of 830.0 ± 93.8 ng/L. Temperatures in control and exposed tanks were maintained at the 27 °C and 32 °C setpoints, respectively. Dissolved oxygen levels in control and exposed tanks were maintained at the 100 % (~8 mg/L) and 50 % (~4 mg/L) setpoints, respectively. The only exception occurred on Day 7 when dissolved oxygen levels in the exposed tank rose to 6 mg/L. Graphs of daily averages of temperature and dissolved oxygen levels as well as raw temperature and dissolved oxygen levels are found in the supplemental files (Appendix C).

3.4.2 mRNA and microRNA expression

Specific mRNA and miRNA transcripts were measured in liver and muscle tissue of zebrafish exposed to multiple stressors (VFX, increased water temperature, decreased dissolved oxygen) for 24 hours. Measurement of these transcript abundances following an acute exposure allows us to understand the initial molecular processes that occurred in response to these sublethal stressors. Due to the role of liver in the stress and detoxification response, transcripts (*hsp70* and *cyp3a65*) and miRNA predicted to regulate stress-responsive mRNAs (miR-214 and miR-181c-5p), were measured. In acutely exposed fish, liver *hsp70* expression was significantly increased 4-fold in exposed fish with no effect of sex ($p = 0.002$, $F = 14.524$; **Figure 3.1A**). Liver *cyp3a65* expression was not affected by treatment but male fish had significantly higher expression than female fish, with 14-fold higher expression in the acute exposure ($p = 4\times10^{-5}$, $F = 31.337$; **Figure 3.1B**). This trend was also observed in the miRNA abundances, with males having higher liver miR-214 and miR-181c-5p expression than females (miR-214: 5-fold higher, $p = 0.001$, $F = 16.1$; miR-181c-5p: 2-fold higher, $p = 0.023$, $F = 6.347$; **Figure 3.1CD**). Due to the importance of muscle in locomotion and overall fitness of the fish, a metabolic transcript (*pgc1a*) and miRNA predicted to regulate it (miR-129-5p and miR-16c-5p), were measured. In acutely exposed fish, there was an interaction between treatment and sex on muscle *pgc1a* expression, with exposed males having significantly higher expression than control

males (2.5-fold increase) but no effect on females (p = 0.014, F = 7.59; **Figure 3.2A**). Male fish had higher muscle expression of miR-129-5p than female fish (1.1-fold higher, p = 0.011, F = 8.2; **Figure 3.2B**). There were no effects of treatment or length of exposure on muscle miR-16c-5p expression (**Figure 3.2C**).

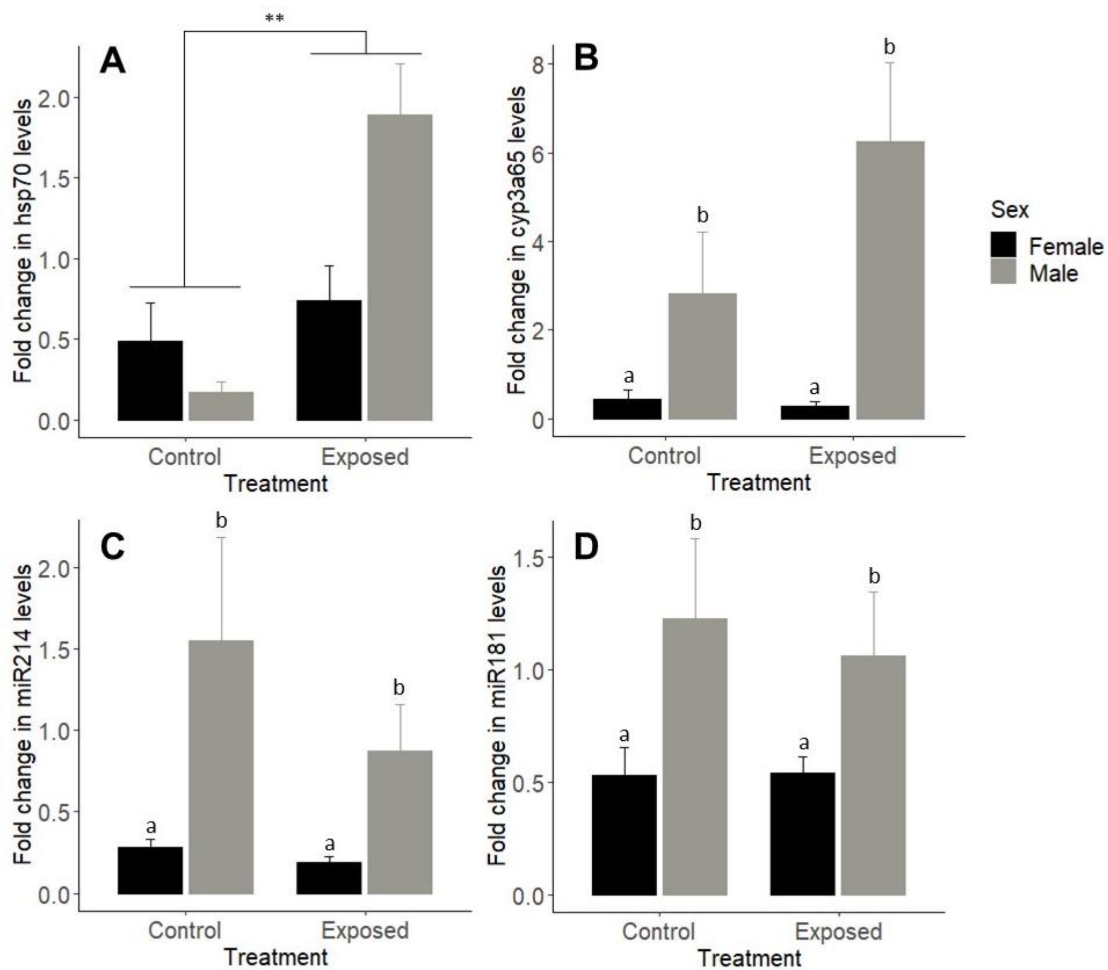

Figure 3.1 Acute exposure to multiple stressors increases *hsp70* transcript levels in zebrafish liver tissue, while other mRNA and microRNA transcripts levels are increased in male liver tissue regardless of exposure.
Mean ± SEM of the fold change of **A)** *hsp70*, **B)** *cyp3a65,* **C)** miR-214, and **D)** miR-181c-5p in liver tissue of zebrafish. Adult zebrafish were either subjected to 0 µg/L venlafaxine, 27 °C, and 100 % dissolved oxygen (control) or to 1 µg/L venlafaxine, 32 °C, and 50 % dissolved oxygen (exposed) for 24 hours. Black bars refer to female fish and grey bars refer to male fish. Asterisks indicate a significant difference between control and exposed treatments, whereas unique letters indicate a significant difference between male and female fish (two-way ANOVA, p < 0.05, n = 10).

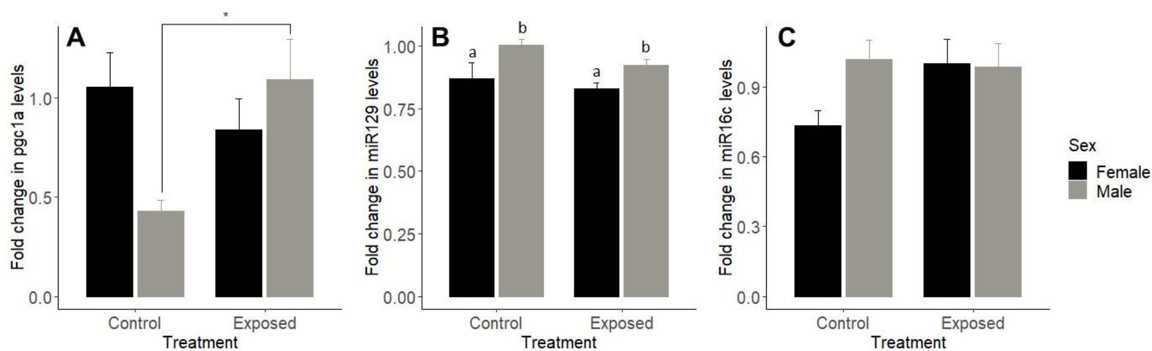

Figure 3.2 Acute exposure to multiple stressors increases *pgc1a* transcript levels in male zebrafish muscle tissue, while miR-129 transcript levels are increased in male muscle tissue regardless of exposure.
Mean ± SEM of the fold change of **A)** *pgc1a*, **B)** miR-129-5p, and **C)** miR-16c-5p in muscle tissue of zebrafish. Adult zebrafish were either subjected to 0 µg/L venlafaxine, 27 °C, and 100 % dissolved oxygen (control) or to 1 µg/L venlafaxine, 32 °C, and 50 % dissolved oxygen (exposed) for 24 hours. Black bars refer to female fish and grey bars refer to male fish. An asterisk indicates a significant difference between two treatments within a sex (Tukey post-hoc test, $p < 0.05$, $n = 5$). Unique letters indicate a significant difference between male and female fish (two-way ANOVA, $p < 0.05$, $n = 10$).

Specific mRNA and miRNA transcripts were measured in liver and muscle tissue of zebrafish exposed to multiple stressors (VFX, increased water temperature, decreased dissolved oxygen) for 21 days. Measurement of these transcript abundances following a chronic exposure allows us to understand part of the long-term molecular processes that occurred in response to these sublethal stressors. Due to the role of liver in the stress and detoxification response, transcripts (*cyp3a65*, *cyp1a*, and *igfbp1a*) and miRNA predicted to regulate them (miR-142a-3p, miR-16c-5p, miR-214, and miR-181c-5p), were measured. In chronically exposed fish, liver *cyp3a65* expression was not affected by treatment but male fish had significantly higher expression than female fish, with 3-fold higher expression in the chronic exposure ($p = 0.000962$, $F = 16.27$; **Figure 3.3A**). Liver expression of *cyp1a* was significantly increased in both male and female fish with a 1.8-fold increase ($p = 0.038$, $F = 5.138$; **Figure 3.3B**). There were no treatment effects on *igfbp1a* liver expression, but male fish had significantly higher expression than female fish (4-fold higher, $p = 0.008$, $F = 9.125$; **Figure 3.3C**). Liver miR-142a-3p expression had a significant interaction between the treatment and sex of the fish with a 970-fold decrease in exposed female fish as compared to control female fish ($p = 6.56\times10^{-6}$, $F = 43.04$; **Figure 3.4A**). Liver miR-16c-5p expression was significantly decreased in

exposed fish with no effect of sex (2.6-fold decrease, p = 0.0494, F = 3.86; **Figure 3.4B**). Liver miR-214 expression was altered by both treatment and sex with control female fish having 4-fold higher expression than exposed female fish (p = 0.02, F = 6.669; **Figure 3.4C**). There were no effects of treatment on miR-181c-5p liver expression (**Figure 3.4D**). Due to the importance of muscle in locomotion and overall fitness of the fish, transcripts (*hsp70* and *pgc1a*) and miRNA predicted to regulate them (miR-16c-5p and miR-129-5p), were measured. In chronically exposed fish, muscle *hsp70* expression was significantly increased 2.5-fold regardless of sex (p = 0.002, F = 13.601**;** **Figure 3.5A**). There was a significant 1.9-fold decrease in muscle *pgc1a* expression in exposed fish regardless of sex (p = 0.0032, F = 8.69; **Figure 3.5B**). Male fish had significantly higher muscle miR-16c-5p and miR-129-5p expression regardless of treatment (miR-16c-5p: 1.8-fold higher, p = 0.001, F = 15.059; miR-129-5p: 1.6-fold higher, p = 0.002, F = 13.775; **Figure 3.5CD**).

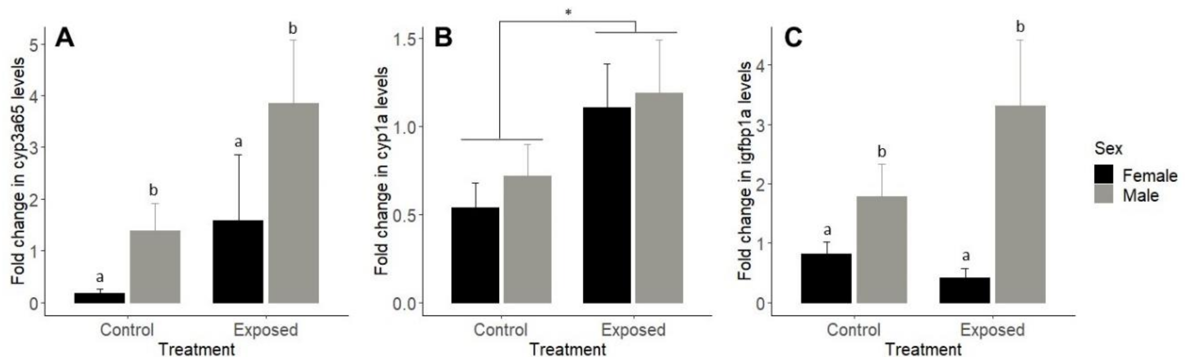

Figure 3.3 Chronic exposure to multiple stressors increases *cyp1a* transcript levels in zebrafish liver tissue, while *cyp3a65* and *igfbp1a* transcript levels are increased in male liver tissue regardless of exposure.
Mean ± SEM of the fold change of **A)** *cyp3a65*, **B)** *cyp1a,* and **C)** *igfbp1a* in liver tissue of zebrafish. Adult zebrafish were either subjected to 0 µg/L venlafaxine, 27 °C, and 100 % dissolved oxygen (control) or to 1 µg/L venlafaxine, 32 °C, and 50 % dissolved oxygen (exposed) for 21 days. Black bars refer to female fish and grey bars refer to male fish. Asterisks indicate a significant difference between control and exposed treatments, whereas unique letters indicate a significant difference between male and female fish (two-way ANOVA, $p < 0.05$, n = 10).

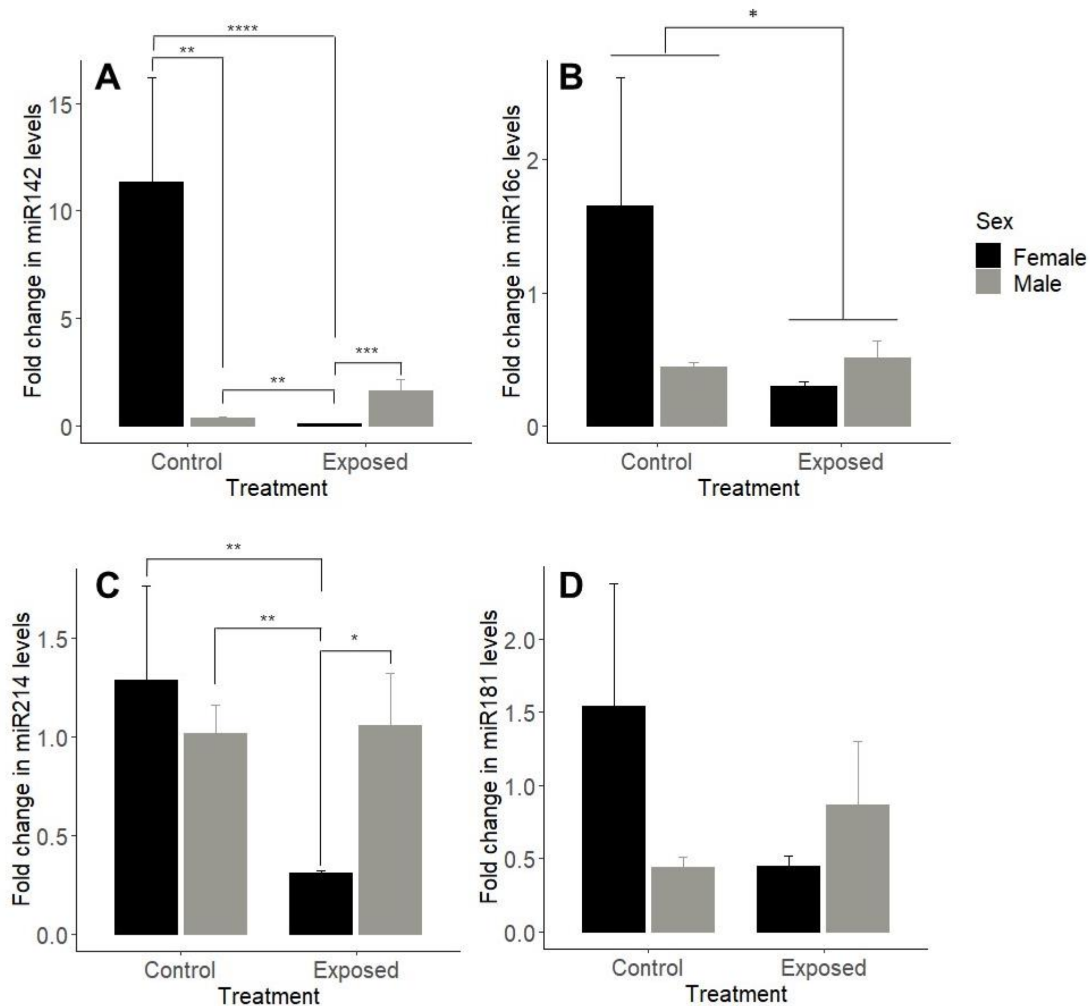

Figure 3.4 Chronic exposure to multiple stressors decreases transcript levels of miR-142, miR-16c, and miR-214 in zebrafish liver tissue.
Mean ± SEM of the fold change of **A)** miR-142-3p, **B)** miR-16c-5p, **C)** miR-214, and **D)** miR-181c-5p in liver tissue of zebrafish. Adult zebrafish were either subjected to 0 µg/L venlafaxine, 27 °C, and 100 % dissolved oxygen (control) or to 1 µg/L venlafaxine, 32 °C, and 50 % dissolved oxygen (exposed) for 21 days. Black bars refer to female fish and grey bars refer to male fish. Asterisks over both bars in one treatment indicate a significant difference between control and exposed treatments (two-way ANOVA, $p < 0.05$, $n = 10$). Asterisks over individual bars indicate a significant difference between treatments within each sex or between sexes within each treatment (Tukey's post-hoc, $p < 0.05$, $n = 5$).

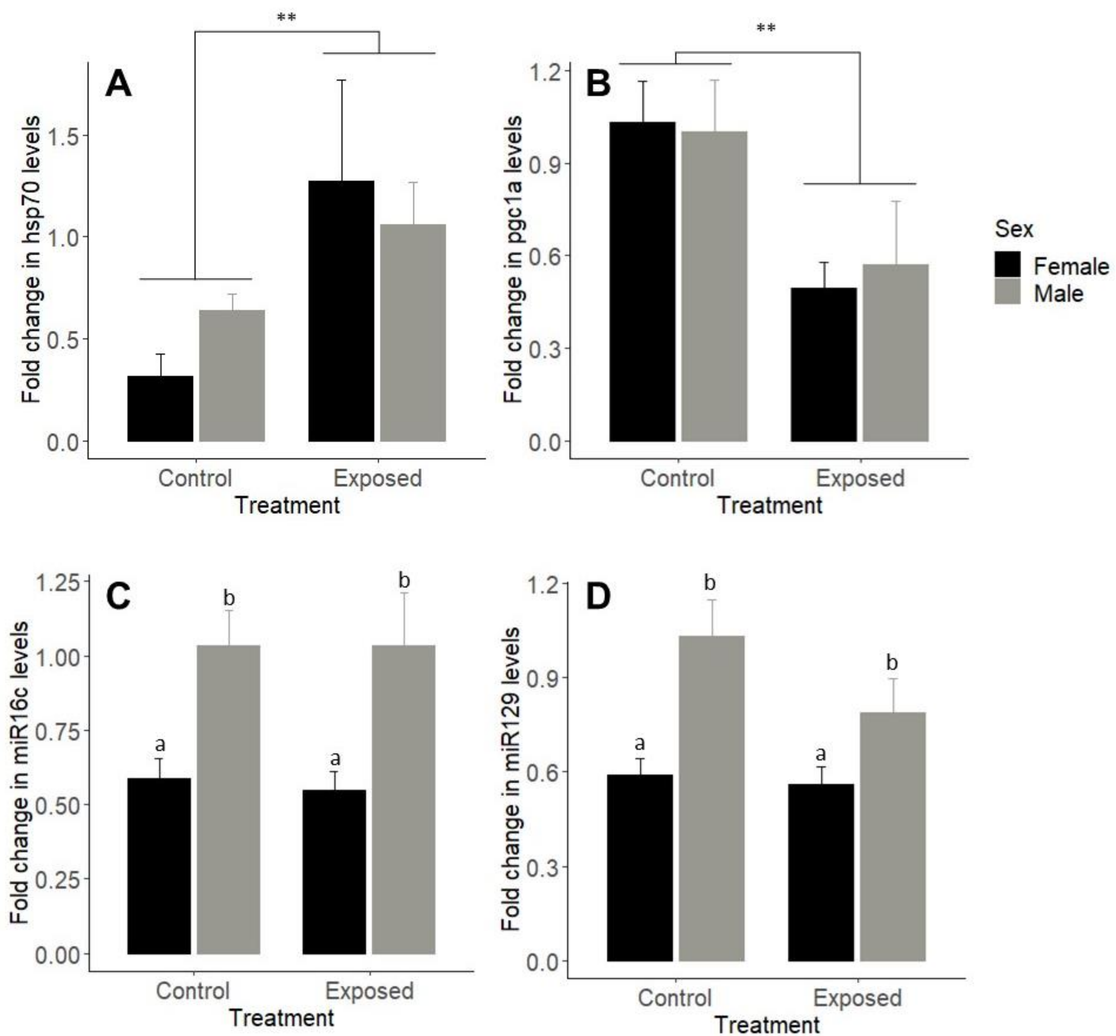

Figure 3.5 Chronic exposure to multiple stressors increases hsp70 transcript levels and decreases pgc1a transcript levels in zebrafish muscle tissue, while miR-16c and miR-129 transcript levels are increased in male muscle tissue regardless of exposure.
Mean ± SEM of the fold change of **A)** *hsp70*, **B)** *pgc1a*, **C)** miR-16c-5p, and **D)** miR-129-5p in muscle tissue of zebrafish. Adult zebrafish were either subjected to 0 µg/L venlafaxine, 27 °C, and 100 % dissolved oxygen (control) or to 1 µg/L venlafaxine, 32 °C, and 50 % dissolved oxygen (exposed) for 21 days. Black bars refer to female fish and grey bars refer to male fish. Asterisks over both bars in one treatment indicate a significant difference between control and exposed treatments, whereas unique letters indicate a significant difference between male and female fish (two-way ANOVA, $p < 0.05$, $n = 10$).

3.4.2.1 Linear regressions between predicted microRNA and mRNA relationships

Since both the mRNA and miRNAs predicted to bind and regulate them were measured in response to this multiple stressor exposure, the presence or absence of inverse relationships between their abundance can be identified using linear regressions. The presence of an inverse relationship between miRNAs and the mRNA they are predicted to target can be used as a preliminary indication that the miRNAs are regulating the mRNA. Of all the predicted miRNA:mRNA pairs tested (**Table 3.1**), only the relationship between miR-16c and *pgc1a* was significantly inversely related in muscle tissue of zebrafish following the acute exposure (**Figure 3.6**). Graphs and statistical results of non-significant linear regressions are found in the supplemental files (Appendix C).

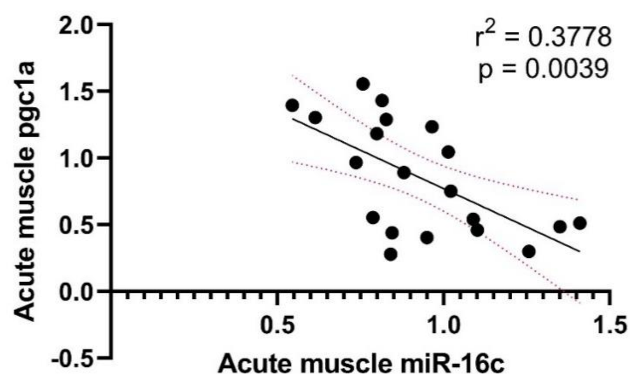

Figure 3.6 Linear regression between relative abundances of miR-16c and *pgc1a* in muscle tissue of zebrafish following acute exposure to multiple stressors.
Adult zebrafish were either subjected to 0 µg/L venlafaxine, 27 °C, and 100 % dissolved oxygen or to 1 µg/L venlafaxine, 32 °C, and 50 % dissolved oxygen for 24 hours. The black line indicates the line of best fit while the dashed red line indicates the 95 % confidence interval. The p-value and correlation coefficient are presented on the graph ($p < 0.05$, $n = 20$).

3.4.3 Protein quantification

A proteomic approach was used to avoid bias in describing the altered phenotype following both acute and chronic exposure to multiple stressors (VFX, increased water temperature, decreased dissolved oxygen. Due to limitations in sample size and only using female for analysis, these data can provide a preliminary understanding of the liver and muscle phenotype following stressor exposure, but conclusions should be made with caution.

3.4.3.1 Proteomics statistics

In liver tissue, 3882 peptides and 1485 unique proteins were identified and in muscle tissue, 7099 peptides and 1115 unique proteins were identified. As expected, the majority of proteins were identified with one unique peptide and were between 20 and 60 kDa (**Figure 3.7**). Equal amounts of peptide spectra matches were identified in each of the ten samples in each tissue which is indicative the lack of measurement bias between samples (**Figure 3.8AB**). Raw files, complete lists of identified peptides and proteins, and run statistics are found in the Supplemental Files (Appendix C).

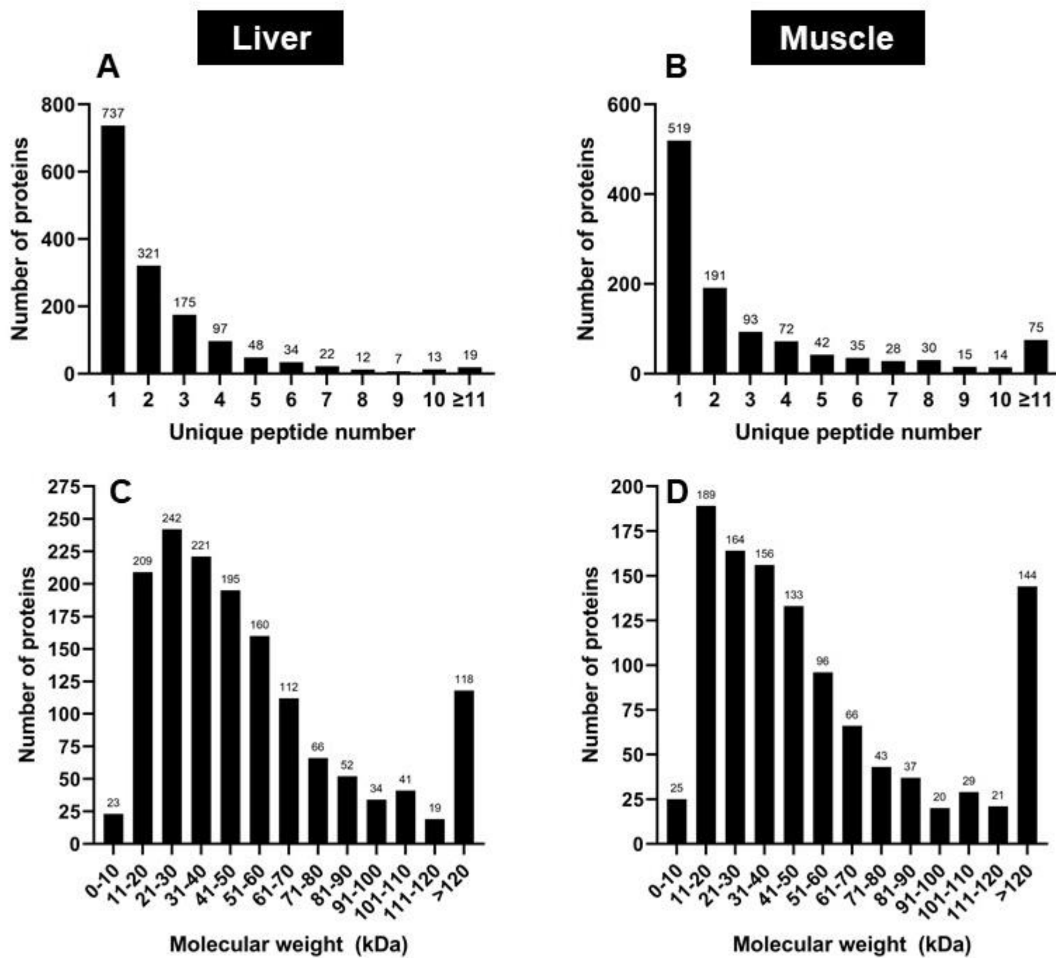

Figure 3.7 Characteristics of proteins identified in liver and muscle tissue.
Frequency distribution of the number of proteins based on the number of unique peptides in **A)** liver and **B)** muscle tissue. Frequency distribution of the number of proteins based on the molecular weight (kDa) in **C)** liver and **D)** muscle tissue.

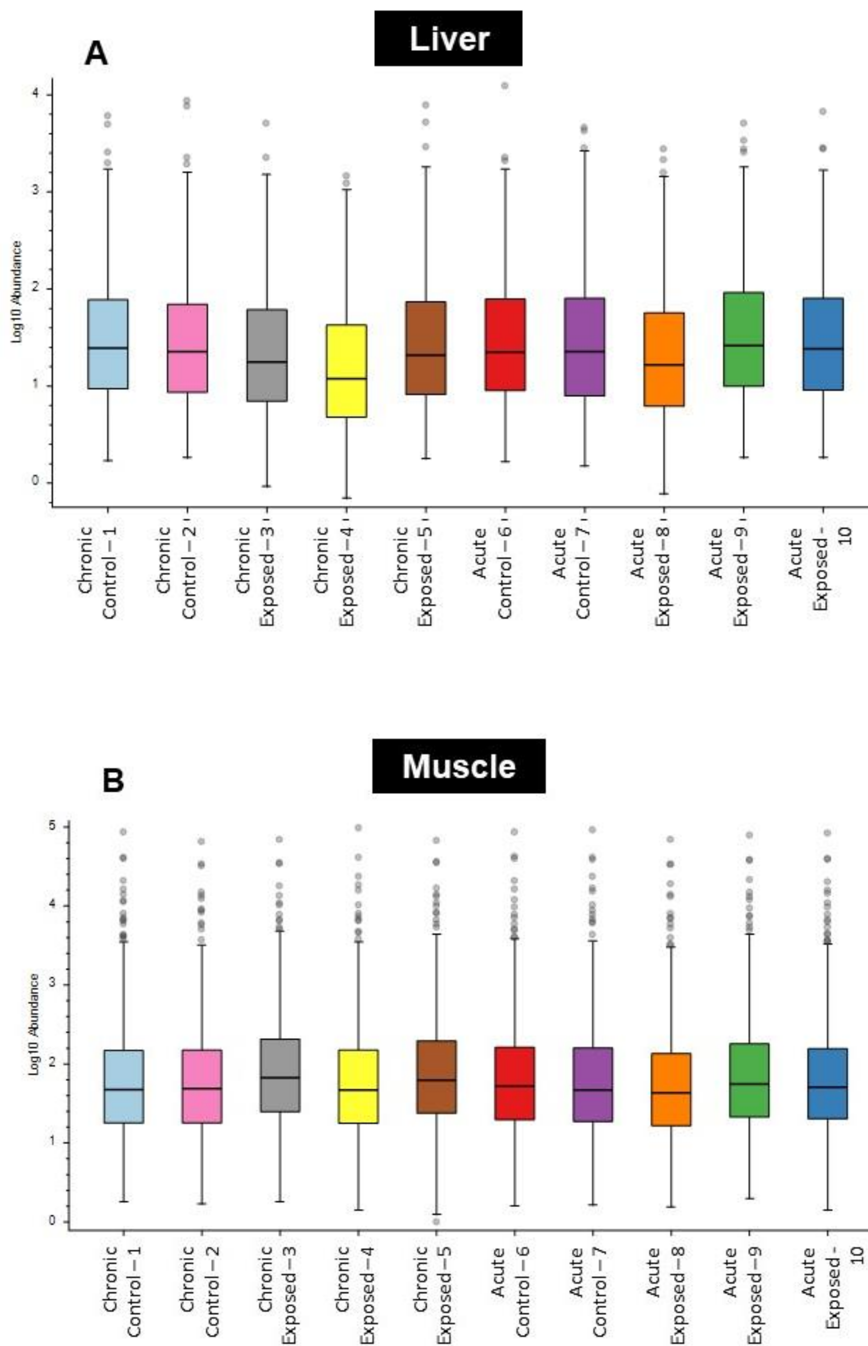

Figure 3.8 Distribution of the log10 peptide spectrum matches intensities.
Box plots of the transformed abundances of peptide spectrum matches for **A)** liver and **B)** muscle tissue samples. Treatment, length of exposure, and sample number are indicated on the x-axis.

3.4.3.2 Differences in protein abundances

Differences between individual protein abundances in zebrafish from the control treatments (acute and chronic controls combined) and chronic exposure treatment were identified in liver and muscle tissue. It is important to note that these differences were identified only in female tissues and with limited power. In liver tissue of female fish, there were three proteins which were downregulated in the chronic exposure (**Figure 3.9**). These were vitellogenin 2 and 3 (**Figure 3.9AB**) which decreased 4.4-fold and 3.5-fold respectively, and Si:dkeyp-46h3.3 (a carbohydrate-binding protein; **Figure 3.9C**) which decreased 3.3-fold in chronically exposed fish. In muscle tissue of female, there was one protein that was upregulated, protein Unc45b, a Hsp90 co-chaperone, which was increased with a fold-change of 2 in chronically exposed female muscle tissue (**Figure 3.10**).

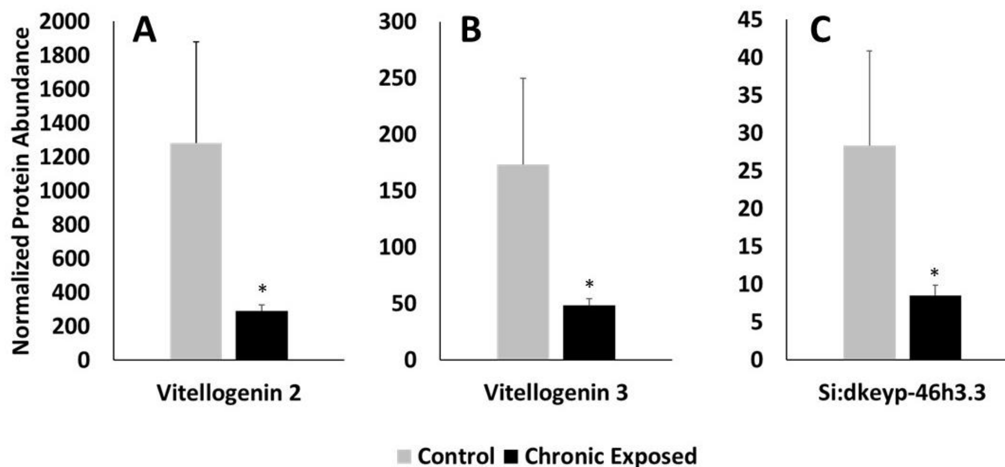

Figure 3.9 Downregulated protein abundances in female liver tissue.
Mean ± SEM of normalized protein abundance quantified using tandem mass tagged-labeled mass spectrometry. Asterisks indicate a significant difference between treatments (limma; $n_{control} = 4$, $n_{exposed} = 3$; $p < 0.05$).

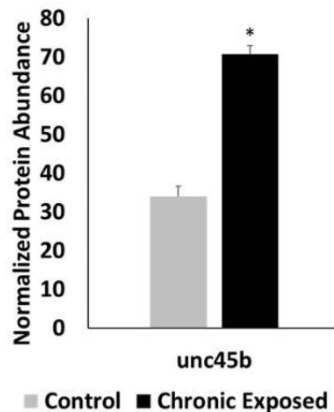

Figure 3.10 Upregulated protein abundance in female muscle tissue.
Mean ± SEM of normalized protein abundance quantified using tandem mass tagged-labelled mass spectrometry. Asterisks indicate a significant difference between treatments (limma; $n_{control} = 4$, $n_{exposed} = 3$; $p < 0.05$).

3.4.3.3 Predicted binding sites and relationships between miRNA and proteins

In silico target prediction between miRNA measured and significantly altered proteins was performed using miRanda. Target prediction only occurred for Vitellogenin 2 (Vtg2), Vitellogenin 3 (Vtg3), and Unc45b, as Si:dkeyp-46h3.3 does not have a known 3′UTR. Two predicted relationships were identified, one between miR-214 and Vtg2 and one between miR-181b and Vtg3 (**Table 3.4**). These predicted miRNA:protein pairs were then used to perform linear regressions to determine if there were significant inverse relationships between the normalized relative expression of the miRNA and abundance of the protein which would be a preliminary indication that the miRNA are regulating the proteins as predicted. Neither predicted relationship was significantly inversely related ($p < 0.05$; Supplemental File; Appendix C).

Table 3.4 Binding sites between microRNA and proteins.
Binding sites identified by miRanda between miRNA (query) and the 3′ untranslated region of the mRNA which codes for the respective protein (ref). Total scores and total energy indicate likelihood of binding occurring (higher score and lower energy indicate higher likelihood).

Binding pair	Binding sites	Total score	Total energy (kCal/mol)
miR-214 Vtg2	Query: 3' gacgGACAGACACGGACGACa 5' :| |||| :|||||| Ref: 5' aacaTTCTCTGAATCTGCTGt 3'	141.00	-18.18
miR-181b-5p Vtg3	Query: 3' gggUGGCUGU----CGUUACUUACaa 5' ||:||||	|||||||| Ref: 5' taaACTGACAACGTGAAATGAATGaa 3'	142.00	-17.57

3.4.3.4 Protein pathway analysis

Gene set enrichment analysis analyzes abundances of significant and non-significantly altered genes or proteins and indicates pathways that are enriched in each treatment. This is a robust tool which analyzes large datasets in an unbiased manner and makes use of all altered proteins which can be useful to measure changes in trends that are not driven by significantly altered proteins. It is important to note the limitation in power for this analysis as there were low n numbers (liver control = 3, liver exposed = 6, muscle control = 4, liver exposed = 6). When analyzing the pathways in the liver tissue, no significantly enriched zebrafish gene ontology gene sets were found. In muscle tissue of control fish, oxidoreductase activity was significantly enriched. In muscle tissue of exposed fish, 44% of the enriched gene sets were responding to steroids/estrogens, 28% were responding to chemicals, 17% were involved in cell movement, and 11% were in other pathways (**Table 3.5**).

Table 3.5 Significantly enriched gene sets in female zebrafish muscle exposed to multiple stressors.
ES refers to the enrichment score, whereas NES refers to the normalized enrichment score. Results are those with a false discovery rate (FDR) q-value less than 0.25. Gene sets are zebrafish gene ontology processes.

Gene Set	ES	NES	FDR q-value
RESPONSE TO LIPID	0.86	1.77	0.078
RESPONSE TO STEROID HORMONE	0.88	1.76	0.045
RESPONSE TO ESTROGEN	0.88	1.75	0.033
RESPONSE TO HORMONE	0.88	1.74	0.031
CELLULAR RESPONSE TO ORGANIC SUBSTANCE	0.88	1.68	0.085
CELLULAR RESPONSE TO CHEMICAL STIMULUS	0.71	1.66	0.095
CELLULAR RESPONSE TO LIPID	0.88	1.66	0.083
CELLULAR RESPONSE TO ORGANIC CYCLIC COMPOUND	0.88	1.65	0.080
CELLULAR RESPONSE TO STEROID HORMONE STIMULUS	0.88	1.65	0.076
CELLULAR RESPONSE TO ENDOGENOUS STIMULUS	0.88	1.65	0.068
CELLULAR RESPONSE TO ESTROGEN STIMULUS	0.88	1.65	0.063
RESPONSE TO ORGANIC SUBSTANCE	0.73	1.64	0.061
CELLULAR RESPONSE TO HORMONE STIMULUS	0.88	1.64	0.057
RESPONSE TO ENDOGENOUS STIMULUS	0.79	1.62	0.075
RESPONSE TO ORGANIC CYCLIC COMPOUND	0.79	1.61	0.081
RESPONSE TO CHEMICAL	0.60	1.55	0.190
CONTRACTILE FIBER PART & SARCOMERE & CONTRACTILE FIBER & MYOFIBRIL	0.87	1.54	0.199
CELL MIGRATION	0.70	1.53	0.226

3.5 Discussion

It is important to determine the sublethal effects of multiple current and future anthropogenic stressors on fish due to their potential to be exacerbated in combination with each other and due to the lack of understanding of the molecular response. The specific objective of this study was to determine the effects of acute and chronic exposure to multiple stressors (VFX, increased water temperature, decreased dissolved oxygen) on miRNA, mRNA, and proteins in zebrafish liver and muscle tissue. Altogether, I found that miRNA (miR-142a-3p, miR-16c-5p, miR-214, miR-129-5p), mRNA (hsp70, cyp3a65, cyp1a, pgc1a), and proteins (Vtg2, Vtg3, Si:dkeyp-46h3.3, Unc45b) were altered in liver and/or muscle tissue exposed to multiple stressors. Additionally, the sex of the fish affected the responses of the measured miRNA and mRNA.

It was hypothesized that due to the exposure to increased temperature, decreased dissolved oxygen, and venlafaxine, stress-responsive mRNA (hsp70, cyp3a65, pgc1a, igfbp1a, cyp1a) levels would be increased in liver and muscle tissue. Transcript levels of hsp70, cyp1a, and pgc1a were increased as expected. It is unknown which of the three stressors is producing the hsp70 response, since heat, hypoxia, and VFX can increase hsp70 transcript levels, but it reinforces the understanding that zebrafish were experiencing cellular stress and that the cellular chaperone response was required following this multiple stressor exposure (Airaksinen et al., 2003; Ekambaram et al., 2016; Yu et al., 2010). This response was still observed in the muscle tissue following chronic exposure indicating a persistence of cellular stress. Liver transcript levels of cyp1a increased following chronic multiple stressor exposure but there was no effect of exposure on the other cytochrome P450 (cyp3a65) transcript measured. This increase in cyp1a transcript levels could be due to its induction by both hypoxia and VFX exposure (Rahman & Thomas, 2012; Saad et al., 2016). The lack of transcript response to the exposure could be due to the large differences in cyp3a65 transcript levels between male and female fish or due to an increase in enzyme activity not reflected at the transcript level. Transcript levels of pgc1a were increased as expected but only in acutely exposed male fish and not female fish. Furthermore, muscle pgc1a transcript levels were decreased in both male and female chronically exposed fish, which was not expected. Since pgc1a levels are indicative of functional impacts on the biogenesis of mitochondria, the increase in pgc1a in males may demonstrate the ability for male fish to respond to the increased energy demands of the multiple stressor exposure (H. Liang & Ward, 2006). The decrease in pgc1a transcript levels following chronic exposure to multiple stressors could indicate that there are long-term energetic impacts and that tradeoffs are being made to

conserve metabolic resources. Lastly, liver igfbp1a transcript levels were not increased as would be expected following exposure to hypoxia; however, this could be due to this only being a mild exposure to hypoxia (Maures & Duan, 2002). Therefore, the transcript response indicates that molecular pathways are responding to the thermal, hypoxic, and contaminant stress.

Of the three miRNA that were altered following exposure to increased temperature, decreased dissolved oxygen, and venlafaxine (miR-142a-3p, miR-16c-5p, miR-214 in liver of chronically exposed fish) none were inversely related to their predicted mRNA targets. This does not mean that these miRNAs are not binding to the mRNA targets and post-transcriptionally regulating them as inverse relationships would only be measured in mRNA if target-mediated decay were to occur (O'Brien et al., 2018). These miRNAs could be binding to the measured transcripts and blocking protein translation or binding to other transcripts as each miRNA can bind to several mRNAs (O'Brien et al., 2018). Even though there are many unknowns about the downstream impacts of these altered miRNA transcript levels, knowing that these miRNAs are responding to the exposure to multiple anthropogenic stressors furthers our understanding of the molecular regulation of the stress response.

The functional impacts of miR-142a-3p, miR-16c-5p, and miR-214 being altered in the liver tissue of chronically exposed fish is unknown; however, due to the conservation of binding of miRNA and targets between organisms, the potential function of these miRNA can be elucidated by comparison to other exposures. Liver transcript levels of miR-142a-3p were decreased in female fish. Functionally, this miRNA could be part of the hypoxic response, due to the alteration of miR-142 in the livers of bass exposed to acute hypoxia (J. L. Sun et al., 2020). MiR-142 could also be responding to heat stress as it has been labelled a thermo-miR in mammals due to its response to changes in temperature (Wong et al., 2016). Transcript levels of miR-16c-5p have not been measured in liver tissue in response to other stressors therefore the potential function of the decrease in both male and female fish is unknown. Transcript levels of miR-214 were decreased in livers female fish and is also known to respond to hypoxic stress in bass (J. L. Sun et al., 2020). Additionally, miR-214 targets and regulate glycogen synthase, AMPK, and lipid metabolism in multiple fish species (Østbye et al., 2020; Zhang et al., 2017, 2019). Therefore, the miRNAs that were decreased in response to multiple stressor exposure have potential functional roles in regulating the thermal, hypoxic and/or metabolic response but further study is required to validate this response.

The liver and muscle proteome of acutely and chronically exposed fish was measured; however, due to only being measured in female fish and the technical issues resulting in low power, minimal conclusions can be made about the phenotypic response to multiple stressors. In liver tissue, two of the three proteins that decreased in abundance were vitellogenin proteins. Typically, vitellogenin is a marker of estrogenic effects of exposure, indicating that this multiple stressor exposure potentially has anti-estrogenic effects on female fish (Martyniuk et al., 2020). Decreased vitellogenin can cause reduced egg production, which can impact fecundity and have negative population effects (Bugel et al., 2011). Luu et al (2021) demonstrated that this type of multiple stressor exposure can impact miRNA and mRNA levels in future generations, and the data presented here tentatively indicates that there may be population impacts, although further investigation is needed. In muscle tissue, there was an increase in the abundance of unc45b, an Hsp90 co-chaperone. This is indicative of a response to hypoxic or general cellular stress which sheds light on the sublethal response to the multiple stressor exposure (Barman et al., 2021). Pathway analysis of the muscle tissue indicated a response to xenobiotics whereas the liver is the site of detoxification; however, this could be useful in development of biomarkers as muscle tissue is much easier to collect using a non-lethal biopsy approach (Henderson et al., 2016). The lack of protein response could be due to the lack of time in the acute exposure to allow for a measurable response at the protein level as other studies have allowed for at least a week to show a quantifiable protein response and/or could be due to the low number of fish used in the proteomic analysis as 103 proteins were differentially abundant in liver tissue of zebrafish exposed to a contaminant stressor (n = 9) (Léger et al., 2021; Simmons et al., 2019). Also, as it is clear from the RNA data, there are many differences in the response to the multiple stressor exposure between sexes so it would be expected that if a proteomic approach was used in male fish, then differences would be observed as well. Future analysis can pool male tissues to ensure that there is sufficient mass for proteomic analysis and to characterize the proteomic response in males in response to multiple stressors. Therefore, there are many limitations to the proteomic data collected, but preliminary data indicate impacts of these multiple stressors on fecundity, the cellular stress response, and detoxification.

Transcript levels of cyp3a65, igfbp1a, miR-214, miR-181c-5p, miR-129-5p, and miR-16c-5p were higher in male fish when compared to female fish, regardless of exposure. Additionally, transcript levels of pgc1a in muscle tissue of acutely exposed fish, and miR-142a-3p and miR-214 in liver tissue of chronically exposed fish were dependent on both sex and exposure. The sex-specific response in

transcript levels provides evidence of a sexually dimorphic response to this multiple stressor exposure, which could be due to differences in the cortisol responses between male and female zebrafish that have previously been characterized or due to impacts that the stressors have on reproductive pathways, as indicated by the impact on the abundance of vitellogenin proteins in female fish (Rambo et al., 2017). Additionally, since both pgc1a and miR-214 are associated with metabolic impacts, future study can address whether female zebrafish have different basal metabolism or metabolic impacts of exposure. Therefore, the transcript response measured following exposure to multiple stressors indicates that male and female fish are impacted uniquely.

Though zebrafish are a vertebrate miRNA toxicity model (Ahkin Chin Tai & Freeman, 2020) they are also very tolerant to changes in temperature and dissolved oxygen levels as they are a tropical fish which experience fluctuations in environmental conditions (Morgan et al., 2019). The stressors measured in this study (increased water temperature, decreased dissolved oxygen, and venlafaxine) are also present in temperate environments which are home to more stenothermal and species that are not as tolerant to environmental stressors (Poesch et al., 2016). Salmonids, such as brook trout, are present in effluent impacted streams and are endangered in anthropogenically disturbed environments (Stranko et al., 2008). Therefore, it is critical to expand the study of the impact of stressors on miRNA and mRNA in salmonids as well.

In conclusion, through the measurement of select miRNA and mRNA transcripts in zebrafish liver and muscle tissue in response to venlafaxine, increased water temperature, and decreased dissolved oxygen, the molecular response to emerging anthropogenic stressors has been further characterized. The impacts of these stressors on initiating cell chaperone, decontamination, and metabolic responses at the transcript level are now known. Specific miRNAs that respond to these stressors were identified and can be further studied to understand their functional role in the stress response.

3.6 References

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Chapter 4

Responses of microRNA and predicted mRNA and enzymatic targets in liver of two salmonids (*Oncorhynchus mykiss* and *Salvelinus fontinalis*) following air exposure

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***Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 256, pp. 110646. 2021.**

4.1 Summary

The acute stress response is well-characterized, with rainbow trout as a teleost model for physiological and molecular responses. Air exposure, which stimulates an acute stress response, modulates liver microRNAs in rainbow trout; however, these highly conserved non-coding RNAs that bind to mRNA and repress translation, have never been measured in brook trout and it is unknown how miRNA expression responds following air exposure in this less studied salmonid. My objective was to characterize the effects of air exposure on rainbow and brook trout liver miRNA expression, as well as the mRNA expression and enzyme activity that the miRNAs are predicted to target. Brook and rainbow trout were sampled pre- and 1-, 3-, and 24-h post- a three-minute air exposure. Plasma cortisol, glucose, and lactate were measured. Relative expression of miR-21a-5p, miR-143-3p, let-7a-5p and relative expression and enzyme activities of five predicted targets (pyruvate kinase, glucokinase, citrate synthase, cytochrome c oxidase, and catalase) were measured in liver. Rainbow and brook trout both had increases in plasma cortisol and lactate, while only rainbow trout had significant post-stress increases in plasma glucose. Furthermore, both trout species had increased miR-143-3p and miR-21a-5p relative expression 24-h post-stress. Four of the five enzymes measured had altered activity following stress. Brook trout miRNAs had inverse relative expression with relative catalase mRNA expression and cytochrome c oxidase enzyme activity, but no relationship was found in rainbow trout. Therefore, I have further characterized the transcriptional and enzymatic response to air exposure in two salmonids.

4.2 Introduction

Salmonids are a critical species for study due to their role in aquaculture, fisheries, and for the ease of use in the lab (Barton, 2000; Pennell and Barton, 1996). Specifically, rainbow trout (*Oncorhynchus mykiss*) have been used as biological models for a variety of subjects, including physiology and molecular biology, and most recently as a model for microRNA research in salmonids (Craig, 2013; Kuc et al., 2017; Ma et al., 2019b; Mennigen and Zhang, 2016). However, this research is often not extended to other salmonids, such as brook trout (*Salvelinus fontinalis*). Therefore, there is a gap in research measuring physiological and molecular responses in both rainbow and brook trout.

When fish are exposed to an acute stressor, such as air, there is a general, conserved adrenergic stress response, where catecholamines are released within 1–3 min and where cortisol is released within minutes to hours (Balasch and Tort, 2019; Tort, 2013; Wendelaar Bonga, 1997). Cortisol and the catecholamines target the gills, intestines, and liver and function to increase oxygen uptake and transfer, mobilize energy via glycogenolysis and gluconeogenesis, and decrease energy for growth, reproduction and the immune response (Cherrington, 1999; Mommsen et al., 1999; Nonogaki, 2000; Wendelaar Bonga, 1997). The increases in plasma cortisol and subsequent increases in plasma glucose and lactate are characteristic of the acute stress response. This response is well conserved and well-studied in both rainbow and brook trout however, molecular mechanisms are still being understood (Barton, 2002, 2000; Martínez-Porchas and Martínez-Córdova, 2009; Pottinger, 2010).

A facet of molecular regulation occurs through microRNA (miRNA), small, non-coding RNA that bind to the 3′ untranslated region (UTR) of mRNA and block translation from occurring (Bartel, 2004). Therefore, there is an inverse relationship between miRNA abundance and the target mRNA/protein abundance. Though miRNA have been measured in many fish species in response to stressors (Best et al., 2018), their role in the acute stress response has only begun to be studied. Following air exposure, three microRNAs (omy-miR-21a-3p, omy-let-7a-5p, and omy-miR-143-3p) decreased in rainbow trout liver and head kidney tissue and increased in circulation (Cadonic et al., 2020). Due to the known impact of air exposures on miRNA in the liver of rainbow trout, these responses can be further studied in brook trout.

Furthermore, since there is a known metabolic impact of acute stress on the liver, and three miRNAs, ssa-miR-21a-5p, ssa-let-7a-5p, and ssa-miR-143-3p, are predicted to target transcripts of metabolic enzymes in the liver (*in silico* predictions performed by authors using miRanda), the

miRNA, mRNA, and enzyme response can be studied following air exposure to expand our understanding of the transcriptional and enzymatic response to air exposure. These three miRNAs are predicted to target citrate synthase (an indicator of aerobic metabolism), pyruvate kinase (last step of glycolysis), glucokinase (initial enzyme in glycolysis), cytochrome c oxidase (last step of the electron transport chain), and catalase (part of the antioxidant defense system) (Michiels et al., 1994). Many of these transcripts are altered in the liver following acute stress, either at the transcript and/or enzymatic level (Gravel and Vijayan, 2007; Ings et al., 2012; López-Patiño et al., 2014; Wiseman et al., 2007; Wiseman and Vijayan, 2011). Therefore, miRNA could be part of the regulation of the transcript and enzyme response to acute stress. Currently, it is unknown what the miRNA, mRNA, and enzyme activity response following air exposure is in the liver of rainbow and brook trout, and if abundances of miRNA are inversely related to abundances of predicted targets.

Our objective was to characterize the liver miRNA, mRNA, and enzyme activity response to air exposure in rainbow and brook trout. Rainbow and brook trout were sampled prior to and 1, 3, and 24 h post exposure to air for three minutes. Plasma cortisol, glucose, and lactate were measured. Relative expression of miR-21a-5p, let-7a-5p, and miR-143-3p, and five transcripts (citrate synthase, pyruvate kinase, glucokinase, cytochrome c oxidase, and catalase) they are predicted to target were measured in the liver. Liver enzyme activities of citrate synthase, pyruvate kinase, glucokinase, cytochrome c oxidase, and catalase were also measured. It was hypothesized that the liver miRNA, mRNA, and enzyme activity response to air exposure would be altered in a time dependent manner post-stress and inverse relationships would exist between miRNAs and predicted targets.

4.3 Methods

4.3.1 Animal husbandry

Immature rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) of mixed sex were procured from a single source, Silver Creek Aquaculture (Erin, ON, Canada). All experimental procedures were approved and conducted per the University of Waterloo and the Canadian Council of Animal Care guidelines (Animal Utilization Project Protocol #40315). Fish were acclimated for 6 to 12 months in the University of Waterloo Aquatic Facility (Waterloo, ON, Canada) in one tank per species at a maximum density of 8 g/L. Trout were maintained at a 12 h:12 h light-dark cycle and fed three times weekly with EWOS Vita (Floating Complete Fish Food for Salmonids) to satiety. Water was maintained at 14 °C, pH ~8.5, ~2000 µS, in well-aerated water within 680 L tanks. Rainbow trout had a mean length of 25.8 ± 0.29 cm, and a mass of 179 ± 7 g. Brook trout had a mean length of 27.2 ± 0.53 cm, and a mass of 224 ± 12 g. There were no significant differences in lengths or weights between treatments for either species.

4.3.2 Experimental setup and tissue collection

Twenty-four fish of each species were housed in a 680 L tank (one species per tank). Rainbow and brook trout were sampled on the same day (rainbow trout sampling began at 9:00 and brook trout sampling began at 13:00). Six fish of each species were sampled prior to the air exposure (control treatment; **Figure 4.1**). The remaining eighteen fish of each species were simultaneously held out of water in scoop nets for three minutes to stimulate the adrenergic stress response before being returned to the same tank. One-, three-, and twenty-four-hours following air exposure, six trout of each species were sampled at random from the tank. It is important to note that tank effects, effects of removing fish from the same tank, and other interindividual factors were not controlled for in this experimental design however this design was used to stimulate the acute stress response in the trout and measure their response over time. The six fish in each treatment group were sampled quickly to ensure fish were collected as close to specified timepoints as possible (max time between first and sixth fish sampled was 17 min, ex. control rainbow trout were sampled within 17 min of each other whereas fish in the 1 h post-stress group were collected 1 h to 1 h and 6 min following the three-minute air exposure). Briefly, two people collected the fish, placed them in a buffered MS-222 solution (0.5 g/L MS-222 in 1 g/L $NaHCO_3$) until fish lost equilibrium and opercular movement was no longer observed, collected weights and lengths, passed the fish onto two blood samplers, who then passed

the fish onto two liver samplers. Blood was collected within one minute of euthanasia with a 23 G needle and 1 mL syringe pre-treated with 0.5 M EDTA from the caudal vein and stored in a 2 mL microcentrifuge tube containing 10 µL of 0.5 M EDTA to prevent coagulation. Blood was stored on ice for less than one hour until centrifugation at 1300 ×g for 10 min at 4 °C to collect plasma. Plasma was aliquoted into 200 µL and stored at −80 °C for cortisol, lactate, and glucose analysis. Livers were sampled, flash frozen in liquid nitrogen, then ground in liquid nitrogen and stored at −80 °C until used for miRNA, mRNA, and enzyme analysis. Only five brook trout livers were available for analysis in the 24-h post-stress treatment group, as one of the livers (brook trout #22) was lost in the grinding process.

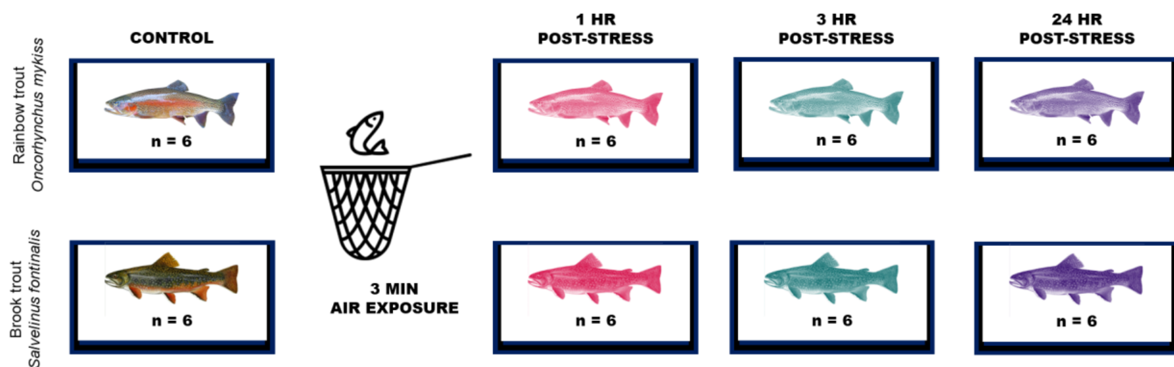

Figure 4.1. Experimental design.
Plasma and liver samples were collected from twenty-four rainbow trout and twenty-four brook trout. Six fish of each species were sampled prior to three minutes air exposure, and six fish were sampled at each timepoint (1-h, 3-h, or 24-h) following air exposure.

4.3.3 Plasma cortisol, glucose, and lactate

To measure plasma cortisol, plasma samples were thawed on ice, diluted by a factor of ten, and cortisol was quantified using the Cortisol Saliva ELISA kit (TECO, Switzerland). To measure plasma lactate and glucose, 200 µL plasma was thawed on ice, samples and standards (0–20 mM lactic acid or glucose) were deproteinized by adding ice cold 8% perchloric acid (PCA) to a final concentration of 0.4 M PCA, vortexing for 30 s, incubating on ice for 10 min, and centrifuging for 5 min at 10,000 × *g* at 4 °C. The supernatant was brought to a pH between 6.5 and 8.0 using 0.4 M potassium hydroxide and 0.8% PCA, incubated on ice for at least 10 min, and centrifuged at 10,000 × *g* for 10

min at 4 °C. To quantify lactate, the supernatant of samples and standards was added to a 96 well plate in triplicate and the reaction cocktail (320 mM glycine, 320 mM hydrazine monohydrate, 2.4 mM NAD^+, 2 U/mL lactate dehydrogenase) was added to a 96 well plate. A kinetic assay at 340 nm was run for an hour until the reaction plateaued and an endpoint reading was collected to quantify lactate by comparing to the standard curve (Bergmeyer, 1974). The lactate method detection limit ranged from 0.18–0.29 mmol/L. To measure plasma glucose, the supernatant of samples and standards was added to the 96 well plate in triplicate and the reaction cocktail (50 mM HEPES pH 7.4, 10 mM $MgCl_2$, 100 mM KCl, 1 mM NADP, 1 mM ATP, 1 U/mL leuconostic glucose-6-phosphate dehydrogenase, 1 U/mL hexokinase) was added to a 96 well plate. A kinetic assay at 340 nm was run for 20 min until the reaction plateaued and an endpoint reading was collected, and glucose was quantified by comparing to the standard curve (Bergmeyer, 1974).

4.3.4 Liver microRNA & mRNA target selection and primer design

MicroRNA targets were selected based on the study conducted previously with the exception of miR-21a-5p, which originates from the same pre-miRNA as miR-21a-3p but is a different mature miRNA which targets the transcripts of interest measured in this study (Cadonic et al., 2020) (**Table 4.1**). Due to the conservation of fish miRNA, the same sequences were used for both brook and rainbow trout. These miRNAs were input into the miRanda target prediction tool and compared against rainbow trout and brook trout 3′UTRs to identify potential targets. Of these targets in one or both species, transcripts for enzymes that are known to be altered in an acute stress response were selected (hexokinase, pyruvate kinase, citrate synthase, cytochrome c oxidase, and catalase; **Table 4.2**). There is no past research indicating that acute air exposure will alter citrate synthase enzyme activity or transcript levels however it was chosen because it is targeted by one of the microRNAs measured.

Table 4.1 MicroRNA and mRNA primers.
The primers used for RT-qPCR are listed with the species they were measured in and their accessions. Arctic char (*Salvelinus alpinus*) sequences were used in place of brook trout (*Salvelinus fontinalis*) sequences where there was no brook trout sequence available. All sequences are listed in the 5′ to 3′ direction.

Trout Species	Target	Accession	Forward sequence	Reverse sequence
Brook & Rainbow	ssa-miR-21a-5p*	MIMAT0032533	TAGCTTATCAGACTGGTGTTGACT	GAATCGAGCACCAGTTACGC
Brook & Rainbow	omy-miR-143-3p & ssa-miR-143-3p**	MIMAT0032366 **	TGAGATGAAGCACTGTAGCT	
Brook & Rainbow	omy-let-7a-5p & ssa-let-7a-5p	MIMAT0032688	TGAGGTAGTAGGTTGTATAGTT	
Rainbow	Citrate synthase	XM_021610150.1	GGCCAAGATTTACCGCAACC	TGAGGTACAGTCGCATGAGC
Brook		XM_023996851.1	CGTGGTCACCATGCTGGATA	TGCAACGCATGGCAGTTTAG
Rainbow	Catalase	XM_021557350.1	AGAAATGGTTTCAGGCCATCG	TCCTTCCCAGGAAACAGTGG
Brook		XM_023984740.1	AAGTTTTGTTTGCACGATGGCT	TTCAGCTTGTCCCCTACCG
Rainbow	Cytochrome c oxidase 5B	XM_021590664.1	CCACGAGCTGCCACACTAAA	CTGTCTAACCCCACTTCACCC
Brook		XM_023984302.1 XM_023969754.1 XM_023987455.2***	CCTTCCGCGATAGAAGGTCC	GGCTCCTAGGCATAAGCAGG
Rainbow	Pyruvate kinase	XM_021622264.1	AGAGTCGCCAGGGAGTACAT	GATCTCTATGCCCAGGTCGC
Brook		XM_024002679.1	TCTGGGATGAACATCGCTCG	AGGCCTCACGCACATTCTTA

Trout Species	Target	Accession	Forward sequence	Reverse sequence
Rainbow	Glucokinase	NM_001124249.2	GATACTGTGC GTCAGCTCCTT	GATCTGCTCC ACCATGATG ACT
Brook		XM_024001499.2	TAGCCTGTAA GATGGCAGCG	CAGAGGCGG ATCACCCTAG A

*Cadonic et al., 2020 measured omy-miR-21a-3p as identified by Juanchich et al. (2016), but this sequence is not fully conserved with ssa-miR-21a-5p although similar (see supplemental methods for alignment; Appendix D)
**ssa-miR-143-3p has an additional C at the 3′ end of the sequence that is not present in the omy-miR-143-3p sequence. The shorter omy-miR-143-3p sequence was used for RT-qPCR.
***These Arctic char CCO5B accessions were not used for primer design but rather to align to the *Salvelinus fontinalis* mitochondrial genome. The conserved portion of the *S. fontinalis* mitochondrial genome was input for primer design (see supplemental methods for the sequence/alignment; Appendix D)

Table 4.2 Predicted microRNA binding sites on stress-related transcripts.
The microRNA sequences listed in the heading are the query, whereas the mRNA sequences are the reference (ref). The species listed is the organism the transcript originates from. miRanda binding scores and other details can be found in supplemental methods (Appendix D).

let-7a-5p

Species	Target	Alignment
Rainbow trout	Cytochrome c oxidase 5B	Query: 3' ttGATATGTTGGATGA-TGGAGt 5' |||| :|||||:|| ||||| Ref: 5' atCTATCTAACCTGCTAACCTCa 3'
Rainbow trout	Catalase	Query: 3' ttGATATGTTGGATGATGGAGt 5' |||| ||| | ||||:|| Ref: 5' tgCTATCCAAGATCCTACTTCt 3'
Rainbow trout	Catalase	Query: 3' uugauaugUUGGAUGAUGGAgu 5' |||| ||||||| Ref: 5' cggctgggAACCAACTACCTgc 3'
Arctic char	Catalase	Query: 3' uuGAUAUGUUGGAUGAUGGAGu 5' |||| ||| | ||||:|| Ref: 5' tgCTATCCAAGATCCTACTTCt 3'
Atlantic salmon*	Citrate synthase	Query: 3' ttGAT-ATGTTGGATGATGGAGt 5' :|| ||| |||| ||||||| Ref: 5' acTTATTAC-ACCT-CTACCTCt 3'

miR-21a-5p

<table>
<tr><td>Rainbow trout</td><td>Catalase</td><td><pre>Query: 3' ucAGUUGUGGUCAGACUAUUCGAu 5'
             | :||:|:| ||||| |||||
Ref: 5' caTAGACGCTA-TCTGAAAAGCTg 3'</pre></td></tr>
<tr><td>Arctic char</td><td>Catalase</td><td><pre>Query: 3' ucaGUUGUGGUCAGACUAUUCGAu 5'
              || : |: ||| |||||||
Ref: 5' tgcCATTCCTTTTCTAATAAGCTg 3'</pre></td></tr>
<tr><td>Rainbow trout</td><td>Glucokinase</td><td><pre>Query: 3' ucaGUUGUGGUCAGACUAUUCGAu 5'
              ||||| | | ||| |||||
Ref: 5' ggaCAACAACTCTATGAAAAGCTg 3'</pre></td></tr>
<tr><td>Arctic char</td><td>Glucokinase</td><td><pre>Query: 3' ucaGUUGUGGUCAGACUAUUCGAu 5'
              ||||| | | ||| |||||
Ref: 5' ggaCAACAACTCTATGAAAAGCTc 3'</pre></td></tr>
</table>

miR-143-3p

<table>
<tr><td>Rainbow trout</td><td>Cytochrome c oxidase 5B</td><td><pre>Query: 3' tcgATGTCACGAAGTAGAGt 5'
              | || | |||:|||||
Ref: 5' ctgTCCAATTCTTTATCTCa 3'</pre></td></tr>
<tr><td>Rainbow trout</td><td>Cytochrome c oxidase 5B</td><td><pre>Query: 3' cuCGAU--GUCACGAAGUAGAGu 5'
             |||: || | |||:|||||
Ref: 5' ttGCTGTCCAATTCTTTATCTCa 3'</pre></td></tr>
<tr><td>Rainbow trout</td><td>Catalase</td><td><pre>Query: 3' tcgATGTCACGAAGTAGAGt 5'
              ||:|||| | ||||||
Ref: 5' cagTATAGTGATCCATCTCt 3'</pre></td></tr>
<tr><td>Arctic char</td><td>Catalase</td><td><pre>Query: 3' cucgAUGUCACGAAGUAGAGu 5'
               ||:|||| | ||||||
Ref: 5' tcagTATAGTGATCCATCTCt 3'</pre></td></tr>
<tr><td>Arctic char</td><td>Pyruvate kinase</td><td><pre>Query: 3' cuCGAUGUCA-CGAAGUAGAgu 5'
             || ||| | || ||||||
Ref: 5' ccGCAACACTGGCATCATCTgc 3'</pre></td></tr>
</table>

*binding site was found by miRanda on the Atlantic salmon (*Salmo salar*) transcript and not specifically on the rainbow or brook trout transcripts, however they are highly conserved sequences.

To design primers for measuring mRNA transcripts of interest in rainbow trout (*Oncorhynchus mykiss*), partial or complete coding sequences were input into NCBI's Primer-BLAST tool. These primers were compared against multiple isoform sequences (if present in the NCBI database) as well before *in vitro* testing described below. To design primers for measuring mRNA transcripts of interest in brook trout (*Salvelinus fontinalis*), since there are no brook trout non-mitochondrial sequences currently present in the NCBI database, sequences from a closely related species, arctic char (*Salvelinus alpinus*), were used. The same methods were followed as detailed for rainbow trout above. The only exception was for cytochrome c oxidase (subunit 5B) as it is a mitochondrial gene, and the brook trout mitochondria is sequenced and publicly available on NCBI. However, it is unannotated, so the predicted brook trout cytochrome c oxidase (subunit 5B) sequence was identified by sequence alignment to multiple isoforms of the arctic char cytochrome c oxidase (subunit 5B) sequence and selecting the conserved section for input into NCBI's Primer-BLAST tool (see Supplemental methods for sequence and alignment; Appendix D).

4.3.5 Liver microRNA & mRNA measurement

Twenty to forty milligrams of ground liver tissue was used to coextract miRNA and mRNA using the Qiagen miRNeasy Kit (Qiagen, Mississauga, ON, Canada). The manufacturer's directions were followed, with the following specifications. Tissues were homogenized on ice for 15 s at 35,000 rpm using an Omni Tip™ tissue homogenizer (OMNI International, Kennesaw, Georgia, USA). Samples were centrifuged at 12,000 × *g*, whenever the speed wasn't specified. The miScript RT II kit was used to reverse transcribe 1 µg of RNA using the HiFlex buffer for mRNA quantification and the HiSpec buffer for miRNA quantification. Optimal annealing temperature and primer specificity were determined by conducting a gradient PCR with annealing temperatures from 55 °C to 65 °C using pooled cDNA made from 3 µL RNA from each sample, and reverse transcribed as mentioned. Primers were also validated by standard curves from pooled cDNA, melt curve analysis, and use of non-template controls to ensure that primer efficiency was between 90 and 110%, no contamination or dimerization of the primers occurred, and no amplification inhibition was present. Gradient qPCR was performed using a master mix containing 5 µL 2× SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario), 1 µL of 5 µM forward primer, 1 µL of 5 µM reverse primer, 2 µL molecular grade water and 1 µL of diluted pooled cDNA sample, whereas standard curves and samples were performed with 1 µL molecular grade water and 2 µL of sample/standard. Samples were analyzed at a 1:40 dilution to fit within the linear portion of the

standard curve. The RT-qPCR analysis was conducted using a BioRad CFX96 Touch Thermal Cycler (Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario), which was set to incubate at 95 °C for 30 s, denature the DNA for 10 s at 95 °C, anneal at 60 °C for 20 s, and then detect the fluorescence (BioRad, CA, USA). The denaturation and annealing processes were repeated for a total of 40 cycles. Afterwards, a melt curve analysis was performed for specificity where the sample increased in temperature from 65 °C to 95 °C in 0.5 °C every 5 s and an image was captured at each temperature. Gradient qPCRs and standard curves were run in duplicate, and samples were measured in triplicate. Only sample Cqs with a standard deviation less than 0.35 between technical replicates were used. Relative quantities of the samples were determined and the fold change relative to the control was calculated by dividing the relative quantity by the average control relative quantity. These values were then normalized using the NORMA-Gene algorithm and expressed as a fold changed compared to the control (Heckmann et al., 2011). Note: the terms "expression" and "relative expression" are used throughout, and all refer to the normalized expression of RNA relative to the control.

4.3.6 Liver enzyme activities

For the enzyme analysis, 10–20 mg aliquots of ground up liver sample were thawed on ice and dissolved in 20× volume of homogenization buffer (pH 7.4; 20 mM HEPES buffer, 1 mM EDTA, 0.1% Triton™ X-100, and 1 tablet/10 mL protease inhibitor tablet (cOmplete™ ULTRA Tablets, Mini, EDTA-free, EASYpack Protease Inhibitor Cocktail, Roche, Mannheim, Germany)). Samples were homogenized on ice for 5 s per sample using an Omni Tip™ tissue homogenizer (OMNI International, Kennesaw, Georgia, USA), and centrifuged at 4 °C at 12,000 × *g* for 10 min. These samples were divided into 40 µL aliquots and stored at −80 °C until enzyme analysis. The following are the reaction buffers used for measuring the specific enzymatic activity listed:

Glucokinase (*GK: E.C. 2.7.1.2*): 5 mM d-glucose, 10 mM $MgCl_2$, 100 mM KCl, 0.5 mM NAD, 5 mM DTT, and 0.5 U/mL glucose-6-phosphate dehydrogenase (from *Leuconostic mesenteroides*) in 50 mM HEPES, pH 7.4. The reaction was started with 80 mM ATP ([final] = 4 mM). Measured at 340 nm.

Citrate synthase (*CS: E.C. 2.3.3.1*): 0.3 mM acetyl Co-A and 0.1 mM DTNB in 50 mM Tris-HCl, pH 8. The reaction was started with 10 mM oxaloacetate ([final] = 0.5 mM). Measured at 412 nm.

Pyruvate kinase (*PK: E.C. 2.7.1.40*): 5 mM ADP, 100 mM KCl, 10 mM $MgCl_2$, 0.15 mM NADH, 10 mM fructose 1,6-bisphosphate, 5 U/mL lactate dehydrogenase in 50 mM imidazole, pH 7.4. The reaction was started with 100 mM phosphoenolpyruvate ([final] = 5 mM). Measured at 340 nm.

Catalase (*CAT: EC 1.11.1.6*): 20 mM K-phosphate buffer, pH 7.4 (16 mM K_2HPO_4 and 4 mM KH_2PO_4). The reaction was started with 400 mM H_2O_2 ([final] = 20 mM). Measured at 240 nm.

Cytochrome c oxidase (*CCO*: EC 1.9.3.1): 20 mM K-phosphate buffer, pH 7.4. The reaction was started with 1 mM reduced cytochrome c oxidase solution (reduced with sodium dithionite; [final] = 0.05 mM). Measured at 550 nm.

All enzymes were recorded at 22 °C in 15 s intervals for 10 min (or until the reaction plateaued) and were followed by a pathlength reading (SpectraMax 190, Molecular Devices, San Jose, CA). Enzyme activities were normalized to the total amount of protein in each homogenized liver sample. Protein amounts were quantified by the BCA assay (Smith et al., 1985).

4.3.7 Statistical analysis

All data were tested for normality, lognormality, and equal variances and were log-transformed or tested using non-parametric tests, as needed. One-way ANOVAs (parametric with equal variances), Welch's ANOVAs (parametric with unequal variances), or Kruskal-Wallis (nonparametric) were used to test for treatment differences within each fish species (in supplemental results; Appendix D). Dunnett's multiple comparison post hoc tests were performed and presented to identify differences between fish sampled post-stress (1, 3, 24 h post-stress treatments) to unstressed (control treatment) fish ($p < 0.05$) to determine the effects of air exposure. Plasma lactate values below the method detection limits were omitted. Linear regressions were performed between normalized relative microRNA expression and normalized relative mRNA expression of transcripts they are predicted to target (**Table 4.2**). Linear regressions were also performed between normalized relative microRNA expression and enzyme activities of transcripts they are predicted to target. They were performed for both species, even if only one species was predicted to be targeted and were used to identify inverse relationships. Linear regressions were performed between relative mRNA expression and respective enzyme activities to determine if direct relationships existed. The correlation coefficient and the measure of whether the slope was significantly non-zero ($p < 0.05$) were calculated. All statistical analysis results can be found in the supplemental materials (Appendix D).

4.4 Results

4.4.1 Plasma cortisol, glucose, and lactate

In order to validate the presence of the acute stress response in the fish, plasma cortisol, glucose, and lactate were measured. In rainbow trout, plasma cortisol increased 12-fold from the control to one-hour post-stress (control: 9.8; 1 h post-stress: 125 ng/mL mean cortisol; $p = 0.0024$) and remained significantly elevated 3- and 24-h post-stress (3 h post-stress: 47.1; 24 h post-stress: 40.3 ng/mL mean cortisol; **Figure 4.2A**; $p_{3hr} = 0.0058$, $p_{24hr} = 0.0055$). In brook trout, plasma cortisol remained below 40 ng/mL throughout the exposure, only significantly increasing at 3- and 24-h post-stress (control: 6.2; 1 h post-stress: 19.5; 3 h post-stress: 24.6; 24 h post-stress: 18.1 ng/mL mean cortisol; $p_{1hr} = 0.0899$, $p_{3hr} = 0.0398$, $p_{24hr} = 0.0428$) with a large variation in response 1 h post-stress (min: 5.8 max: 34.7 ng/mL cortisol; **Figure 4.2A**). Therefore, there was a significant increase in plasma cortisol post-stress in both rainbow and brook trout.

In rainbow trout, there was a significant 6-fold increase in plasma glucose 1- and 3-h post-stress ($p_{1hr} = 0.0009$, $p_{3hr} = 0.0031$), which was back to control levels 24-h post-stress ($p_{24hr} = 0.9548$**, Figure 4.2B**). A similar trend can be observed within brook trout, however there is large variation within the control treatment (min: 4.2 max: 23 mmol/L; **Figure 4.2B**). Therefore, plasma glucose was only significantly altered post-stress in rainbow trout.

In rainbow trout there was a significant 7 to 8-fold increase in plasma lactate 1- and 3-h post-stress ($p_{1hr} = 0.0002$, $p_{3hr} < 0.0001$), which returned to control levels 24-h post-stress ($p_{24hr} = 0.1528$, **Figure 4.2C**). The same trend can be observed within brook trout, with a nonsignificant 2-fold increase in plasma lactate 1-h post stress ($p_{1hr} = 0.0637$) and a significant 2.5-fold increase 3-h post-stress ($p_{3hr} = 0.0154$), which returned to control levels 24-h post-stress ($p_{24hr} = 0.6562$, **Figure 4.2C**). Therefore, there was a significant increase in plasma lactate post-air exposure in both rainbow and brook trout.

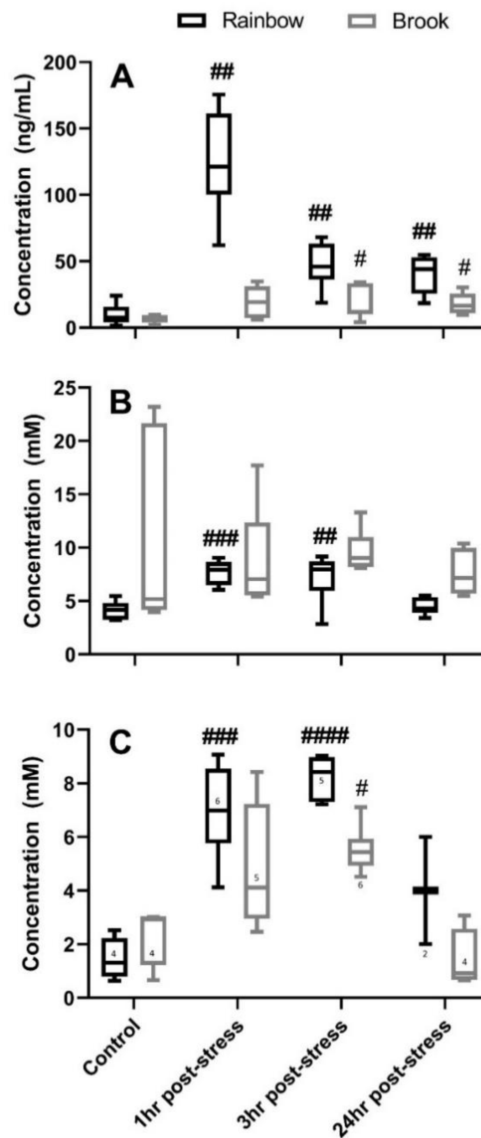

Figure 4.2 Plasma cortisol, glucose, and lactate of trout exposed to air for three minutes. Trout were subjected to air for three minutes and sampled one, three, or twenty-four hours post stress. Box plots of the concentration of **A)** plasma cortisol (ng/mL), **B)** plasma glucose (mM), and **C)** plasma lactate (mM) in rainbow and brook trout. Minimum, first quartile, median, third quartile, and maximum are indicated by the horizontal lines (when reading from bottom to top). Plasma lactate values below the method detection limits were omitted (n numbers are indicated on the graph). Differences between each timepoint post-stress and the control were determined for each species separately and are indicated by the # symbol (Dunnett's post-hoc, n=6 unless otherwise noted, $p < 0.05$). A single symbol indicates a p-value (or adjusted p-value) between 0.01 and 0.05, double symbols indicate a p-value between 0.001 and 0.01, triple symbols indicate a p-value between 0.0001 and 0.001, and quadruple symbols indicate a p-value less than 0.0001.

4.4.2 Liver microRNA

Three microRNA (miR-143-3p, let-7a-5p, miR-21a-5p) were measured in rainbow and brook trout liver following air exposure to characterize the response (**Figure 4.3**). Expression of miR-143-3p significantly increased at 24-h post-stress in rainbow trout ($p = 0.0497$, **Figure 4.3A**). Also, miR-143-3p expression was significantly decreased 1-h post-stress and increased at 24-h post-stress in brook trout ($p_{1hr} < 0.0001$, $p_{24hr} = 0.0075$). Expression of let-7a-5p was significantly increased at 3-h post-stress ($p = 0.0443$) in rainbow trout whereas it was significantly increased at 24-h post-stress in brook trout ($p = 0.0003$, **Figure 4.3B**). In rainbow trout, expression of miR-21a-5p was significantly increased at 3- and 24-h post-stress ($p_{3hr} = 0.0309$, $p_{24hr} = 0.0179$, **Figure 4.3C**). In brook trout, expression of miR-21a-5p was only significantly increased 24-h post-stress ($p = 0.0024$). Therefore, these three miRNAs are altered in trout liver following air exposure.

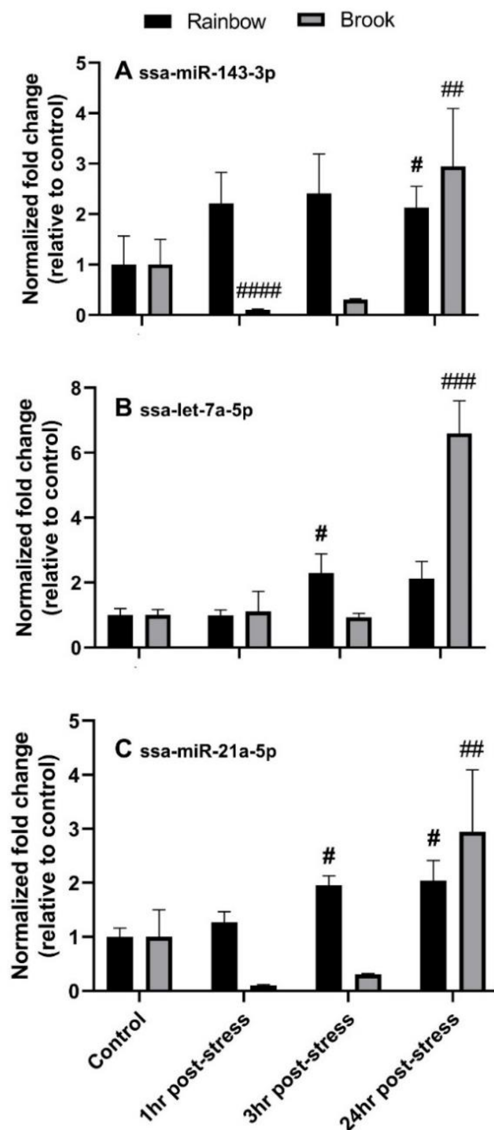

Figure 4.3 Normalized fold change relative to the control of rainbow and brook trout liver microRNA.

Mean ± SEM of fold change of microRNA (**A**: miR-143-3p, **B**: let-7-5p, **C**: miR-21a-5p) of rainbow and brook trout that were subjected to air for three minutes and sampled one, three, or twenty-four hours post stress. Values were log transformed to normalize where needed for statistical analysis, however untransformed data are presented here. Differences between each timepoint post-stress and the control were determined for each species separately and are indicated by the # symbol (Dunnett's post-hoc, $n = 6$ ($n_{Brook@24hr} = 5$), $p < 0.05$). A single symbol indicates a p-value (or adjusted p-value) between 0.01 and 0.05, double symbols indicate a p-value between 0.001 and 0.01, triple symbols indicate a p-value between 0.0001 and 0.001, and quadruple symbols indicate a p-value less than 0.0001.

4.4.3 Liver mRNA

Five mRNA (CS, GK, PK, CCO5B, CAT) predicted *in silico* to be targeted by the miRNA analyzed were measured in rainbow and brook trout liver following air exposure to characterize the response (**Figure 4.4**). There were no significant effects of air exposure on CS expression in both brook and rainbow trout (**Figure 4.4A**). There was a significant decrease in rainbow trout GK expression 24-h post-stress ($p = 0.0025$, **Figure 4.4B**). PK expression did not differ for rainbow or brook trout following air exposure (**Figure 4.4C**). CCO subunit 5b expression was decreased 24 h post stress in brook trout ($p = 0.0256$, **Figure 4.4D**). CAT expression was decreased 24 h post stress in brook trout ($p = 0.0103$, **Figure 4.4E**).

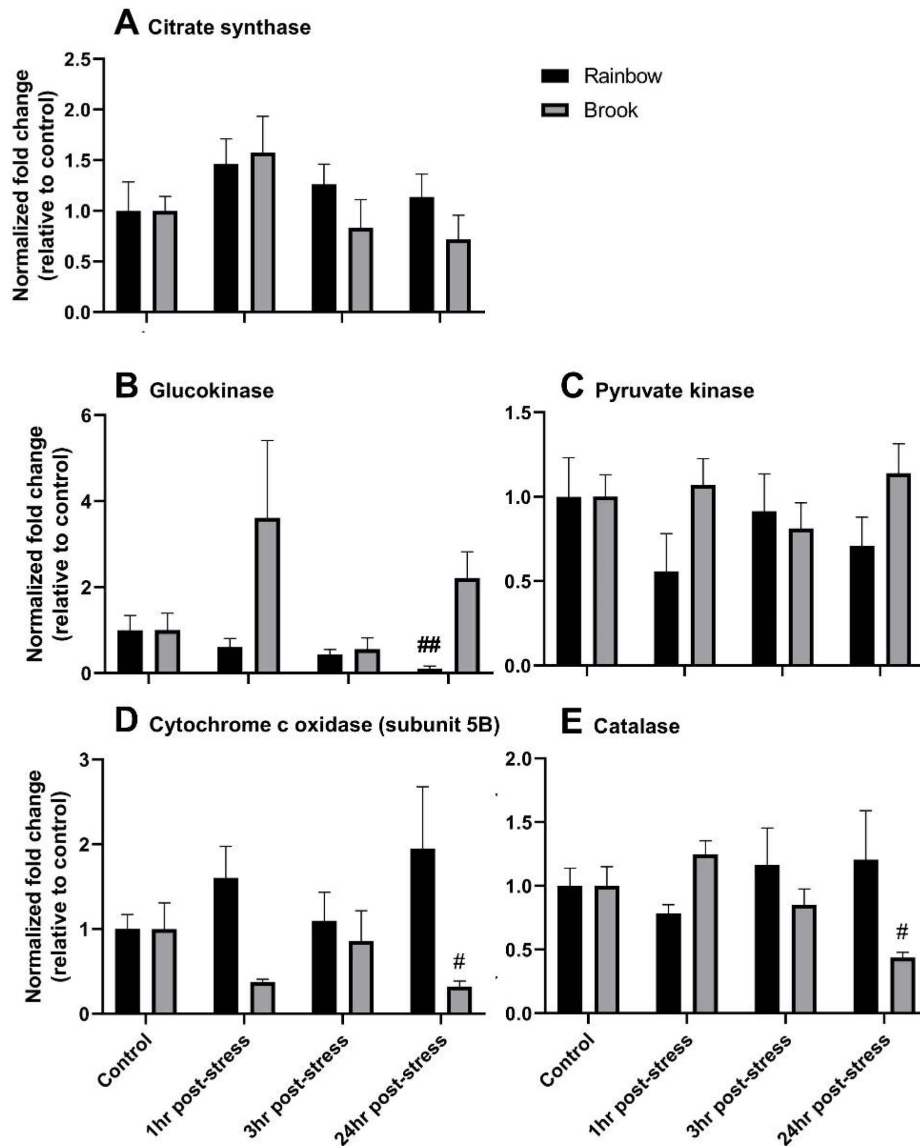

Figure 4.4 Normalized expression of rainbow and brook trout liver mRNA.
Mean ± SEM of fold change of liver **A)** citrate synthase, **B)** glucokinase, **C)** pyruvate kinase, **D)** cytochrome C oxidase, and **E)** catalase mRNA expression of rainbow and brook trout that were subjected to air for three minutes and sampled one, three, or twenty-four hours post stress. Values were log transformed to normalize where needed for statistical analysis, however untransformed data are presented here. Differences between each timepoint post-stress and the control were determined for each species separately and are indicated by the # symbol (Dunnett's post-hoc, $n = 6$ ($n_{Brook@24hr} = 5$), $p < 0.05$). A single symbol indicates a p-value (or adjusted p-value) between 0.01 and 0.05 and double symbols indicate a p-value between 0.001 and 0.01.

4.4.4 Liver enzyme activities

Activities of five enzymes (CS, GK, PK, CCO, CAT) predicted to be targeted by the miRNA were measured in rainbow and brook trout liver following air exposure to characterize the response (**Figure 4.5**). Rainbow trout liver CS activity decreased 1- and 24-h post-stress ($p_{1hr} = 0.0348$, $p_{24hr} = 0.0056$, **Figure 4.5A**). GK activity was not significantly altered between treatments for both brook and rainbow trout (**Figure 4.5B**). PK activity did not differ for rainbow trout following air exposure (**Figure 4.5C**). However, liver PK was significantly decreased specifically 3-h post-stress in brook trout ($p = 0.0394$). For rainbow trout, CCO activity was decreased 3- and 24-h post stress ($p_{3hr} = 0.0153$, $p_{24hr} = 0.0066$, **Figure 4.5D**). CAT activity was increased at all timepoints post stress in rainbow trout ($p_{1hr} = 0.0208$, $p_{3hr} = 0.0040$, $p_{24hr} = 0.0086$, **Figure 4.5E**). Altogether, liver enzyme activities of CS, PK, CCO, and CAT were altered following air exposure in brook and/or rainbow trout.

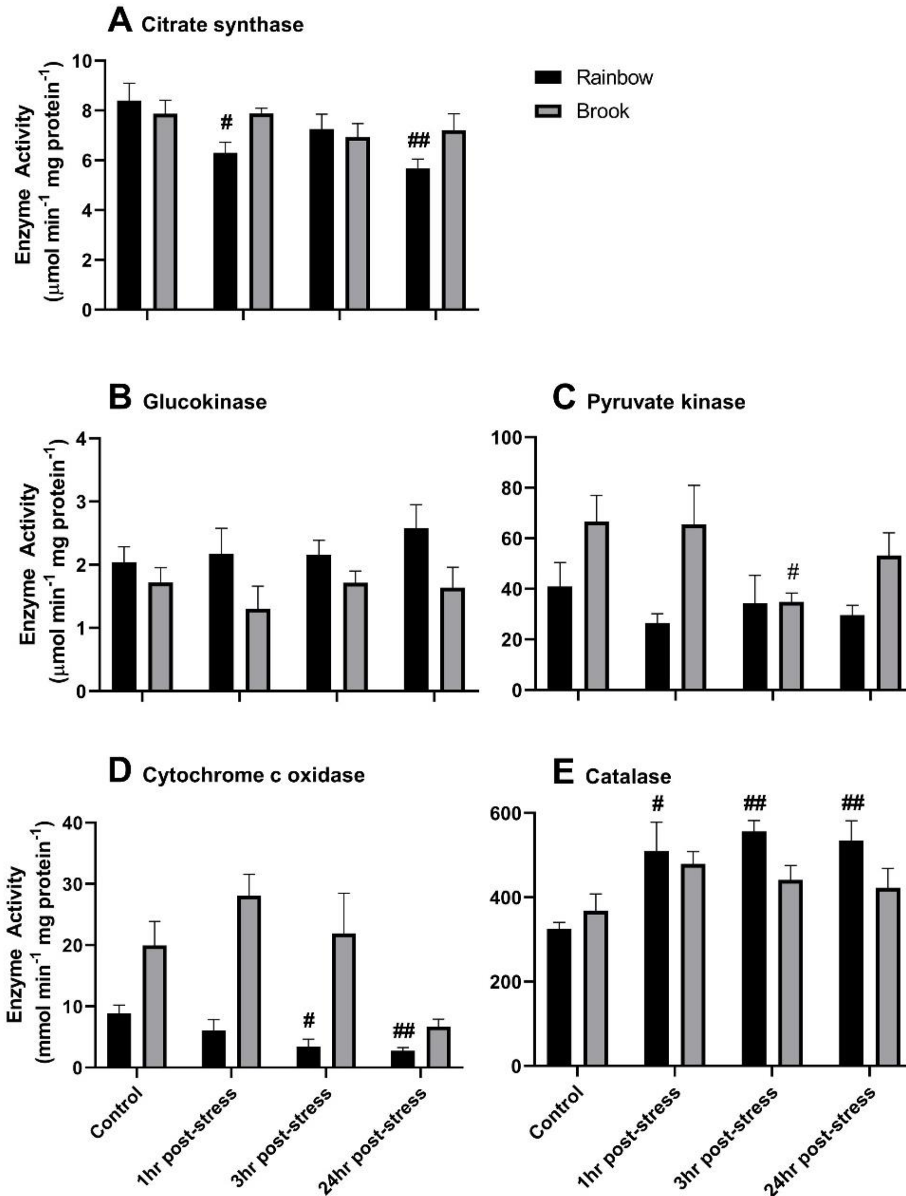

Figure 4.5 Activities of rainbow and brook trout liver enzymes.
Mean ± SEM of liver **A)** citrate synthase, **B)** glucokinase, **C)** pyruvate kinase, **D)** cytochrome C oxidase, and **E)** catalase enzyme activities of rainbow and brook trout that were subjected to air for three minutes and sampled one, three, or twenty-four hours post stress. Values were log transformed to normalize where needed for statistical analysis, however untransformed data are presented here. Note that units differ between enzymes to provide readable values. Differences between each timepoint post-stress and the control were determined for each species separately and are indicated by the # symbol (Dunnett's post-hoc, $n = 6$ ($n_{Brook@24hr} = 5$), $p < 0.05$). A single symbol indicates a p-value (or adjusted p-value) between 0.01 and 0.05 and double symbols indicate a p-value between 0.001 and 0.01.

4.4.5 Linear regressions

To determine if there were any inverse relationships between the relative expression of miRNA measured and relative expression or activities of their predicted mRNA and enzyme targets, linear regressions were performed. Only the significant relationships and the regressions are presented. Non-significant regressions can be found in the supplemental results (Appendix D). The correlation between mRNA transcript expression and enzyme activity were also measured to determine if they were directly related. However, no statistically significant correlation between mRNA expression and enzyme activities were observed.

4.4.5.1 Between miRNA and mRNA

Of all the predicted relationships between miRNA and mRNA expression measured, there were only three significant relationships (**Figure 4.6**). The expression of miRNA and mRNA transcripts that are inversely correlated with each other are miR-143-3p, let-7a-5p, and miR-21a-5p and CAT, and are only observed in brook trout.

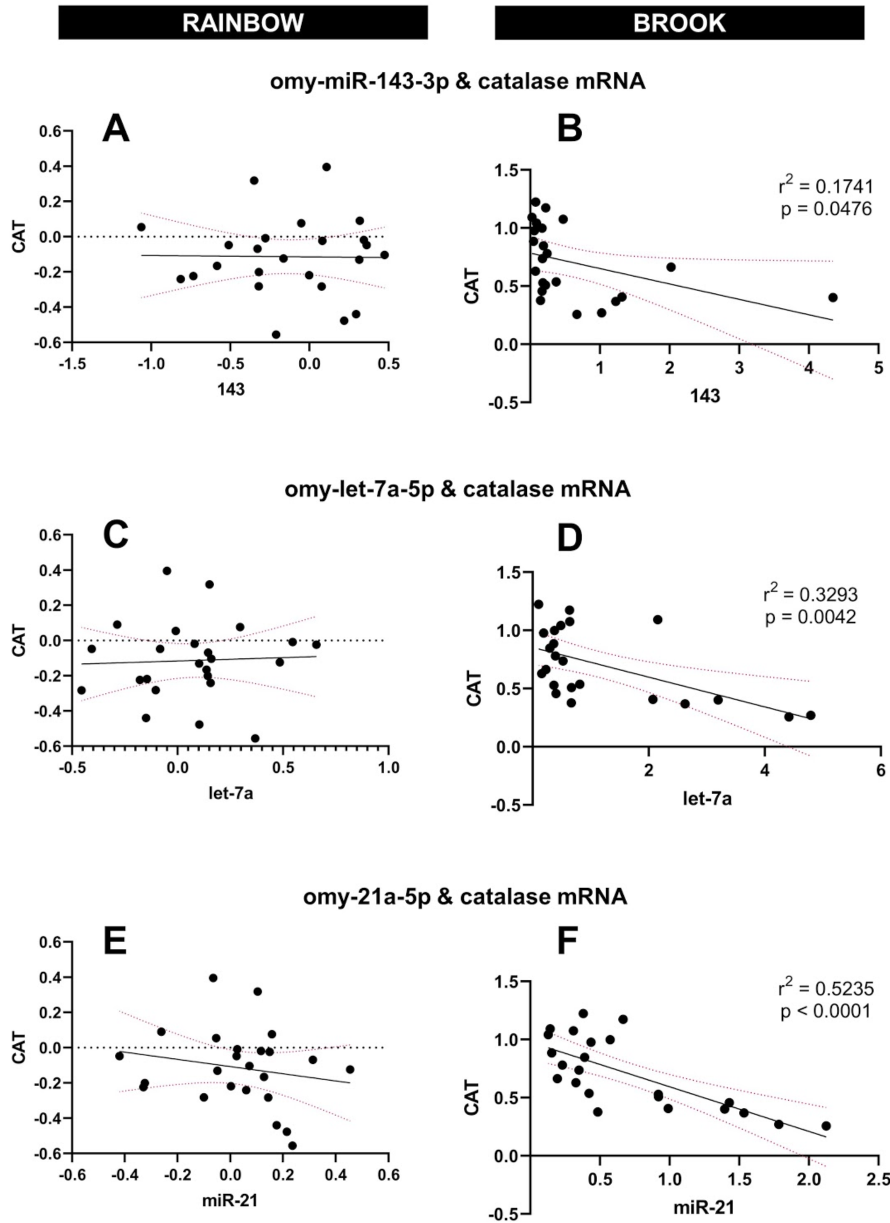

Figure 4.6 Linear regression of microRNA and the predicted target mRNA transcript expression.

Expression of microRNA and their predicted target mRNA expression were compared within each species (Rainbow: **A**, **C**, **E**; Brook: **B**, **D**, **F**). Values were log transformed to normalize where needed and applicable. Only significant relationships and the results in the other species are presented. P-values and correlation coefficients are presented on each graph where a significant inverse relationship exists ($p < 0.05$, $n_{rainbow} = 24$, $n_{brook} = 23$).

4.4.5.2 Between miRNA and enzymes

Of all the predicted relationships between miRNA expression and enzyme activities measured, there were only two significant relationships (**Figure 4.7**). The miRNA expression and enzyme activities that are inversely correlated with each other are miR-143-3p and let-7a-5p and *CCO* activity and are only observed in brook trout.

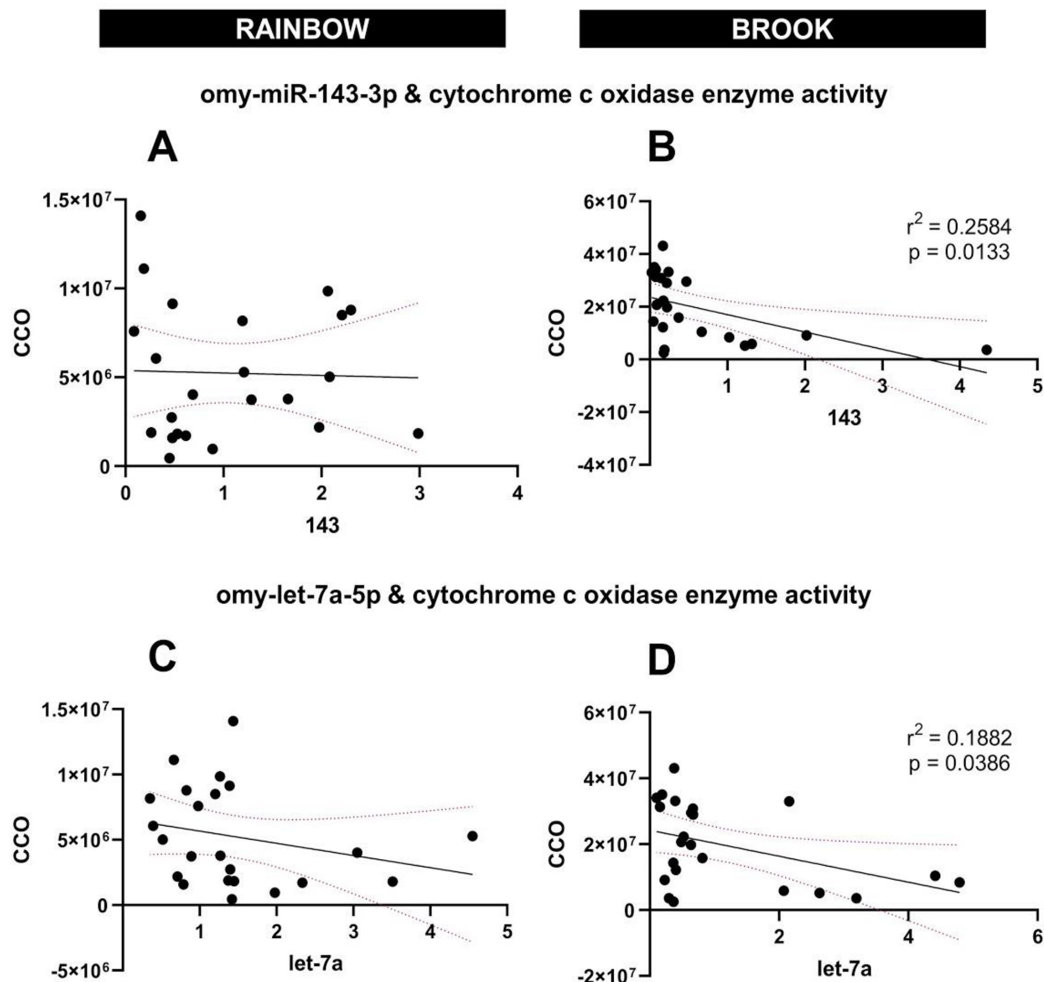

Figure 4.7 Linear regression of microRNA and the predicted target enzyme activity. Expression of microRNA and their predicted target enzyme activities were compared within each species (Rainbow: **A** & **C**; Brook: **B** & **D**). Only significant relationships and the results in the other species are presented. P-values and correlation coefficients are presented on each graph where a significant inverse relationship exists ($p < 0.05$, $n = 23$).

4.5 Discussion

Our aim was to further characterize the molecular and enzymatic response following air exposure in the liver of rainbow and brook trout. These trout are members of sister genera within the salmonid phylogeny and are estimated to have diverged approximately 20 million years ago (Lecaudey et al., 2018). Overall, rainbow and brook trout had altered plasma metabolites following exposure to air but differed in the magnitude of their plasma cortisol, glucose, and lactate response. Furthermore, the trout species shared increases in miR-143-3p and miR-21a-5p expression 24-h post-stress. There were few mRNA alterations post-stress, but four of the five enzymes measured had altered activity in one or both trout species following stress. There were inverse relationships measured only in brook trout between miRNA and catalase mRNA expression, as well as miRNA and cytochrome c oxidase enzyme activity.

The plasma cortisol, glucose, and lactate response following air exposure has been well-characterized in both rainbow trout and brook trout and were assessed as physiological markers of acute stress following air exposure. In this study, rainbow trout cortisol, glucose, and lactate all significantly increased in a time-dependent manner post-stress, as previously characterized (Alderman et al., 2012; Gravel and Vijayan, 2007; Ings et al., 2012; López-Patiño et al., 2014; Pottinger, 2010; Wiseman et al., 2007; Wiseman and Vijayan, 2011). In brook trout, cortisol significantly increased, but only at 3- and 24-h post-stress. This was unexpected due to brook trout historically responding to air exposure with a higher magnitude cortisol response (~80 ng/mL) than measured here (~25 ng/mL) (Barton, 2000). This could be due to the decrease in cortisol response during the reproductive season (Aug – Dec for brook trout; Pottinger et al., 1995; Pottinger and Carrucj, 2000). Though our trout were immature, I did sample mid-December. Furthermore, the cortisol response can vary based on source of the fish, which was different than brook trout previously measured (Barton, 2002, 2000; Romero and Beattie, 2021). This response could also be due to uncontrolled factors, such as sampling from the same tank, as these trout were exposed to air to induce a stress response and not to only measure the effect of air exposure. Plasma lactate increased in brook trout post-air exposure as expected (Pottinger, 2010). Plasma glucose appears to increase following air exposure however two fish had much higher pre-stress plasma glucose levels. This could be due to hormonal factors, such as melatonin levels or variation that occurs throughout the day (Mommsen et al., 1999; Polakof et al., 2012; Vera et al., 2014). Further intraspecies measurements of plasma metabolites in different populations and at different times of the day could shed light into the blunted cortisol and varied

baseline glucose measurements measured in brook trout. Though brook trout had a different cortisol response to the air exposure than typically expected, the transcriptional and enzymatic responses measured here align to the common stressor of an air exposure and provide relevant information into the molecular and enzymatic response to air exposure in these two fish species.

MiRNAs have only been measured in fish following air exposure twice previously and both were in rainbow trout (Cadonic et al., 2020; Ikert et al., 2021). In a similar study, two of the miRNAs measured here (miR-143-3p and let-7a-5p) were also altered 1-, 3-, and 24-h post air-stress in rainbow trout liver (Cadonic et al., 2020). These miRNAs decreased in liver following air exposure, whereas in this study, both miR-143-3p and let-7a-5p are increased 24-h post-stress. These differences could be due to the size differences between trout (past study: 660 g mean weight; current study: 179 g mean weight). Due to the high intraspecies variation in the liver miRNA response in rainbow trout exposed to the same stressor, no comparison can be made about the miRNA post-air exposure response between rainbow and brook trout. Future studies can determine if size, differences in fish source, or technical differences such as normalization techniques, cause miRNA variation within a species. Therefore, miRNA expression has been further characterized following air exposure in rainbow trout, highlighting intraspecies variation, and the miRNA response has now been measured in the less studied brook trout.

All three miRNAs measured in brook and rainbow trout liver were altered following air exposure and it is important to understand the functional implications of these alterations. Briefly, in rainbow trout, relative expression of miR-143-3p was increased 24 h post stress, let-7a-5p was increased 3 h post stress, and miR-21a-5p was increased at both 3 and 24 h post stress. In brook trout, relative expression of miR-143-3p was decreased 1 h post stress and increased 24 h post stress, let-7a-5p and miR-21a-5p were increased 24 h post stress. MiRNAs are post-transcriptional regulators that can target several mRNA and decrease transcript stability or translation (Bartel, 2004). Therefore, miRNA expression is inversely related to target mRNA transcript expression and/or protein abundance or enzyme activity, meaning that the targets measured would mostly decrease post-stress due to the increase in miRNA expression. Therefore, by determining what the miRNA will target, computationally and/or through functional validation, the functional implications of altered miRNA transcript levels can be determined. *In silico* predictions were performed and the transcript expression and enzyme activities of five of the predicted targets were measured. Inverse relationships were found between miRNAs and CAT transcript expression and between miRNAs and CCO activity in brook

trout. These inverse relationships are helpful indicators of potential regulation, though 3′UTR luciferase assays would need to be conducted to validate these relationships (Jin et al., 2013). Furthermore, there are no known binding sites for the miRNAs measured in Arctic char (closest sequenced relative to brook trout) CCO 3′UTR (*in-silico* analysis using miRanda; supplemental methods; Appendix D). This could mean that this inverse relationship is purely correlative or that no binding site has yet to be identified, and sequencing of the non-coding regions of the brook trout CCO gene itself would be useful. Therefore, these inverse relationships can be used to understand potential functional impacts as well as to develop future studies of miRNA responses and regulation in brook and rainbow trout.

Functional implications of these altered miRNA can also be elucidated by identifying conditions where these miRNAs were also altered. miR-21a-5p decreased post-infection and played a role in the inflammatory response to bacteria in grass carp (Tao et al., 2019), and increased following exposure to substances which play a role in the antioxidant response in fishes (Ma et al., 2019a; Tang et al., 2016). As previously mentioned, let-7a-5p and miR-143-3p were decreased in rainbow trout liver following air exposure (Cadonic et al., 2020). miR-143-3p increased 4 h post refeeding and following microcystin exposure but decreased following oxidative stress in sliver carp and in spleen following an immune stressor (Feng et al., 2017; Ma et al., 2019a; Mennigen et al., 2012; Xu et al., 2016). Additionally, let-7a increased in older fish and increased in slow growth fish as compared to fast growth fish (Zhao et al., 2017). Due to the sequence conservation of miRNA between vertebrates, the mammalian response can also be investigated. These three miRNAs are involved in glucose and lipid metabolism (Calo et al., 2016), and in the oxidative stress response in mammals (Cai et al., 2020; Chang et al., 2020; Cheng et al., 2009; Engedal et al., 2018; Hong et al., 2020; Simone et al., 2009). Overall, there are a variety of conditions where these miRNAs are altered in fish and other vertebrates, but they especially play a role in the oxidative stress response. In fact, miR-21a-5p is known to target VEGF, and knockout of this miRNA affected reactive oxygen species production in tilapia (Zhao et al., 2016). Both brook and rainbow trout were responding to oxidative stress in our air exposure due to the significant increase in catalase activity in rainbow trout liver and the upward trend in brook trout liver. Therefore, with the inverse relationships between the miRNA and catalase expression, the alteration of these miRNA in response to oxidative stress in other species, and the increase in catalase activity post-air exposure, there is a good indication these altered miRNAs could have a functional role in the molecular regulation of oxidative stress.

Since enzyme activities were measured post air exposure in the liver of brook and rainbow trout, the physiological response of these targets can be understood. The five targets can be divided by function into either glycolytic enzymes (glucokinase and pyruvate kinase), or being involved in aerobic metabolism, including oxidative phosphorylation and the antioxidant response (citrate synthase, cytochrome c oxidase, and catalase). These are not all the pathways or enzymes that could be altered following air exposure, but they provide a targeted understanding of the effects of air exposure in these two trout species. Also, transcripts of these enzymes were measured to understand the molecular regulation of the response to air exposure and to determine if they were inversely related to miRNA expression, but the changes are less functionally relevant as there were no correlation between transcript expression and enzyme activity.

Following air exposure, it is theoretically expected that *GK* and *PK*, as the first and last steps in glycolysis, respectively, would be predicted to have lowered or unchanged activity following exposure to air and activation of the acute stress response. This is because gluconeogenesis is occurring instead and is mobilizing glucose out of the liver (Mommsen et al., 1999; Wendelaar Bonga, 1997). However, these enzymes could potentially increase post stress as there is an increase in plasma glucose that can be metabolized, and increased hexokinase (general form of *GK*) activity can indicate increased liver capacity for glucose uptake (Moon, 2001). Past exposures to air or other acute stressors (ex. chasing or handling) have had mixed effects on *GK* and *PK* activity. Liver *GK* activity decreased 45 min post chasing but increased 2-h following 5 min chasing or handling stress (Ings et al., 2012; López-Patiño et al., 2014). Activity of liver *PK* increased 4- and 24-h post-handling (Ings et al., 2012), decreased 4-h post-chasing (López-Patiño et al., 2014), and remained unchanged following a netting/chasing stressor (Wiseman and Vijayan, 2011). These past results do show decreases following acute stress, as theorized, with some post-stress increases depending on the stressor type. Following air exposure, no change occurred in *GK* and *PK* enzyme activity in rainbow trout and no change occurred in brook trout *GK* but a decrease in *PK* activity 3-h post-stress. There was no increase in activity during recovery as with other acute chasing or handling stressors but since those were more exercise-driven stressors than air exposure, the increase in glycolytic capacity could be more essential during recovery. Therefore, though the lack of change in *GK* activity and decrease in *PK* activity in brook trout liver is expected, it is indicative that there is a small decrease in glycolytic capacity and no increase to potentially recover from the stressor. Whether this is due to a lack of ability or lack of need to respond to the stressor can be studied further.

Following air exposure, it would be expected that due to the initial lack of oxygen, reoxygenation, and overall stress response, that aerobic metabolism and oxidative phosphorylation, and therefore *CS* and *CCO* activity would be decreased but perhaps increase during recovery (Kadenbach et al., 2000; Mommsen et al., 1999). Furthermore, due to the limited oxygen supply and reperfusion of oxygen in tissues following air exposure, an increased presence of reactive oxygen species (ROS) triggering an antioxidant response, increasing *CAT* activity, is expected (Martínez-Álvarez et al., 2005; Paital, 2013; Pereira-da-Silva and Oliveira, 2017). There is also a known relationship between stress, decreased *CCO* activity, and increased ROS, which would increase *CAT* activity (Martínez-Álvarez et al., 2005; Ramzan et al., 2020). Previously, *CAT* activity increased in the liver of lambari following air exposure however the other two enzymes have not previously been measured (de Oliveira et al., 2019). *CS* activity was decreased 1- and 24-h post stress in rainbow trout only. *CCO* activity also decreased 3- and 24-h post stress in rainbow trout only. *CAT* activity was significantly increased at all timepoints post stress in rainbow trout and trending upwards in brook trout. Therefore, in rainbow trout, the predicted decrease in *CS* and *CCO* liver activity, as well as an increase in *CAT*, was measured. Functionally, aerobic metabolism was decreased in rainbow trout post-stress, indicating a potential reliance on anaerobic metabolism. Also, the decreased *CCO* activity in rainbow trout could be contributing to the increased *CAT* activity, through the production of ROS. However, this is likely only partially contributing as the increased *CAT* activity is observed before the decrease in *CCO*. In brook trout, only an upward trend in *CAT* activity was measured following air exposure, potentially due to ROS only being produced in response to the air exposure/reperfusion as there was no decrease in *CCO* to produce ROS. Furthermore, the lack of effect of air exposure on *CS* and *CCO* activity in liver could indicate that aerobic metabolism was recovered more quickly potentially due to the lack of ability or need to respond to the stressor.

In conclusion, measurement of liver miRNA, mRNA, and enzyme activities in brook and rainbow trout has expanded our understanding of the response to air exposure, especially in the less-studied brook trout, but also highlights many gaps in understanding. The functional implications and roles of the miRNA, transcripts and enzymes measured need to be further studied. Their role in the acute stress response could be elucidated using cortisol implants/injections and/or cell cultures to remove the variation measured within and between species following air exposure. The predicted miRNA-target relationships can also be validated to further understand molecular and epigenetic regulation of the acute stress response to air exposure.

4.6 Thank you

Thanks to Nathan Bennoit, Ivan Cadonic, Nathanael Harper, Rhiannon Hodgson, Ivy Luu, Patricija Marjan, and Karyn Robichaud who helped sample the trout analyzed in this study.

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Chapter 5

High Throughput Sequencing of MicroRNA in Rainbow Trout Plasma, Mucus, and Surrounding Water Following Acute Stress

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***Frontiers in Physiology,* 11, pp. 1821. 2021.**

5.1 Summary

Circulating plasma microRNAs (miRNAs) are well established as biomarkers of several diseases in humans and have recently been used as indicators of environmental exposures in fish. However, the role of plasma miRNAs in regulating acute stress responses in fish is largely unknown. Tissue and plasma miRNAs have recently been associated with excreted miRNAs; however, external miRNAs have never been measured in fish. The objective of this study was to identify the altered plasma miRNAs in response to acute stress in rainbow trout (*Oncorhynchus mykiss*), as well as altered miRNAs in fish epidermal mucus and the surrounding ambient water. Small RNA was extracted and sequenced from plasma, mucus, and water collected from rainbow trout pre- and 1 h-post a 3-min air stressor. Following small RNA-Seq and pathway analysis, differentially expressed plasma miRNAs that targeted biosynthetic, degradation, and metabolic pathways were identified. I successfully isolated miRNA from trout mucus and the surrounding water and detected differences in miRNA expression 1-h post air stress. The expressed miRNA profiles in mucus and water were different from the altered plasma miRNA profile, which indicated that the plasma miRNA response was not associated with or immediately reflected in external samples, which was further validated through qPCR. This research expands understanding of the role of plasma miRNA in the acute stress response of fish and is the first report of successful isolation and profiling of miRNA from fish mucus or samples of ambient water. Measurements of miRNA from plasma, mucus, or water can be further studied and have potential to be applied as non-lethal indicators of acute stress in fish.

5.2 Introduction

Fish experience acute stress when they are exposed to air. This causes an acute stress response releasing catecholamines within seconds to minutes and releasing cortisol within minutes to hours (Wendelaar Bonga, 1997; Tort, 2013; Balasch and Tort, 2019; Cadonic et al., 2020). Molecular regulation of this stress response is well studied in fish, however, the role of miRNA in this response is a nascent field of study. MicroRNAs are short, 22-nucleotide long, non-coding RNA that decrease stability and translation of mRNA (Lee et al., 1993; Ruvkun et al., 1993; Bartel et al., 2003). MicroRNAs regulate the response of fish in a tissue-specific manner to a variety of stressors, such as hypoxia in tilapia (Qiang et al., 2020), contaminant exposure in carp (Liu et al., 2020a, b), and overcrowding in rainbow trout (Gonçalves et al., 2020). Additionally, miRNAs are measurable in blood plasma of fish and specific miRNAs are differentially expressed following exposure of rainbow trout to air (Cadonic et al., 2020). However, the entire plasma miRNA response to acute stress has not yet been characterized. It is also unknown how changes in profiles of miRNAs in blood plasma of fishes target known genes and molecular pathways of the acute stress response.

Non-lethal sampling of plasma from fish can conservatively occur up to a maximum of 1 mL per kg of body mass (Canadian Council on Animal Care, 2005; Hawkins et al., 2011). Therefore, non-lethal measurements of miRNA in blood plasma of large fishes can be made. However, when measuring miRNA from smaller or endangered species, an alternative method would be more appropriate. Epidermal mucus of fishes has been used as a sampling location for indicators of acute stress. These include cortisol, stress-related proteins, enzymes, glucose, and lactate levels (Easy and Ross, 2010; Guardiola et al., 2016; Fernández-Alacid et al., 2019). Furthermore, miRNAs are known to be excreted from tissue and fluids in exosomes (Valadi et al., 2007; Skog et al., 2008; Zernecke et al., 2009; Kosaka et al., 2010; Zhang et al., 2010), associated with proteins (Wang et al., 2010; Arroyo et al., 2011; Turchinovich et al., 2011; Vickers et al., 2011), or by passively leaking from a site of injury (Chen et al., 2008; Mitchell et al., 2008). Therefore, in order to provide a representation of the stress status of smaller fishes, mucus is a potential matrix for non-lethal sampling of miRNA. However, miRNAs have never been measured in mucus of fishes and if present, it is unknown whether patterns of relative concentrations of miRNAs change following acute stress, as it does in blood plasma.

Collection of mucus requires fish to be handled and animal guidelines recommend minimizing the removal of epidermal mucus (Canadian Council on Animal Care, 2005). Therefore, to measure changes in miRNA both non-lethally and non-invasively, one of the study objectives was to determine if miRNA can also be measured from ambient water samples. Nucleic acids, in the form of environmental DNA (eDNA), can be measured from water samples and that DNA originates from epithelia, blood, urine, feces, and gametes of fishes, which are all locations that contain miRNA (Höss et al., 1992; Valiere and Taberlet, 2000; Ficetola et al., 2008; Jerde et al., 2011). In humans, profiles of relative concentrations of miRNA in tissues are reflected in excreted fluids (Cui and Cui, 2020; Park et al., 2020). Therefore, profiles of miRNA present in water external to fishes might be related to miRNA profiles in blood. If this supposition is correct, there might be no need to capture fish or collect samples of blood or mucus to assess status and trends in the physiological state of stress. A concern when measuring RNA in the environment is its instability, as mRNA have an average half-life of 5 min (Moran et al., 2013). However, miRNAs have a half-life of 5 days and are protected by exosomes or associated proteins (Gantier et al., 2011). Therefore, there is potential for miRNA to be measured in water and their abundance to be altered following stress, however, this has not yet been measured.

The objective of this study was to determine how miRNA profiles of rainbow trout are altered in non-lethal samples in response to acute stress. First, it was hypothesized that the pattern of relative concentrations of miRNAs in plasma of rainbow trout would be altered following acute stress and this response would be linked to known molecular responses to stress. Second, it was hypothesized that rainbow trout miRNAs would be present and measurable in epidermal mucus and water and miRNA abundances would be altered following acute stress. Third, it was hypothesized that there would be differentially expressed miRNAs in common in plasma, mucus, and water, which would provide evidence that external profiles of miRNA are representative of internal changes in miRNA. To test these hypotheses, samples of blood plasma, epidermal mucus, and water were collected from rainbow trout (*Oncorhynchus mykiss*) prior to and following exposure to 3 min of air. RNA was extracted and differentially expressed miRNAs identified in all three matrices, by use of high throughput sequencing, followed by pathway analysis of altered plasma miRNA targets, and qPCR validation. MicroRNA was further developed as a non-lethal measure of stress in fishes and the foundation was laid for further development of mucus and water miRNA as non-lethal measures of fish stress.

5.3 Materials and Methods

5.3.1 Animals and Experimental Design

Rainbow trout (*Oncorhynchus mykiss*) were procured from Silver Creek Aquaculture (Erin, ON, Canada) or donated by the Ontario Ministry of Natural Resources and Forestry. All experimental procedures were approved and conducted per the University of Waterloo and the Canadian Council of Animal Care guidelines (Animal Utilization Project Protocol #40315). Fish were housed in the University of Waterloo Aquatic Facility (Waterloo, ON, Canada), with a 12 h:12 h light-dark cycle, and fed daily with EWOS Vita (Floating Complete Fish Food for Salmonids) to satiety. Water was maintained at 13.5 ± 0.7 °C, pH 8.85, ~2000 μS, in well-aerated water. Fish were maintained at a density of four fish per 200 L tank. Experimental fish had a mean length of 30.5 ± 3.9 cm and a mass of 322 ± 138 g. Water flow was maintained at 3.6 ± 0.1 L/min. Flow through loading of the tanks was 0.350 ± 0.057 kg/L/min.

Control samples of plasma and epidermal mucus were collected from two rainbow trout and then water samples were collected from a tank containing two remaining fish (control samples). The remaining two rainbow trout were held out of water for 3 min. One hour following the 3-min in air, water was collected from the tank containing the stressed fish (1 h post-stress water sample). Immediately following collection of water, blood plasma and epidermal mucus were obtained (1 h post-stress mucus and blood samples). To ensure that both catecholamines and cortisol had been released or were being released, samples were collected 1-h post stress. Also, due to the water flow maintained in the tanks, an entire tank turnover was completed after 1 h. Three experimental replicates of the air stress experiment were performed (**Figure 5.1**).

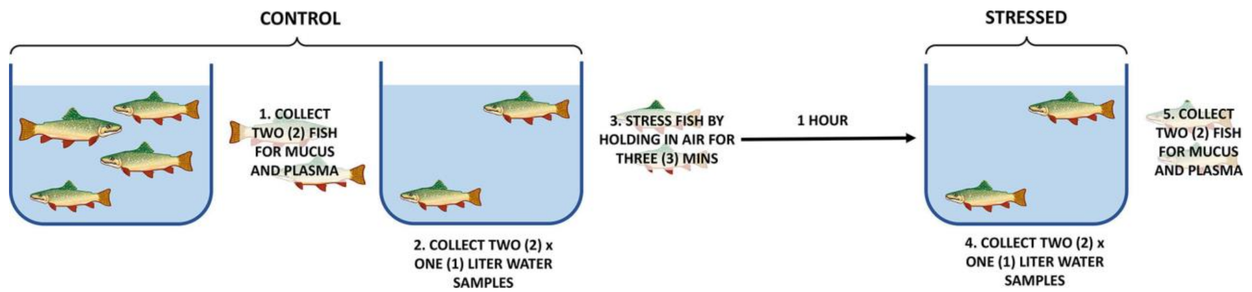

Figure 5.1 Experimental design.
This experiment was performed in triplicate. Plasma and mucus from one pre-stress fish and one post-stress fish were sequenced from each experimental replicate. One pre-stress water sample and one post-stress water sample were sequenced from each experimental replicate. The experimental order of sample collection was organized so that the same density of fish was present in the tank when the water collections occurred.

For collection of blood and mucus, fish were euthanized in a buffered MS-222 solution (0.5 g/L MS-222 in 1 g/L $NaHCO_3$). Mucus was collected by scraping epidermal mucus from the left side of rainbow trout, being cautious not to introduce any foreign DNA or RNA. This was accomplished by using a new pair of gloves cleaned with 70 % ethanol, using one hand to secure the trout by holding by the mouth and gill operculum, and using the other hand to scrape the mucus into a 2 mL cryotube. Mucus was placed on dry ice and stored at −80 °C until analysis. Blood was collected by caudal puncture using a 23 G needle fitted onto a 1 mL syringe and pre-coated with 0.5 M EDTA. One mL of blood was added to a 2 mL microcentrifuge tube containing 10 µL of 0.5 M EDTA to prevent coagulation. Blood was stored on ice until centrifugation at 1,300 × *g* for 10 min at 4 °C to collect plasma. Plasma was aliquoted into 200 µL and stored at −80 °C until RNA extraction. Only samples with no visible lysis were used for RNA extraction and sequencing.

Water was collected in 1 L amber bottles sterilized with 10 % bleach for 20 min, rinsed three times with ultrapure water, and autoclaved. Care was taken to collect water from the tank without introducing DNA or RNA. For example, the net used to capture and move fish during the 3 min air stress was placed into the water before control water samples were collected so that there would be no new nucleic acids introduced from the net. Samples of water were stored at 4 °C until filtered within 3 h of collection. Samples were filtered onto 0.45 µm cellulose nitrate filters (Thermo Scientific™ 1450045) in a sterilized laminar flow hood using a peristaltic pump. Filters were placed in 5 mL tubes on dry ice and stored at −80 °C.

5.3.2 Stress assessment

To validate that rainbow trout experienced a physiological response to the acute stress treatment, cortisol, lactate, and glucose in plasma were measured. To measure plasma cortisol, plasma samples were thawed on ice, diluted by a factor of ten, and cortisol was quantified using the Cortisol Saliva ELISA kit (TECO, Switzerland). To measure plasma lactate, plasma was thawed on ice, samples and standards were deproteinized by adding 200 µL ice cold 6 % perchloric acid, incubating on ice for 5 min, and centrifuging for 2 min at 18,000 × *g* at 4 °C. The supernatant was brought to a pH between 6.5 and 8.0 using potassium hydroxide and centrifuged at 18,000 × *g* for 15 min at 4 °C. The supernatant was added to the 96 well plate in triplicate and the reaction cocktail (320 mM glycine, 320 mM hydrazine monohydrate, 2.4 mM NAD+, 2 U/mL LDH) was added to a 96 well plate. A kinetic assay at 340 nm was run and lactate quantified (Bergmeyer, 1974). To measure plasma glucose, plasma was brought to room temperature, 4 µL was placed on a cleaned glass slide and an Accu-Chek Aviva Nano glucose monitor was used per the manufacturer's directions. Duplicate measurements were conducted on each sample. Data were tested for normality and equal variance. One-tailed t-tests were conducted on this data comparing control and stressed plasma samples. These data were tested with 95% confidence ($n = 3$).

5.3.3 MicroRNA Extraction and Sequencing

MicroRNAs were extracted from blood plasma by use of the Norgen Plasma/Serum RNA Purification Mini Kit (CAT 55000). Directions from the manufacturer were followed with a few modifications. Aliquots of plasma were thawed on ice and centrifuged for 2 min at 400 × *g* and 200 µL of cleared supernatant was extracted. During elution, 25 µL of Solution A was applied to the column and the eluate was transferred to the column again for maximum recovery of RNA. Following this, the extracted RNA was further processed using the Norgen RNA Clean-Up and Concentration Micro-Elute Kit (CAT 61000). With this kit, 75 µL RNase free water was added to the 25 µL of extracted RNA to bring the starting volume to 100 µL. Samples were vortexed for 10 s and spun at 14,000 × *g*, where no specified directions were given. Two separate elutions were performed using 15 µL of eluate. This ensured maximum recovery. RNA quantification occurred using the SoftMax SpectraDrop microplate.

MicroRNAs in mucus were extracted using the Qiagen miRNeasy Serum/Plasma Advanced Kit (CAT 217204). The manufacturer's directions were followed and 200 µL of mucus was used for the

extraction. For any non-specified centrifuge speed, 12,000 × *g* was used. RNA quantification was performed by Norgen Biotek using the RiboGreen™ RNA Assay on a fluorescent microplate reader.

Environmental miRNAs were extracted from water by use of the Norgen Water RNA/DNA Purification Kit (CAT 26480). The manufacturer's directions were followed with the following exceptions. Beads were transferred to the 5 mL tube where filters had been stored, and 1 mL lysis buffer E was added. Tubes were vortexed for 5 min on a flatbed vortex mixer at maximum speed. The initial 1 min spin at 20,000 × *g* was replaced with a 7 min spin at 3000 × *g* (maximum allowable speed for 5 mL tubes in centrifuge). Due to this modification, visual separation of lysate and filter was ensured before removing the supernatant for use in the remainder of the protocol. The optional DNase I treatment was applied (Norgen RNase-Free DNase I Kit – CAT 25710) to remove potential DNA contamination prior to sequencing. An optional addition of 50 µL elution solution to maximize yield was performed. RNA quantification was performed by Norgen Biotek using the RiboGreen™ RNA Assay on a fluorescent microplate reader.

Library prep and sequencing were performed by Norgen Biotek (Thorold, ON, Canada) using the Norgen Biotek Small RNA Library Prep Kit (CAT 63600). Samples (n = 3 for plasma, mucus, and water at both control and 1 h post-stress = total 18 samples) were sequenced using the Illumina NextSeq500 with a minimum 10 million read depth. The NextSeq 500/550 High Output Kit v2 (51 cycles using a 75-Cycle Kit) (Illumina, CAT FC-404-2005) was used as the sequencing reagent.

5.3.4 Bioinformatic Analysis

Bioinformatic analysis was accomplished using the miARma-Seq analysis workflow (version 1.7.2; Andrés-León et al., 2016; Andrés-León and Rojas, 2019). However, adapter removal, quality filtering, and differential expression analysis were performed independently of the miARma-Seq tool. Adapter removal and quality filtering, performed by cutadapt (version 2.10; used under python3.8), was conducted separately from the miARma-Seq tool due to the incompatibility of the version of cutadapt present in the miARma-Seq pipeline with the two-color chemistry of the Illumina NextSeq (Martin, 2011). The differential expression analysis was performed independently from the miARma-Seq tool because two methods of differential expression analysis, DESeq2 and edgeR, were used. edgeR is included within the miARma-Seq tool but not DESeq2, which was therefore performed independently. Similarly, additional analysis options were needed in edgeR than those included within the miARma-Seq tool, so this analysis was also performed separately. The overall

bioinformatic analysis followed the miARma-Seq tool workflow but only sequence quality checks, alignment, annotation, and read counting were performed using the miARma-Seq tool directly.

5.3.4.1 Quality Control of Raw Reads

To ensure that there were no issues with the raw fastq sequencing reads, quality control was performed using FastQC (version 0.11.9, Andrews, 2019). Metrics that were required to pass in this step for the reads to be included in downstream analysis were "per base sequence quality," "per tile sequence quality," "per sequence quality scores," "per base N content," and "sequence length distribution." FastQC metrics that were expected to fail for raw reads or for RNA-Seq were "per base sequence content," "per sequence GC content," "sequence duplication levels," "overrepresented sequences," and "adapter content." Highly abundant sequences in RNA-Seq caused the percentage of each base to deviate from the expected 25% frequency (Sheng et al., 2017). The per sequence GC content was narrower due to overexpression of certain sequences, a characteristic of RNA-Seq. More than one peak in GC content was observed as there are different types, and therefore lengths, of small RNA (Sheng et al., 2017). The miARma-Seq tool only annotates miRNAs therefore other small RNA were excluded from our analysis (Sheng et al., 2017). The level of duplication of sequences and overrepresented sequences metric failed as expected due to the large sequence overrepresentation and few unique sequences present in RNA-Seq reads. Refer to the Supplementary Methods for the configuration file used to run this analysis (precutadapt_fastqc_configfile.ini).

5.3.4.2 Adapter Removal and Quality Filtering of Raw Reads

Adapter removal and filtering was performed using cutadapt (version 2.10; used under python3.8; Martin, 2011). The adapter was removed and any bases less than a quality score of 28 (high quality) were trimmed (refer to may7_cutadapt2.10adapter_removal_and_trimming.sh in the Supplementary Methods). Next, sequencing files were further filtered to remove any sequences shorter than 18 bases or longer than 35 bases, as they are not considered useful in miRNA analysis (Andrés-León and Rojas, 2019; refer to may7_cutadapt2.10sizetrim.sh in the Supplementary Methods).

5.3.4.3 Quality Control of Trimmed Reads

FastQC analysis was performed again on the trimmed reads to ensure that only sequences with quality scores greater than 28, with lengths between 18 and 35 bases, and with no adapters were present. The

configuration file is available in the Supplementary Methods (Appendix E, postcutadapt_fastqc_configfile.ini).

5.3.4.4 Alignment, Annotation, and Read Counting

The *de novo* approach of the miARma-Seq pipeline was used since no rainbow trout miRNAs are present in the mirbase.org database. Reads were aligned to the rainbow trout genome, annotated using *Salmo salar* miRNA (closest salmonid relative in mirbase.org), due to the conservation in miRNA sequence identity between fish species (Salem et al., 2010), and counted. Viral miRNAs were excluded from differential expression analysis though they can share a seed sequence with eukaryotic miRNAs, as there is not full sequence similarity (Kincaid et al., 2012; Guo and Steitz, 2014; Mishra et al., 2020). Therefore, they would not be annotated and counted. Alignment was performed within miARma-Seq using bowtie1 (version 1.1.1) and annotation and read counting using mirDeep2 (2.0.1.2) (Langmead et al., 2009; Friedländer et al., 2012). Workflow and parameters are outlined in the miARma-Seq configuration file (Supplementary Methods – miARmaseq_denovo_ssa_configfile.ini).

For alignment and annotation, a bowtie1 index was created from the rainbow trout genome (GCF_002163495.1_Omyk_1.0_genomic.fna; downloaded April 9, 2019). The bowtie index is available at https://doi.org/10.6084/m9.figshare.12459905.v2 (Ikert, 2020a). Additional bowtie parameters were added to allow for only one mismatch (default is two) and to only report the best alignment, according to recommendations by Tam et al. (2015). The miARma-Seq script defaults were used for all remaining miRDeep2 tools (for description of arguments https://github.com/rajewsky-lab/mirdeep2). Also, it is crucial to note that miRDeep2 will count reads that originate from different precursors twice. miARma-Seq corrects for this by averaging the number of counts of mature miRNA from different precursors so that all the tags (miRNAs) are unique in the read count file. This allows for more accurate differential expression analysis. Quality control of the alignment, annotation, and read counting was performed by referring to the stats and log files, ensuring that no errors were present, and that the percentage of reads aligned versus unaligned were reasonable.

5.3.4.5 Differential Expression of MicroRNA

In order to identify miRNAs that were differentially expressed, the read count file from miARma-Seq was analyzed by using both edgeR and DESeq2 (Robinson and Smyth, 2007, 2008; Robinson and Oshlack, 2010; Robinson et al., 2010; McCarthy et al., 2012; Chen et al., 2014; Love et al., 2014; Zhou et al., 2014; Lun et al., 2016). These are tools recommended and validated for differential expression analysis for studies with a sample size of three and are able to detect effect sizes of a log-fold change greater than two (2.0) (Schurch et al., 2016). Since they both have well-developed models for analyzing RNA-Seq files and to avoid missing true positives, which is a concern in multiple comparison testing, both tools were used to create a list of differentially expressed miRNA for each matrix (Yendrek et al., 2012). It was expected that each tool would produce unique results because each conduct their analysis differently. edgeR normalizes sequencing reads by using the TMM (trimmed mean of m-values) method and filters using a nominal, user-defined CPM (counts-per-million) (Robinson and Oshlack, 2010). DESeq2 normalizes sequencing reads using a median of ratios method and filters by determining the optimal CPM threshold from several tested threshold values (Anders and Huber, 2010; Love et al., 2014). Scripts used for each of these tools and versions of packages for each are found in the Supplementary Methods (Appendix E, script_deseq2.R and script_edgeR.R).

For edgeR analysis, the read count file was split by sample type so that library normalization would not be affected by types of matrices. Samples were normalized by treatment groups and internally calculated lowly expressed genes were filtered. MDS plots were used to ensure that samples separated by treatment. Exact edgeR tests were conducted for mucus and plasma because they have two treatment groups (control vs. stressed) and samples are not paired since they were collected from separate fish in each treatment (**Figure 5.1**). For water, since the control and stressed samples were collected from the same tank of water, samples were paired. Therefore, data for water were analyzed by use of a generalized linear model using the fish number as the blocking factor. All results were exported after being sorted by false discovery rate (FDR) to identify significantly differentially expressed miRNA.

For analysis using DESeq2, the read count file was split by sample type so that library normalization would not be affected by types of samples. Following this, the DESeq2 data set was created from the matrix of read counts. This step filters and normalizes the read counts. For plasma and mucus, the design was solely based on the treatment (control vs. stressed), but due to the paired

nature of the water samples, the fish number was added in as the blocking factor. These DESeq2 datasets were used to conduct differential expression analysis. Results were obtained and ordered via adjusted p-value (equivalent to false discovery rate) and exported to identify significant results.

Statistical significance for differentially expressed miRNA was defined as miRNA with p values ≤ 0.05, adjusted p values or false discovery rates (FDR) < 0.1, and log fold changes greater than 2 and less than -2. These cutoffs were chosen based on acceptable thresholds for RNA-Seq data and by the amount of false positives that were allowed to ensure reliable data while minimizing false negative results (Renthal et al., 2018). The data produced in this study have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE151138 (Edgar et al., 2002; Barrett et al., 2013).

5.3.4.6 Target Prediction and Pathway Analysis of Differentially Expressed Plasma MicroRNA

The differentially expressed miRNAs in plasma samples were used to predict 3′UTR targets in silico for Salmo salar (the closest related species present in KEGG and DAVID databases) using the miRanda command line tool (Enright et al., 2003; Berthelot et al., 2014; Lien et al., 2016). miRanda uses sequence alignment based on complementarity and RNA stability of the binding (Zuker and Stiegler, 1981; McCaskill, 1990; Hofacker et al., 1994; Enright et al., 2003). Each known, significantly differentially expressed miRNA sequence in plasma was input into the miRanda tool and compared against a curated list of *Salmo salar* 3′ untranslated regions (UTRs) (Lynch, 2020; https://doi.org/10.6084/m9.figshare.12461969.v1). Since predicted miRNAs were not validated, novel differentially expressed miRNAs were omitted to improve reliability of the *in-silico* predictions. Only mRNA with a complementarity score greater than 140 and an energy score (Δ G) less than -20 were used for pathway analysis (Kostyniuk et al., 2019; Cadonic et al., 2020). The script can be accessed in the Supplementary Methods (one-miRNA-all-3UTR.miranda_analysis.sh; Appendix E).

All mRNA targets identified using miRanda were combined into a common list and converted to UniProt IDs (https://www.uniprot.org/uploadlists/). This list was submitted to KEGG Search & Color pathway (specifying *Salmo salar* and UniProt IDs; Kanehisa and Goto, 2000; Kanehisa, 2019; Kanehisa et al., 2019). These results were analyzed for known responses to acute stress and to curate a list of percentages of proteins targeted in pathways to identify enriched pathways. The combined Salmo salar UniProt ID list was also uploaded to the DAVID Functional Annotation tool (Huang et

al., 2009a, b; https://david.ncifcrf.gov/). The recommended options for functional clustering and medium stringency, as well as a cutoff of an enrichment score of −1.3 or less (equivalent to $p \leq 0.05$) were used (Huang et al., 2007). This analysis was only performed using altered plasma miRNA, as the biological role of mucus and water miRNA is unknown and pathway analysis of targets would be speculative.

5.3.5 Validation of Differentially Expressed MicroRNA Using RT-qPCR

The known, differentially expressed microRNA in water samples identified using RNA-Seq and subsequent analysis were validated by measuring expression via RT-qPCR. RNA from separate, non-sequenced plasma, mucus, and water samples was extracted using the RNA extraction techniques previously mentioned with the exception of the addition of 3.5 µL of 1.6×10^8 copies of RNA spike-in (cel-miR-39, Qiagen) following the lysis step. MicroRNA was quantified using the Qubit microRNA Assay kit (Thermo Fisher Scientific). To synthesize cDNA, two methods were used, converting a common volume of extracted miRNA to cDNA (common volume cDNA) and converting a common amount of miRNA to cDNA (common amount cDNA) to assess different methods of gene expression normalization (endogenous control U6, cel-39 spike-in RNA, or total amount of small RNA). For common volume cDNA, 12 µL extracted plasma RNA, 5 µL extracted mucus RNA, and 12 µL extracted water RNA were added to their respective reactions (HiSpec buffer; Qiagen miScript II RT kit). For common amount cDNA, 3 ng plasma miRNA and 140 ng mucus miRNA were added to their respective reactions (HiSpec buffer; Qiagen miScript II RT kit). No common amount cDNA was made for water samples. The qPCR reactions consisted of 5 µL 2× Bio-Rad SsoAdvanced SYBR, 1 µL of 5 µM forward primer (see **Table 5.1**), 1 µL of 5 µM Qiagen Universal Reverse Primer, 2 µL RNase-free water, and 1 µL 1:10 diluted cDNA performed in triplicate. The qPCR protocol consisted of a one-time polymerase activation step at 95 °C for 30 s, followed by denaturing at 95 °C for 10 s, annealing and extension at 60 °C for 20 s, and a plate read, which was repeated for a total of 40 cycles. Each qPCR run also included a melt curve analysis which was performed by increasing the temperature from 65 to 95 °C every 5 s in 0.5 °C increments with a plate read at each increment. Non-template controls were used to ensure no contamination or primer-dimerization occurred. Expression was calculated by using the relative quantity (as calculated by the Bio-Rad Maestro software) and then, as appropriate, normalized to one of the following: amount of miRNA input (only possible for common volume cDNA), synthetic cel-39 spike-in, and/or the relative quantity of endogenous control U6. Statistical analysis included testing for normality and

equal variances and performing a one-tailed t-test ($p < 0.05$, $n = 3$). For miR-26a-5p, no statistical analysis was performed as it was a presence/absence test. Pearson correlation coefficients were calculated to compare RNA-Seq and qPCR results. These values were calculated between the normalized read counts from the tool (DESeq2, edgeR) that identified the miRNA as differentially expressed and the RT-qPCR expression values.

Table 5.1 Primers used to measure microRNA expression via RT-qPCR.
The sample within which the targets were measured are indicated by the sample column. Where applicable, the miRbase.org or NCBI accession number is included. The miRNA sequences were all used as forward primers, with the Qiagen Universal Reverse primer as the reverse primer.

Name	Sample	Accession	Sequence (5′ to 3′)
ssa-miR-16b-5p	Plasma	**MIMAT0032408**	TAGCAGCACGTAAATATTGGTG
ssa-miR-30b-5p	Water, Mucus	**MIMAT0032604**	TGTAAACATCCCCGACTGGAAGCT
ssa-miR-26a-5p	Water, Mucus	**MIMAT0032563**	TTCAAGTAATCCAGGATAGGCT
U6 - Forward	Plasma, Mucus	**XR_005037161.1**	CTCGCTTCGGCAGCACATA
U6 – Reverse			AGGAACGCTTCACGAATTTGC
Ce_miR-39_1 miScript Primer Assay		**MIMAT0000010**	n/a
Qiagen Universal Reverse Primer			GAATCGAGCACCAGTTACGC

5.4 Results

5.4.1 Validation of Stress Response

To demonstrate that 3 min out of water in ambient air caused a physiological response in rainbow trout, plasma cortisol, lactate, and glucose in blood plasma were measured. One-hour post air exposure, a statistically significant 5-fold increase in plasma cortisol, a significant 7-fold increase in plasma lactate, and a significant 3-fold increase in plasma glucose were measured (one-tailed t-test, $n = 3$, $p < 0.05$; **Figure 5.2**). Data were normally distributed and had equal variance.

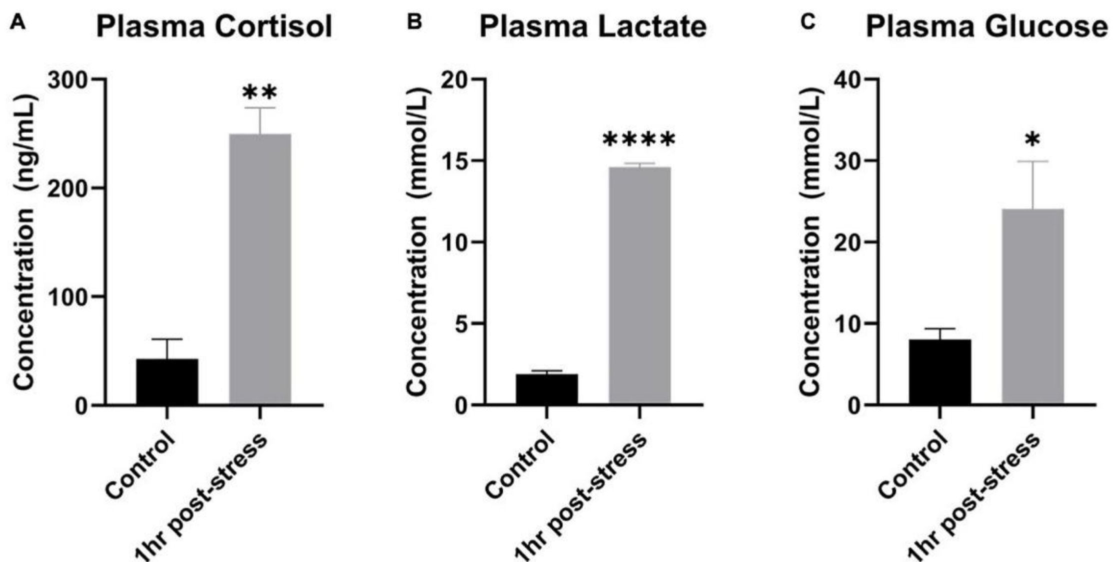

Figure 5.2 Plasma cortisol, lactate, and glucose increased following acute stress.
Mean ± SEM of plasma cortisol (**A**), lactate (**B**), and glucose (**C**) before (control) and 1 h after a 3-min air exposure stressor (1 h post-stress) in rainbow trout. Asterisks indicate a significant difference in the measure ($n = 3$; one-tailed t-test). A single asterisk indicates $p \leq 0.05$, double asterisks indicate $p \leq 0.01$, and four asterisks indicate $p \leq 0.0001$.

5.4.2 Sequencing Metadata and *in silico* Validation

Eighteen samples were sequenced, which includes six samples of plasma, six samples of mucus, and six samples of water (n = 3 for each treatment). Water had less total RNA than did plasma or mucus. However, there was no significant difference (two-way ANOVA; $n_{sample} = 6$, $n_{treatment} = 3$; $p < 0.05$; **Figure 5.3A**) between samples from control or stressed individuals. Sequencing depth of the samples was between 12 and 30 million reads and did not differ based on sample type or treatment

(two-way ANOVA; $n_{sample} = 6$, $n_{treatment} = 3$; $p < 0.05$; **Figure 5.3B**). Detailed RNA concentrations and read counts for each sample are available in the Supplementary Results (RNA concentrations and Read counts tabs; Appendix E). Heat maps for each sample type and analysis are provided (Heat maps_edgeR and Heat maps_DESeq2 tabs). Quality analysis was performed on raw and processed samples and the sequences passed all requirements for use in this analysis. Complete FastQC reports can be accessed in the Supplementary Quality Control File (Appendix E).

All sample types were mapped to the rainbow trout genome, with some variation in the amount mapped by sample type (**Figure 5.3C**). Overall, there were between 94 and 182 unique known miRNAs annotated in each sample (Supplementary Results – Number of microRNA tab; Appendix E). The miRNAs had a median read length of 23 nucleotides (**Figure 5.3D**). The log file from the alignment, annotation, and read counting through miARma-Seq indicated that all steps occurred without any errors. Complete stats and log files from the alignment, annotation, and read counting using miARma-Seq (which uses bowtie1 and miRDeep2) are available in the Supplementary Results (Stats file and Log file tabs; Appendix E).

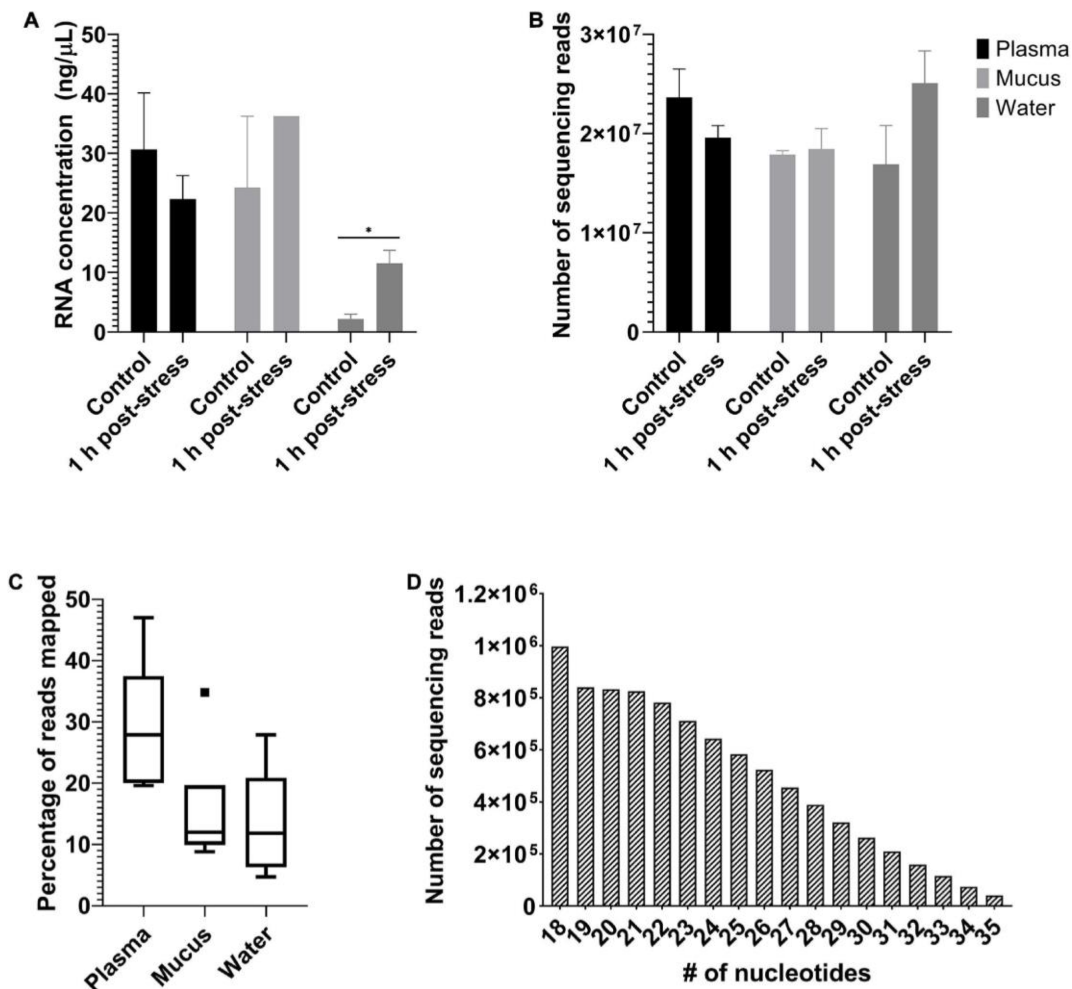

Figure 5.3 Sequencing metadata.
Mean ± SEM of (**A**) extracted RNA concentrations (ng/µL) and (**B**) number of sequencing reads from plasma, mucus, and water samples. Extracted RNA concentrations did not differ between treatments within each sample, but did differ as a function of sample type, with water being significantly less than those of plasma and mucus. Number of reads did not differ among treatments or sample type. Significant differences among samples indicated by an asterisk (two-way ANOVA, $n_{sample} = 6$, $n_{treatment} = 3$, $p \leq 0.05$). Box plot (**C**) of the percentage of sequencing reads mapped to the rainbow trout genome by miRDeep2. Minimum, first quartile, median, third quartile, and maximum are indicated by the horizontal lines (when reading from bottom to top). Outliers are indicated by a filled square. Histogram (**D**) of the number of sequencing reads at each read length between 18 and 35 nucleotides.

5.4.3 Identification of Differentially Expressed MicroRNA Using High Throughput Sequencing

High throughput sequencing and bioinformatic analysis were used to identify altered miRNA in rainbow trout plasma, mucus, and water in which the fish were held, following acute stress. The statistical tools used, edgeR and DESeq2, identified differentially expressed miRNA and though there were some shared results, most differentially expressed miRNAs identified by each tool are unique to that tool. The read sequences for all known and novel miRNAs identified in this study are deposited in figshare (Ikert, 2020b; https://doi.org/10.6084/m9.figshare.12974792.v1).

5.4.3.1 Differentially Expressed Plasma MicroRNA and *in silico* Target Prediction

Ten miRNAs were significantly altered in plasma following air exposure (**Table 5.2**). Plasma miRNAs increased with a log 2-fold change between 11 and 21 and miRNAs decreased with a log 2-fold change of −21. Both edgeR and DESeq2 identified miR-16b-5p as significantly altered with an increase of approximately 12-fold following acute stress.

Table 5.2 List of novel and known differentially expressed microRNA (miRNA) in plasma. The miRNAs referred to as *Oncorhynchus mykiss* genomic locations are novel and miRNAs referred to as ssa-miR-## are known. MicroRNAs that increase following the stressor are indicated by UP (yellow or lighter shade background) and those that decrease following the stressor are indicated by DOWN (blue background or darker shade background). If the differentially expressed miRNA was identified using edgeR or DESeq2, there is a log2 fold change and false discovery rate (FDR) indicated in their respective column. Expression of microRNAs with data in both edgeR and DESeq2 were determined to be significantly altered using both tools (FDR < 0.1). MicroRNAs are ordered from smallest to largest FDR.

		edgeR		DESeq2	
microRNA	Up/Down	log2FC	FDR	log2FC	FDR
NC_035085.1:26541150..26541213:+	UP			20.8	1.18E-05
NC_035088.1:67280345..67280412:-	UP			20.8	1.18E-05
NC_035088.1:75894388..75894432:-	DOWN			-21.4	1.18E-05
NC_035091.1:58934884..58934935:+	UP			20.8	1.18E-05
NC_035091.1:58942466..58942517:+	UP			20.8	1.18E-05
NC_035093.1:33779799..33779854:+	DOWN			-21.4	1.18E-05
NC_035096.1:21083292..21083351:+	DOWN			-21.4	1.18E-05
NC_035100.1:16889950..16889990:-	UP			20.8	1.18E-05
ssa-miR-16b-5p	UP	12.2	9.93E-02	11.7	1.72E-02
ssa-miR-16c-5p	UP			12.8	9.15E-02

Two known miRNAs, ssa-16b-5p and ssa-16c-5p, were upregulated with an approximate log 2-fold change of 12 following stress (**Table 5.2**). These two known miRNAs were used to predict mRNA targets *in silico*. The miRanda algorithm predicted that miR-16b-5p targeted 2517 mRNA and miR-16c-5p targeted 3171 mRNA. These results were combined for gene enrichment (DAVID) and pathway analysis (KEGG) as both miRNAs were increased so the targeted mRNA would all be predicted to decrease. DAVID functional enrichment analysis indicated a statistically significant decrease in transmembrane proteins and their activity (Supplementary Results – DAVID results tab; Appendix E). Biosynthetic, degradation, and metabolic KEGG pathways were predicted to be targeted by altered plasma miRNAs (**Table 5.3**). Thirteen mRNA in the adrenergic signaling in cardiomyocytes KEGG pathway were targeted. Specifically, the beta-2-adrenergic receptor, which binds adrenaline and noradrenaline, was predicted to be downregulated via miR-16b-5p and miR-16c-5p. Complete results from miRanda target prediction, KEGG pathway analysis, and DAVID functional enrichment analysis can be found in the Supplementary Results (miRanda results 16b-5p, miRanda results 16c-5p, miRanda combined UniProt, KEGG results, KEGG percentages of pathways tabs; Appendix E).

Table 5.3 Altered plasma miRNAs target biosynthetic, degradation, and metabolic KEGG pathways.
The number of targeted mRNA in each KEGG pathway was compared to the total number of mRNA in each pathway to identify pathways with large percentages of mRNA targeted. Only pathways with five percent or more of pathway targeted where included.

KEGG pathway	% of pathway targeted	Number of mRNA targeted	Number of mRNA in pathway
sasa00280 Valine, leucine and isoleucine degradation	30.00	3	10
sasa00232 Caffeine metabolism	25.00	1	4
sasa00514 Other types of O-glycan biosynthesis	16.36	9	55
sasa05132 Salmonella infection	9.81	21	214
sasa00515 Mannose type O-glycan biosynthesis	9.72	7	72
sasa00533 Glycosaminoglycan biosynthesis - keratan sulfate	9.43	5	53
sasa00601 Glycosphingolipid biosynthesis - lacto and neolacto series	8.99	8	89
sasa00072 Synthesis and degradation of ketone bodies	7.14	1	14
sasa00563 Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	6.25	2	32
sasa00260 Glycine, serine and threonine metabolism	6.10	5	82
sasa00511 Other glycan degradation	5.88	2	34
sasa00512 Mucin type O-glycan biosynthesis	5.62	5	89
sasa00534 Glycosaminoglycan biosynthesis - heparan sulfate / heparin	5.13	4	78
sasa00603 Glycosphingolipid biosynthesis - globo and isoglobo series	5.08	3	59

5.4.3.2 Differentially Expressed MicroRNA in Mucus

Abundances of sixteen miRNAs were significantly altered in mucus following the stress caused by being out of water (**Table 5.4**). Seven known miRNAs were altered following stress (**Table 5.4**). Differentially expressed mucus miRNAs increased with a magnitude between a log2 fold change of 8 and 30 and decreased with a magnitude between a log2 fold change of −9 and −28. One miRNA, miR-26a5p, was identified by both edgeR and DESeq2 to increase by a log2 fold change of 15.

Table 5.4 List of novel and known differentially expressed microRNA (miRNA) in mucus. The miRNAs referred to as *Oncorhynchus mykiss* genomic locations are novel and miRNAs referred to as ssa-miR-## are known. MicroRNAs that increase following the stressor are indicated by UP (yellow or lighter shade background) and those that decrease following the stressor are indicated by DOWN (blue background or darker shade background). If the differentially expressed miRNA was identified using edgeR or DESeq2, there is a log2 fold change and false discovery rate (FDR) indicated in their respective column. Expression of microRNAs with data in both edgeR and DESeq2 were determined to be significantly altered using both tools (FDR < 0.1). MicroRNAs are ordered from smallest to largest FDR.

		edgeR		DESeq2	
microRNA	Up/Down	log2FC	FDR	log2FC	FDR
NC_035085.1:61423757..61423832:-	UP			29.8	3.73E-14
ssa-miR-30b-5p	DOWN			-27.7	7.88E-13
NC_035101.1:13572153..13572243:-	DOWN			-22.7	3.94E-07
NC_035092.1:51254681..51254742:+	DOWN			-22.2	6.41E-07
NC_035097.1:4654575..4654638:+	DOWN			-21.9	8.55E-07
NC_035083.1:7009417..7009490:+	UP			21.6	1.02E-06
NC_035078.1:25630487..25630534:-	UP			21.4	1.23E-06
ssa-miR-26a-5p	UP	15.2	6.38E-03	14.4	2.40E-05
ssa-let-7b-5p	DOWN			-11.6	2.03E-02
ssa-miR-125a-5p	DOWN			-11.4	2.06E-02
ssa-miR-24a-3p	UP			10.4	5.42E-02
ssa-miR-142a-5p	DOWN			-10.0	7.52E-02
NC_035083.1:76251544..76251598:-	DOWN			-9.6	7.81E-02
NC_035094.1:43812635..43812699:-	UP			8.3	8.09E-02
NC_035097.1:4654529..4654594:+	DOWN			-9.2	9.91E-02
ssa-miR-26d-5p	DOWN	-9.6	7.76E-02		

5.4.3.3 Differentially Expressed MicroRNA in Water

Abundances of thirty-seven miRNAs were significantly altered in water following stress (**Table 5.5** & **Table 5.6**). Twelve known miRNAs were upregulated following stress (**Table 5.5**). Differentially expressed miRNAs in water increased between a log2 fold change between 7 and 20 and decreased with a log2 fold change between −3 and −30. Six miRNAs, five known and one novel, were identified by both edgeR and DESeq2 to be differentially expressed.

Table 5.5 List of known differentially expressed microRNA (miRNA) in water. MicroRNAs that increase following the stressor are indicated by UP (yellow or lighter shade background) and those that decrease following the stressor are indicated by DOWN (blue background or darker shade background). If the differentially expressed miRNA was identified using edgeR or DESeq2, there is a log2 fold change and false discovery rate (FDR) indicated in their respective column. Expression of microRNAs with data in both edgeR and DESeq2 were determined to be significantly altered using both tools. MicroRNAs are ordered from smallest to largest FDR.

		edgeR		DESeq2	
microRNA	**Up/Down**	**log2FC**	**FDR**	**log2FC**	**FDR**
ssa-miR-30b-5p	UP	14.50	2.75E-05	11.54	2.36E-03
ssa-miR-26a-5p	UP			12.62	9.07E-03
ssa-miR-128-4-5p	UP	13.29	4.83E-05	10.34	9.93E-03
ssa-miR-125b-5p	UP			10.15	1.12E-02
ssa-miR-27c-3p	UP	13.10	4.83E-05	10.14	1.12E-02
ssa-miR-27c-5p	UP	13.10	4.83E-05	10.14	1.12E-02
ssa-miR-16a-5p	UP			9.40	2.33E-02
ssa-miR-128-2-5p	UP			8.83	3.71E-02
ssa-miR-128-3-5p	UP			8.83	3.71E-02
ssa-miR-26b-5p	DOWN	-5.69	1.47E-07	-5.77	8.51E-02
ssa-let-7d-3p	UP	8.71	4.46E-03		
ssa-miR-29a-5p	UP	7.41	4.17E-02		

Table 5.6 List of novel differentially expressed microRNA (miRNA) in water. MicroRNAs that increase following the stressor are indicated by UP (yellow or lighter shade background) and those that decrease following the stressor are indicated by DOWN (blue or darker shade background). If the differentially expressed miRNA was identified using edgeR or DESeq2, there is a log2 fold change and false discovery rate (FDR) indicated in their respective column. Expression of microRNAs with data in both edgeR and DESeq2 were determined to be significantly altered using both tools. MicroRNAs are ordered from smallest to largest FDR.

		edgeR		DESeq2	
microRNA	**Up/Down**	**log2FC**	**FDR**	**log2FC**	**FDR**
NC_035091.1:38285981..38286031:-	DOWN			-30.00	4.48E-15
NW_018553268.1:46749..46815:-	DOWN			-30.00	4.48E-15
NW_018553268.1:50634..50700:-	DOWN			-30.00	4.48E-15
NC_035103.1:8461012..8461090:-	DOWN			-29.53	1.03E-14
NC_035088.1:59475872..59475914:-	DOWN			-17.86	4.69E-07
NW_018586303.1:4270..4365:-	UP			20.25	8.26E-07
NC_035077.1:28628390..28628451:+	UP			15.46	1.37E-05
NC_035077.1:28628392..28628451:+	DOWN			-17.71	1.37E-05
NC_035078.1:58142056..58142117:+	UP			15.46	1.37E-05
NC_035078.1:58142058..58142117:+	DOWN			-17.71	1.37E-05
NC_035087.1:193452..193514:+	DOWN			-16.40	5.81E-05
NC_035078.1:79936510..79936599:+	DOWN			-13.04	2.64E-03
NC_035093.1:40341222..40341275:+	DOWN			-10.61	2.33E-02
NC_035105.1:28003733..28003771:+	DOWN			-10.53	2.33E-02
NC_035093.1:20336754..20336817:-	DOWN			-9.83	4.38E-02
NC_035081.1:25546959..25547017:-	UP	11.30	6.95E-05	8.31	6.90E-02
NC_035093.1:20338603..20338666:-	DOWN			-8.91	9.65E-02
NC_035091.1:24851800..24851884:+	DOWN			-8.82	9.88E-02
NC_035077.1:6400391..6400472:-	DOWN	-3.66	3.50E-04		
NC_035085.1:41468002..41468063:+	UP	6.99	7.08E-02		
NC_035086.1:39319785..39319840:-	UP	8.03	1.62E-02		
NC_035088.1:63936169..63936225:-	UP	7.69	2.65E-02		
NC_035097.1:19576344..19576407:+	UP	7.37	4.17E-02		
NC_035098.1:18940944..18941013:+	DOWN	-3.25	2.99E-03		
NC_035100.1:18059421..18059494:+	DOWN	-3.42	8.85E-04		

5.4.3.4 Comparison of Known Differentially Expressed MicroRNA in All Sample Types

Differentially expressed miRNAs were compared across sample types (plasma, mucus, water) to understand whether altered miRNA profiles are conserved between sample types. No altered miRNAs were shared between plasma and the other sample types (**Figure 5.4**). Only two differentially

expressed miRNAs, miR-30b-5p and miR-26a-5p, were shared between water and mucus (**Figure 5.4**). Mucus and water had inverse expressions of miR-30b-5p (decreased in mucus, increased in water), whereas miR-26a-5p was increased in both mucus and water samples taken from 1 h post air-stressed fish (**Table 5.4** & **Table 5.5**).

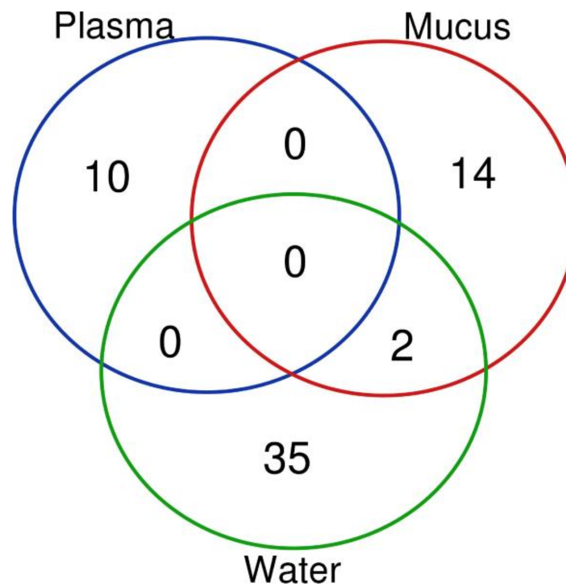

Figure 5.4 A Venn diagram of all differentially expressed microRNA in each sample type. Differentially expressed miRNAs ($p \leq 0.05$, FDR < 0.1, log fold change < |2|) shared between each sample type are indicated by the overlap of the circle and by the number of miRNAs that are shared. Altered miRNA profiles were mostly unique to matrix type.

5.4.4 Validation of Differentially Expressed MicroRNA Using RT-qPCR

Differentially expressed miRNAs identified via RNA-Seq were measured via RT-qPCR in un-sequenced samples to validate the RNA-Seq results and to develop a RT-qPCR assay for measuring miRNA expression changes in water. Several normalization methods for miRNA expression were tested during RT-qPCR validation. Expression of ssa-mir-16b-5p in plasma samples was normalized by using a common miRNA input amount (3 ng) into the cDNA reaction. Expression of ssa-miR-30b-5p in mucus samples was normalized by using a common miRNA input amount (140 ng) into the cDNA reaction and normalizing to cel-miR-39 expression (corrected for input volume of spike-in).

Expression of ssa-miR-30b-5p and ssa-miR-26a-5p in water samples was normalized by using a common miRNA volume input (12 µL) and normalizing to miRNA input (quantified by Qubit).

In plasma samples, ssa-miR-16b-5p expression increased by a log2 fold change of 1.01 1-h post-stress ($p = 0.24$; **Figure 5.5A**). Correlation between RNA-Seq and RT-qPCR values in plasma was 0.76 (DESeq2) and 0.89 (edgeR). In mucus samples, ssa-miR-30b-5p expression decreased by a log2 fold change of −1.40 1-h post stress ($p = 0.054$; **Figure 5.5B**). Correlation between RNA-Seq and RT-qPCR values in mucus was −0.17 (DESeq2). In water samples, ssa-miR-30b-5p expression increased by a log2 fold change of 2.05 ($p = 0.0061$) 1-h post-stress (**Figure 5.5C**). In addition, ssa-miR-26a-5p was only measured in the stressed samples (present) and was absent in the control samples (**Figure 5.5D**). Correlation between RNA-Seq and RT-qPCR values in water was 0.93 (DESeq2) and 0.94 (edgeR) for miR-30b and 0.98 (DESeq2) for miR-26a. Relative expression and calculations are found in the Supplementary Results (qPCR_results tab; Appendix E).

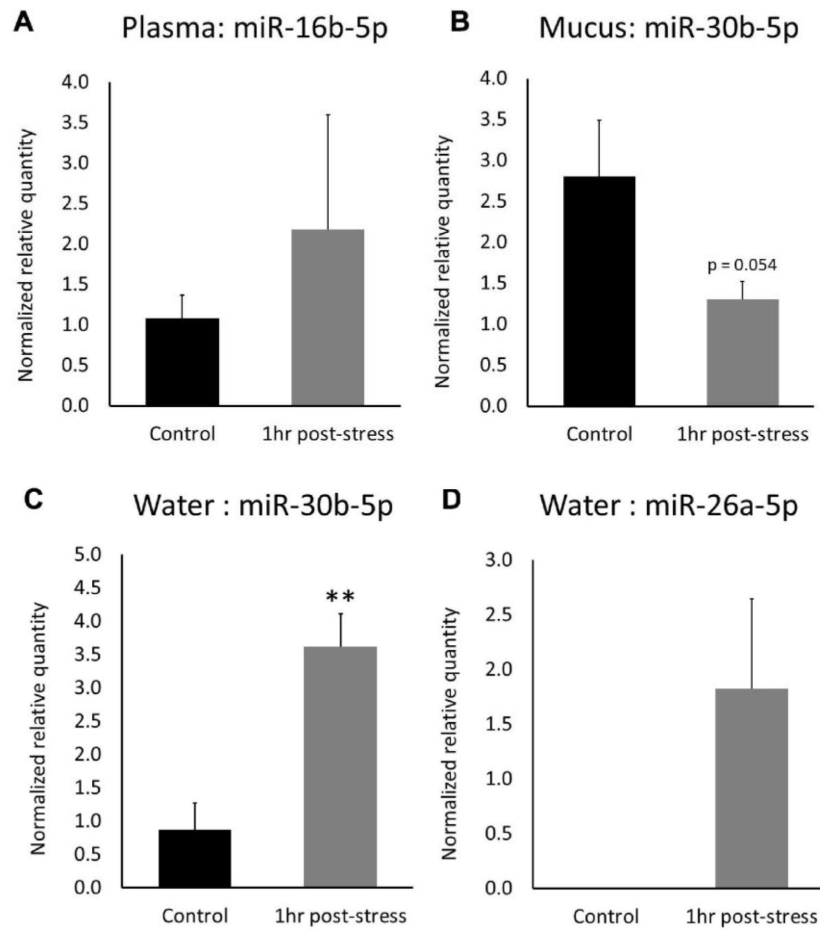

Figure 5.5 RT-qPCR expression of differentially expressed miRNAs.
Mean expression ± SEM of (**A**) ssa-miR-16b-5p in plasma, (**B**) ssa-miR-30b-5p in mucus, (**C**) ssa-miR-30b-5p in water, and (**D**) ssa-miR-26a-5p in water. Significant differences between treatments are indicated by asterisks (one-tailed t-test, $n = 3$, $p < 0.05$).

5.5 Discussion

The objective of this study was to understand how microRNAs of rainbow trout are altered in plasma, mucus, and water in response to acute stress. The changes in plasma miRNA expression 1-h post stressor were determined. MicroRNAs were isolated from epidermal mucus and surrounding water. Alterations in relative amounts of miRNAs were observed in both mucus and water following acute stress. One-hour after acute stress there were only two significantly altered miRNAs that were identified in both mucus and adjacent water.

5.5.1 Role of Plasma MicroRNA in Physiological Responses to Stress

Following acute stress, seven miRNAs in plasma were upregulated, and three miRNAs were downregulated. This is the first identification of all miRNAs in blood plasma following acute stress of a teleost. The current study was restricted to examining the profile of miRNAs in plasma at a single time. Additional studies are required to further describe the dynamics or responses of miRNAs to stressor time points within 1 h, when cortisol is increasing, and after 1 h, as the stress response is decreasing.

The altered profile of miRNAs in plasma was used to perform pathway analyses *in silico* to identify putative targets of miRNA. That analysis identified targeted pathways of biosynthesis, degradation, and metabolism. None of the targets are associated with well-known molecular stress response signaling, such as the glucocorticoid receptor (Ducouret et al., 1995). However, miR-16b-5p and miR-16c-5p were predicted to cause a decrease in thirteen targets of the adrenergic signaling in cardiomyocytes, including receptors for adrenaline and noradrenaline, the catecholamines that increase within seconds to minutes of the acute stress response, which would be predicted to be less active at 1-h post stress (Randall and Ferry, 1992; Wendelaar Bonga, 1997; Tort, 2013). These results are *in silico* predictions and the ability of miR-16b-5p and miR-16c-5p to target the mRNA would need to be validated by use of 3′UTR luciferase assays (Zeng and Cullen, 2003). Predictions made *in silico* during the current study are useful in developing future hypotheses to further understanding of how acute responses to stress are regulated in fishes.

5.5.2 Benefits and Limitations of Each Non-lethal Sampling Location

MicroRNAs in plasma, mucus, and water can all be collected non-lethally, but each sample type has benefits and limitations. These are discussed here to provide applications and advise areas of future

study of altered miRNAs in each of these sample types. For both plasma and mucus, miRNA can be associated with specific fish being sampled. However, fish must be caught and handled, which is invasive and time consuming and can confound the acute stress response (Brydges et al., 2009). Measurements of miRNA in plasma have the benefit of being associated with internal tissue responses as biomarkers of disease states (Cui and Cui, 2020). However, non-lethal sampling of blood from fish for identification and quantification of miRNA conservatively requires a minimum fish mass of 500 g. This is due to the most conservative, non-lethal blood sampling guideline to take no more than 1 mL blood per 1 kg of total fish mass and the requirement of 0.5 mL of blood to collect the 0.2 mL plasma required to extract miRNA (Canadian Council on Animal Care, 2005; Hawkins et al., 2011; Lawrence et al., 2020). Additionally, many sentinel species are small-bodied fish, which would require lethal sampling to collect blood plasma, so less invasive methods are advised (Bahamonde et al., 2015; Thornton et al., 2017; Arlos et al., 2018). Mucus can be collected non-lethally from fish smaller than 500 g, though the amount of mucus that can be collected from a fish without causing unacceptable stress or resulting in infections is not yet defined (Canadian Council on Animal Care, 2005). Though changes in miRNAs in mucus following acute stress have been suggested as a safe and effective way to monitor for stress responses, the biological roles of miRNAs associated with mucus are not yet understood. Collection of water in which fish have resided is completely non-invasive, requires limited training, and can be collected regardless of season. However, miRNA measured in the water column are not associated with one organism and can only be associated with a particular fish species if there is only a single species present in the water. Within environmental samples, plant and animal miRNA can be differentiated easily based on sequence (Hertel and Stadler, 2015). However, due to conservation of miRNA between fish and other vertebrates, measurement of miRNA from the aquatic environment would be indicative of the entire fish or vertebrate community (Hertel and Stadler, 2015). Though this could be detrimental when attempting to study the health status of a particular fish species, this can be leveraged as a method to understand the health status of the entire aquatic community; removing the need to use only fish or a particular fish species as sentinels of environmental health.

5.5.3 Unknown Sources of MicroRNAs in Mucus and Water

Two of the sixteen differentially expressed mucus miRNAs also had altered levels in water, which indicates mucus is a possible source of some of the miRNAs. This was expected since one source of DNA in water (eDNA) is mucus of fishes, but the actual source or sources of miRNAs in water is not

known and mucus could be only one source of the 37 altered miRNA in water. None of the altered plasma miRNA profile is shared with the mucus or water miRNA profiles indicating that perhaps secretion of miRNA from plasma into mucus or water does not occur immediately. These unique profiles of miRNA indicate that the source of altered miRNA in water is not known 1 h after acute stress. Sources of mucus itself are goblet, sacciform, and club cells (Fasulo et al., 1993; Shephard, 1993; Zaccone et al., 2001). MicroRNAs in mucus could be packaged in exosomes or associated with proteins and secreted from these cells or epidermal cells themselves. eDNA of fishes originates from feces, urine, gametes, mucus, scales, blood, and secretions (Höss et al., 1992; Valiere and Taberlet, 2000; Ficetola et al., 2008; Jerde et al., 2011). Therefore, miRNA can be sampled from these locations and compared to profiles of miRNA in mucus and water to better understand sources of environmental miRNA. Differentially expressed miRNA in each of the samples could be fluorescently labeled to track paths of secretion (Manca et al., 2018). Identifying the destination of plasma miRNA and the source of mucus and water miRNA would allow us to understand the role of miRNA in responding to stress and facilitate the development of miRNA as a reliable, non-lethal biomarker of stress.

5.5.4 Considerations for RNA-Seq Analysis of MicroRNA and RT-qPCR Validation

To identify differentially expressed miRNA in sequenced samples from rainbow trout, the miARma-Seq pipeline was applied (Andrés-León et al., 2016; Andrés-León and Rojas, 2019). This pipeline and the results that it produced have been tested *in silico*. Since rainbow trout are not a model organism and their miRNAs are not present in miRbase, even though they have been sequenced (Juanchich et al., 2016), alternative references and indexes had to be curated for this analysis and can be used in future analyses. The customized options have been outlined in the methods and resources are available in the Supplementary Material (Appendix E). These options and resources can be useful to others analyzing rainbow trout miRNA via RNA-Seq.

A concern when analyzing these RNA-Seq data was the low mapping percentages of reads to the rainbow trout genome and Atlantic salmon miRNA. When comparing plasma mapping percentages, it was found to be within range of human plasma miRNA mapping (Dufourd et al., 2019). The mapping percentages were less for mucus and water and because miRNA in these samples had not been measured previously, there is no comparison to ensure this is acceptable. The observed lesser percentages can be attributed to other environmental sources of RNA present. Therefore, future

sequencing of environmental samples could focus on enriching miRNA prior to sequencing as well as further *in silico* filtering of extraneous RNA. The unmapped RNA can be identified and analyzed to determine if other small RNA are altered following acute stress. These mapping percentages of miRNA present in mucus and water can be used as a threshold for future research.

To validate the RNA-Seq results and the miRNAs as biomarkers, miRNAs (ssa-miR-16b-5p, ssa-miR-30b-5p, ssa-miR-26a-5p) were measured via RT-qPCR in un-sequenced plasma, mucus, and water samples. The RNA-Seq and RT-qPCR results are highly correlated when comparing statistically significant results and using Pearson's correlation coefficient. However, though miRNA expression has extensively been measured in plasma (Mitchell et al., 2008; Wu et al., 2012; Sourvinou et al., 2013; Brunet-Vega et al., 2015; Vigneron et al., 2016; Zhao et al., 2016; Scrutinio et al., 2017; Binderup et al., 2018; Poel et al., 2018), and has been measured once in mucus (Zhao et al., 2020) using RT-qPCR, there is still much debate as to the appropriate methods of normalization. A number of methods (common miRNA input amount and volume, synthetic spike-in, and a small RNA reference gene) were tested during our validation. However, more robust methods should be established. Here, miRNA expression has been measured from water samples via RT-qPCR for the first time and determined that normalizing to miRNA input amount is a successful method in this sample type. Therefore, the RT-qPCR validation confirms the RNA-Seq results, extending the usefulness of these miRNAs as biomarkers since the qPCR validation was performed on separate samples, and highlighting the potential to develop more robust methods of measuring miRNA expression in plasma, mucus, and water samples.

5.5.5 Future Development of MicroRNA as Non-lethal Indicators of Stress

Since changes in expression patterns of miRNA in blood, mucus, and water following acute stress in rainbow trout were observed, this work can be expanded by measuring changes in miRNA in response to other stressors and in other aquatic organisms to test the conservation of the miRNA response. Due to the ability to non-lethally measure miRNA and the potential functional relationships to internal mRNA changes, blood, mucus, and/or water miRNA could be developed as biomarkers of stress in fish and the aquatic environment. The benefit to developing miRNA as indicators of fish stress is that in tissues, specific miRNAs are altered in response to specific stressors (ex., Gonçalves et al., 2020; Liu et al., 2020a, b; Qiang et al., 2020). Therefore, if this is true in non-lethal samples, non-lethally collected miRNA can provide not only an early indicator of fish stress but also provide

an indicator of what type of fish stress (ex., temp, metals) is being experienced, allowing for earlier, targeted remediation to be implemented. Due to the current collection and analysis of DNA in the water being termed environmental DNA (eDNA), we propose that measurements of miRNA in water be termed environmental miRNA (e-microRNA or e-miRNA). E-miRNA can currently be extracted from the same water samples or filter used in eDNA collection and can potentially be developed as a method of simultaneously measuring fish stress as well as fish species present.

5.6 Manuscript Details

5.6.1 Data Availability Statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: the NCBI Gene Expression Omnibus (GSE151138).

5.6.2 Thank you

The authors thank Nathan Bennoit, Ivan Cadonic, Huming Guo, Dilpreet Matharu, Nicolas Mourad, Tina Papazotos, and Karyn Robichaud for their help conducting the rainbow trout air exposures.

5.6.3 References

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Chapter 6

General Conclusions

6.1 Overall objectives and findings

The overall objective of my thesis was to further understand how fish miRNAs are regulated in response to acute and environmental stress. Specifically, the aim was to further understand the miRNA response at different timepoints, in different tissues (liver, muscle, gonad) or samples (plasma, mucus, water), and in different species of fish (zebrafish, brook trout, rainbow trout) following stress. Also, the mRNA, protein, and enzyme response that the miRNAs were predicted to be regulating were measured to identify if an inverse relationship existed (preliminary indication of miRNA regulation) and to further characterize the molecular and physiological response to stress.

It was found that VFX on its own or in combination with increased water temperature altered zebrafish gonad miRNAs (**Figure 6.1**). VFX, increased water temperature, and decreased dissolved oxygen altered zebrafish liver and muscle miRNA, mRNA, and proteins. Acute air exposure altered rainbow and brook trout liver miRNA, mRNA, and enzyme activities. Acute air exposure altered rainbow trout miRNA in plasma, mucus, and surrounding water samples. Altogether, the miRNA response to stress depended on the tissue type, was described in different species. Furthermore, the mRNA, protein and enzyme responses were described in response to stressor exposure.

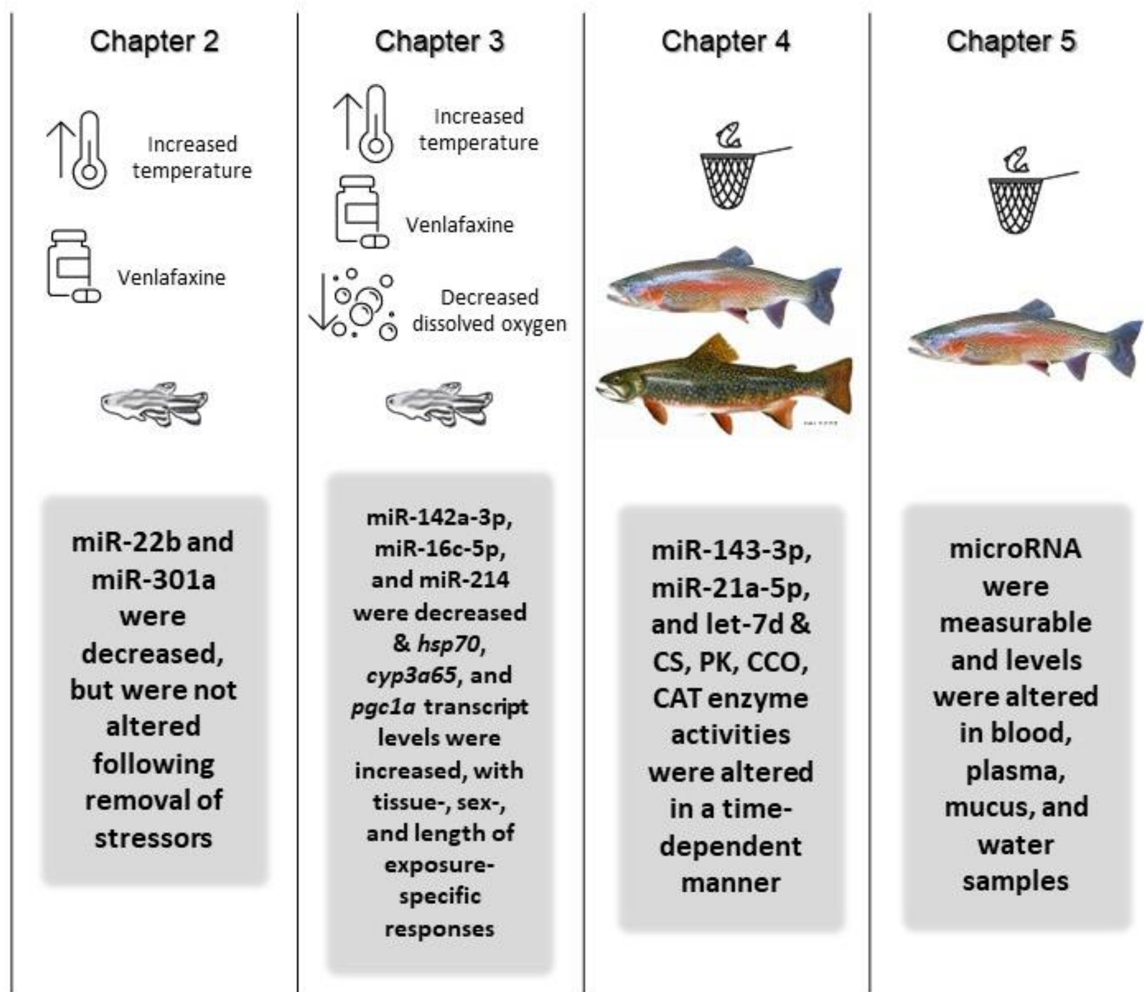

Figure 6.1 Graphical summary of thesis research findings.
The stressors, fish species, and findings measured are depicted under the respective chapter heading. In Chapters 2 and 3 miRNA changes were measured in zebrafish following anthropogenic stressors and in Chapters 4 and 5 miRNA changes were measured in salmonids (Chapter 4: rainbow and brook trout; Chapter 5: rainbow trout) following acute air exposure. The time-sensitiveness of the miRNA responses was measured in Chapters 2, 3, and 4. Downstream molecular responses (ex. mRNA) were measured in Chapters 3 and 4. The ability to measure altered miRNA levels in non-lethal samples was measured in Chapter 5.

6.2 Strengths and limitations of microRNA binding predictions

Throughout my thesis, I leveraged genetic resources and miRNA target prediction algorithms to identify miRNA that would be predicted to respond to air exposure or anthropogenic stressors. These miRNAs were used in hypothesis development and to understand their potential regulatory role in the

stress response. However, as observed by only three of the ten miRNAs responding to multiple stressor exposure in Chapter 3, and the lack of correlation to predicted mRNA and protein targets in Chapters 3 and 4, this method was minimally successful. This indicates that there are limitations with current *in silico* miRNA target prediction algorithms and highlights the complexity of how miRNAs post-transcriptionally regulate.

Alternative methods of predicting which miRNAs will respond to stress in fish are available and could be used in place of or alongside *in silico* prediction methods to improve their robustness. Prediction of miRNA that will be altered in response to different stressors can be achieved by identifying miRNAs that are altered in similar stress responses in other organisms. This is possible due to the sequence conservation of miRNAs and conservation of some of the 3′UTR sequences (Kuc et al., 2017). Also, the ability of several miRNAs to target and regulate specific mRNAs has been validated (ex. Johnston et al., 2019), so using validated relationships in hypothesis building can increase the robustness of the predicted miRNA response. Sequencing can also be used to identify miRNAs being altered in response to stress in unstudied conditions or targets, though the cost of analysis can be prohibitive. Therefore, alternative methods of predicting and identifying miRNA that respond to stress can be used on their own or in combination with *in silico* tools.

The miRNA target prediction algorithm workflows that I employed in my thesis can still be useful but are perhaps more robust when employed in a slightly different way. Instead of selecting only the miRNA that has the highest likelihood of binding to each mRNA of interest, all of the miRNAs that are computationally predicted to bind and regulate the mRNA can be identified and measured. Or more realistically, at least the top ten miRNAs predicted to bind can be selected and measured using a RT-qPCR approach (potentially with a customized PCR panel, such as the miRCURY LNA miRNA Custom PCR Panel). In this way, a targeted, more affordable approach can be used while ensuring a greater likelihood of identifying whether one or more miRNAs are responding to the stressor of interest. In some instances, more than one miRNA is needed to post-transcriptionally regulate a particular mRNA (ex. Zhou et al., 2016), which could be identified in this approach. Therefore, using miRNA target prediction algorithms to identify all miRNAs predicted to bind to a particular mRNA of interest could be a beneficial approach to identifying miRNA involved in a variety of stress responses.

6.3 MicroRNA as biomarkers: Strengths, weaknesses, and current applications

MiRNAs are potential biomarkers of acute and environmental stress in fish. Biomarkers are early warning signals related to negative biological impacts of the stressor and ideally are easy to measure, specific to the length and severity of the stressor, and can be non-lethally or non-invasively sampled (Kaviraj et al., 2014; Peakall & Walker, 1994; van der Oost et al., 2003). Now that I have measured the miRNA response to acute and environmental stress in zebrafish, rainbow, and brook trout, I have increased insight into the strengths and weaknesses of using miRNA as biomarkers, which leads to areas of future study and potential current applications of use of miRNAs as biomarkers.

MiRNAs show potential to be used as biomarkers of stress in fish as they can be easily and specifically measured using both RT-qPCR and RNA sequencing (Ch. 2-5). In fact, miRNAs were able to be quantified in brook trout and water samples, a species and sample type in which no miRNAs had been previously characterized (Ch. 4&5). Therefore, not only can miRNAs be measured with robust techniques, but these techniques can also be used in new species and a variety of sample types. The ability to measure miRNAs non-lethally and especially non-invasively provides an exciting new avenue of research and would greatly add to the usefulness of miRNAs as a biomarker since fish health could be monitored without needing to catch and/or euthanize fish (Ch. 5). Furthermore, a preliminary study that I conducted to determine if miRNAs could be measured from field samples and if stress-responsive miRNAs were increased downstream of the Kitchener (Ontario, Canada) wastewater treatment plant highlights the potential for miRNA to be used as a biomarker in the natural environment. Two of the three stress-responsive miRNAs (miR-30b-5p and miR-26a-5p; identified in Ch.5) were able to be measured and miR-30b-5p showed increased levels (**Figure 6.2**; Student’s t-test, $p = 0.092$). The usefulness of miRNAs as biomarkers is further evidenced by the ability of miRNAs to respond to both acute and environmental stress in a time-dependent manner (Ch. 2-4). This was indicated by the plasticity of the miRNA response in gonads of zebrafish following the removal of stressors, the difference in response when acute or chronic exposure to environmental exposure occurred, and the change in miRNAs levels in the hours following air exposure. MiRNA responses are also specific to both the tissue and sex of the fish measured which allows for increased resolution when using as biomarkers (Ch. 3).

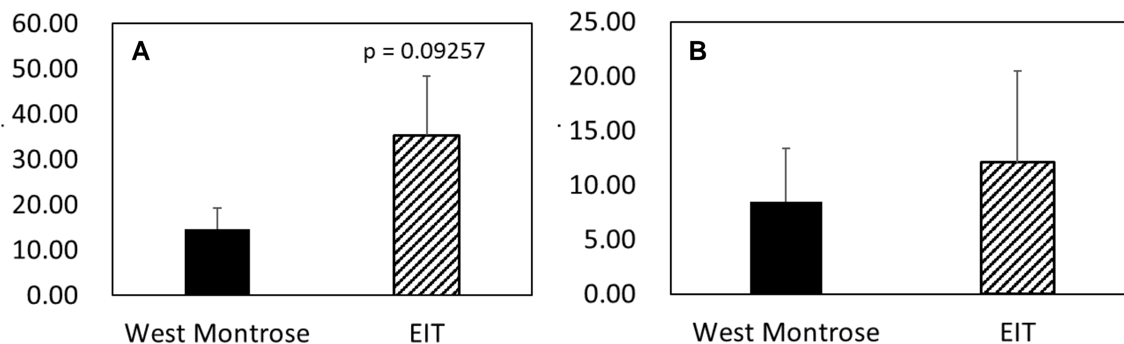

Figure 6.2 Relative abundance of microRNAs sampled in the Grand River up and downstream of the Kitchener wastewater treatment plant (Ontario, Canada).
Mean ± SEM of the relative abundance of **A**) ssa-miR-30b-5p and **B**) ssa-miR-26a-5p with relative abundance measured on the y-axis. West Montrose is the site upstream of the Kitchener wastewater treatment plant (WWTP) indicated by the black bars, and EIT (Economical Insurance Trailway) is downstream of the Kitchener WWTP, indicated by the line-filled bars. These microRNAs were extracted from water samples collected from the Grand River and filtered on 0.45 µM filters and quantified via RT-qPCR. Differences between sites were identified using Student's t-test (n = 4).

However, limitations to the widespread use of miRNAs as biomarkers of fish stress still exist and were identified throughout my research. The miRNA response is not always specific to the stressor, as was observed by the same miRNA being altered following exposure to either VFX or increased water temperature (Ch.2). This lack of resolution would make it difficult to determine which stressor to mitigate. Furthermore, though the response of miRNAs to environmental stressors have been measured in previous studies (ex. Craig et al., 2014), and in response to multiple, emerging environmental stressors in this thesis (Ch. 2&3), the miRNA response has only been characterized following specific stressors. It is unrealistic to consider measuring the effect of every stressor on miRNA levels. This means that the functions of miRNAs altered in response to stress need to be measured and directly related to the level of adverse biological impacts. This would allow for the identification of a suite of miRNAs that could be used as to be indicators of severe environmental stress that would result in population-level impacts. Though I measured the miRNA response following stressors and correlated it with predicted or measured downstream molecular impacts, it is unknown if these miRNA responses correspond to population-level effects (Ch.2-5). Furthermore, though miRNAs can be easily identified using RT-qPCR and RNA sequencing, there are still technical difficulties that exist in quantifying miRNAs robustly and consistently in low abundance

samples, such as mucus and water. Therefore, though miRNAs show promise as biomarkers of fish stress, there are several limitations that have yet to be addressed.

To consider the usage of fish miRNA as biomarkers, future study must be undertaken to address the concerns listed. The miRNA response needs to be measured in more species across different life stages to determine if the response is conserved or specific. More technical development of miRNA quantification can occur, leveraging the methods used to measure viral RNA from wastewater samples that have been developed during the COVID-19 pandemic (D'Aoust et al., 2021a, 2021b; Wurtz et al., 2021). Furthermore, collaboration with members of conservation groups can be leveraged to determine which health indices (ex. changes in vitellogenin) are important in fish biomonitoring. Measurement of altered miRNA transcript levels in conjunction with health indices, as well as the validation of miRNA targets to the molecular components of the health indices, will further strengthen the usage of miRNA as biomarkers.

With the current status of understanding of the role and response of miRNA to stress in fish, I am cautious to recommend the use of miRNA as biomarkers. However, I believe that miRNAs could potentially be used as biomarkers in aquaculture. Disease in aquaculture negatively impacts stocks and is predicted to continue to negatively impact the growth rate of global aquaculture production (FAO, 2020). Current end-user tests are being produced to indicate the presence of aquatic diseases within farms (ex. Gaskiya Diagnostics). Though the impact of disease on miRNA levels was not studied in this thesis, many miRNAs have been implicated in the immune stress response and have recently been measured in the mucus of fish (Schyth et al., 2015; Yan et al., 2015; Zhao et al., 2020; 2021). Measurement of miRNAs within fin clips, blood, or epidermal mucus of fish in aquaculture facilities could be reasonably developed as a current biomarker of disease. Additionally, due to the usage of eDNA sampling in current biomonitoring processes, miRNA can be currently extracted from the same filter (Beng & Corlett, 2020). Though specific miRNAs correlated with adverse health outcomes in fish have yet to be discovered, samples can be used for method development and can also be archived for analysis if stress-responsive indicator miRNAs are been identified. Therefore, though miRNAs may not be fully developed as reliable biomarkers, they can be currently applied in aquaculture and eDNA biomonitoring settings.

6.4 Need for functional and molecular validation of microRNA targets

While the predicted and measured phenotypic responses to stress associated with altered miRNA transcript levels can be preliminarily investigated with inverse relationships between miRNA and predicted mRNA, these relationships need to be functionally validated. Validation of the miRNA targets will expand our understanding of the mechanism of action of miRNAs in fish, as well as increase our understanding of the role of miRNA in regulating the acute or environmental stress response. To do this, knockdown of miRNAs using CRISPR/cas9 or antisense mimics and overexpression using synthetic miRNAs can be used to validate the effects the altered miRNA transcript levels on the downstream phenotype (Chang et al., 2016; Johnston et al., 2019; Müller-Deile et al., 2016). To validate the ability of miRNA to target and regulate specific mRNA, 3′UTR luciferase assays can be performed (Jin et al., 2013). These assays should be performed on the miRNAs and predicted targets and this validation can begin with the miRNA:mRNA pairs that showed inverse relationships.

6.5 Fish response to stress: What is now known

The impacts of multiple, emerging anthropogenic stressors on fish miRNA and downstream responses were characterized, allowing for greater understanding of the overall impacts of these stressors on fish health. Exposure to venlafaxine and increased water temperature were predicted to impact oocyte meiosis, and drug metabolism, and ovarian pathologies in zebrafish gonads (Ch. 2), whereas exposure to venlafaxine, increased water temperature, and decreased dissolved oxygen resulted in the measured increase in liver and muscle *hsp70*, *cyp1a*, *pgc1a* levels, indicative of metabolic, toxic, and cell stress responses (Ch. 3). Furthermore, multiple stressor exposure resulted in preliminary indication of protein level changes in vitellogenin and cell chaperones, indicative of endocrine and cell stress impacts. Taken together, there is evidence across fish tissues and endpoints which indicate that fecundity of fish could be adversely impacted by these environmental stressors. The molecular impacts have also been measured across multiple generations, including in un-exposed offspring of zebrafish (Luu et al., 2021). Therefore, though the anthropogenic stressors studied do not cause mortality in fish, there is a threat of long-term impacts on fish populations.

I have also further characterized the acute stress response in rainbow trout and brook trout. It is evident that this stress response increased levels of plasma cortisol, glucose, and lactate, as previously characterized (Iwama, 1998). However, there was high species-specific variation in this response,

highlighting the importance of not relying on only one measure when confirming that fish are stressed. This concept was introduced by Martínez-Porchas et al. (2009) and it is demonstrated once again with the varied plasma metabolite response measured in brook trout (Ch. 4). The increase in citrate synthase, cytochrome c oxidase, and catalase activities in the liver of rainbow trout following air stress also increase our understanding of the impacts of air exposure. Furthermore, during the response to acute stress, miRNAs were measured externally to the fish in the mucus and surrounding water. Though, it is unknown whether this is occurring passively or actively, I wonder if external miRNAs are used in signaling between fish, or even other organisms due to the sequence conservation of miRNA between vertebrates (Bartel et al., 2009). Therefore, I have increased our understanding of the physiological repercussions of acute stress via air exposure and opened avenues for potential research into the role of miRNAs present in the natural environment.

6.6 Future directions

It is evident that miRNAs respond to stress experienced by fish, however the mechanisms of how miRNAs are driving the stress response remains undiscovered. By using knockout and knockdowns of miRNAs during exposures to stressors, the tissue-specific and organism-level roles of miRNAs in coordinating the stress response can be characterized. The identification of miRNAs crucial to allowing fish to respond to and cope with stress (stress-miRs) could be critical indicators of when fish have been exposed to overwhelming amounts of stress which need to be mitigated. Furthermore, the identification of miRNAs in species or individuals within species that are highly adapted to certain environments can be used to determine if miRNAs are part of the molecular mechanisms that allow for increased adaptation or tolerance to certain stressors.

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Chapter 2

Chronic exposure to venlafaxine and increased water temperature reversibly alters microRNA in zebrafish gonads (Danio rerio)

Author: Heather Ikert,Paul M. Craig

Publication: Comparative Biochemistry and Physiology Part D: Genomics and Proteomics

Publisher: Elsevier

Date: March 2020

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BACK CLOSE WINDOW

Chapter 4

Responses of microRNA and predicted mRNA and enzymatic targets in liver of two salmonids (Oncorhynchus mykiss and Salvelinus fontinalis) following air exposure

Author: Heather Ikert,Slava Osokin,Joshua R. Saito,Paul M. Craig

Publication: Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology

Publisher: Elsevier

Date: October–December 2021

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BACK CLOSE WINDOW

Chapter 5

frontiers
in Physiology

ORIGINAL RESEARCH
published: 13 January 2021
doi: 10.3389/fphys.2020.588313

High Throughput Sequencing of MicroRNA in Rainbow Trout Plasma, Mucus, and Surrounding Water Following Acute Stress

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Circulating plasma microRNAs (miRNAs) are well established as biomarkers of several diseases in humans and have recently been used as indicators of environmental exposures in fish. However, the role of plasma miRNAs in regulating acute stress responses in fish is largely unknown. Tissue and plasma miRNAs have recently been associated with excreted miRNAs; however, external miRNAs have never been

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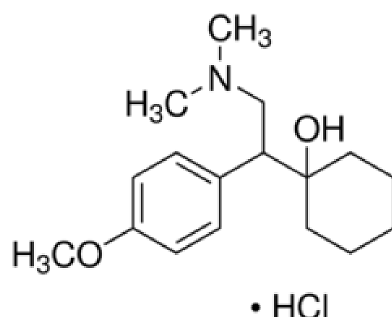
Appendix A – Properties of venlafaxine



Structure of venlafaxine hydrochloride.
The structure of venlafaxine in the form in which venlafaxine is prescribed as an antidepressant (also the form of venlafaxine used in the exposures; Chapters 2 & 3).

Chemical properties of venlafaxine hydrochloride. Various chemical properties of venlafaxine hydrochloride compiled by and accessed from National Center for Biotechnology Information (2021). PubChem Compound Summary for CID 62923, Venlafaxine hydrochloride. https://pubchem.ncbi.nlm.nih.gov/compound/Venlafaxine-hydrochloride.

Property Name	Property Value	Reference
LogP	0.43	EPA DSSTox, https://comptox.epa.gov/dashboard/DTXSID8047397
Molecular Weight	313.9	Computed by PubChem 2.1 (PubChem release 2021.05.07)
Hydrogen Bond Donor Count	2	Computed by Cactvs 3.4.8.18 (PubChem release 2021.05.07)
Hydrogen Bond Acceptor Count	3	Computed by Cactvs 3.4.8.18 (PubChem release 2021.05.07)
Rotatable Bond Count	5	Computed by Cactvs 3.4.8.18 (PubChem release 2021.05.07)
Exact Mass	313.1808568	Computed by PubChem 2.1 (PubChem release 2021.05.07)
Monoisotopic Mass	313.1808568	Computed by PubChem 2.1 (PubChem release 2021.05.07)
Topological Polar Surface Area	32.7 Å²	Computed by Cactvs 3.4.8.18 (PubChem release 2021.05.07)
Heavy Atom Count	21	Computed by PubChem
Formal Charge	0	Computed by PubChem
Complexity	279	Computed by Cactvs 3.4.8.18 (PubChem release 2021.05.07)
Isotope Atom Count	0	Computed by PubChem
Defined Atom Stereocenter Count	0	Computed by PubChem
Undefined Atom Stereocenter Count	1	Computed by PubChem
Defined Bond Stereocenter Count	0	Computed by PubChem
Undefined Bond Stereocenter Count	0	Computed by PubChem
Covalently-Bonded Unit Count	2	Computed by PubChem
Compound Is Canonicalized	Yes	Computed by PubChem (release 2021.05.07)

Appendix B – Supplementary data for Chapter 2

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbd.2019.100634. Supplementary material contains venlafaxine extraction and quantification details, all mRNA targets of the miRNA in this study, DAVID enrichment groups, KEGG pathways (list and percentage of total), and a list of cytochrome P450s predicted to be targeted by the miRNA in this study. These are presented as separate tabs in the Excel file.

Appendix C – Supplementary data for Chapter 3

There are several folders and files in the supplemental data folder which can be accessed on figshare (10.6084/m9.figshare.16660849). This document describes the structure and contents. Folders are bolded and files are italicized. The folders and files are listed in alphabetical order here.

DO_temp – contains dissolved oxygen and temperature readings collected over the exposures. The .cmbl files are the raw files created by LoggerPro and the .txt files are the extracted values from the LoggerPro software (organized by date(s) collected). The data is combined and summarized (including graphs) in the DO_Temp_summary_stats_updated.xlsx file.

microRNA_mRNA_data – contains all raw and analysed microRNA and mRNA data. See file and folder breakdown below:

> *NORMA-Gene_Macro V1.1 … .xls* files contain the relative quantities of the RNA in the Data tab, the statistics used by the algorithm to normalize in the Fitting results tab, and the normalized RNA data in the Normalization results tab.
>
> *Five .xlsx* files named for the tissue and length of exposure contain the normalized RNA data in a format to be used for statistical analysis in R. Files named in a way to be used by the R scripts.
>
> *treatment_sex_zebrafish_multi_stressor_RNA_analysis.R* is the R script used to perform the two-way ANOVAs.
>
> *session_info_2021-07-30_15-26-21.txt* is the output file produced by running the last part of the R script analysis above which outputs a text file containing the packages installed and versions of packages used in R.
>
> **two_way_ANOVA_results** is a folder containing the .txt files of the two-way ANOVA results produced by R. Consult the ‘Two-way ANOVA .txt file legend.docx’ file to understand the contents of the .txt files.
>
> **linear_regressions** is a folder containing the statistical and graphical results of the simple linear regressions performed between microRNA and mRNA.

protein_data – contains the raw and analysed protein data in the following file and folders:

forpub… Gsea folders contain the gene set enrichment analysis (GSEA) results for the liver and muscle data with zebrafish gene symbols. Consult the GSEA manual at https://www.gsea-msigdb.org/gsea/doc/GSEAUserGuideFrame.html for descriptions of file types.

miRanda_target_analysis contains all the files used to run the target prediction analysis between microRNA and proteins (bash script, list of all mature zebrafish microRNA from miRbase.org, fasta files of the 3'UTRs of vtg2, vtg3, and unc45b, .tab and excel files of the results), as well as the results of the linear regressions between the predicted binding pairs (tests for normality, linear regression statistics, and graphs).

20191015_UVPD_SVS_IkertH_ …. .txt (6 files) received from SPARC biocentre containing the peptide, protein, and run statistics from the identification and quantification of the proteins in the liver and muscle samples. The 'Proteins' files were used for analysis in limma and GSEA.

de_analysis_protein_(liver OR muscle)_cleaned.R are the two R scripts used to run the limma analysis to identify proteins that were significantly altered in the exposed fish tissues.

(liver OR muscle)_sig_proteins.txt are the output files from the limma analysis in R identifying significantly up or downregulated proteins.

Heather Ikert Methods TMT October 2019.docx – contains the detailed methods used by SPARC BioCentre to identify and quantify the TMT-labelled peptides extracted from the muscle and liver tissue.

VFX_extraction_quantification.xlsx – contains the detailed methods used to extract and quantify the venlafaxine from the exposure tanks.

zebrafish_weights.xlsx – contains the weights and sexes of the fish used for analysis.

Appendix D – Supplementary data for Chapter 4

There are several folders and files in the supplemental data folder which can be accessed on figshare (10.6084/m9.figshare.14417453). This document describes the structure and contents. Folders are bolded and files are italicized. The folders and files are listed in alphabetical order here.

- *miR-21a-3p_vs_5p.mas* – alignment of omy-miR-21a-3p to ssa-miR-21a-5p and ssa-miR-21b-5p. This is a MEGA file (software can be downloaded here https://www.megasoftware.net/).
- **miRanda_target_analysis**
  - **miRNA_to_mRNA_targets** – contains folders and files of microRNA sequences, bash scripts, and results of miRanda analysis of mRNAs predicted to be targeted by the three miRNAs measured in this study. The data is split up by which 3′UTR database was used for analysis (ensembl.org or genoscope).
  - **mRNA_to_miRNA_targets** – contains files of mRNA sequences, bash scripts, and results of miRanda analysis of miRNAs predicted to target the five mRNAs measured in this study.
- **non_sig_linear_regression_graphs** – contains folders of figures of non-significant linear regressions. The figures are split into miRNA-mRNA, miRNA-enzyme, and mRNA-enzyme regressions.
- **NORMA-Gene_files** – contains four excel files of the normalization performed using the NORMA-Gene algorithm (excel macro). There is a separate file for each species and each RNA type (miRNA and mRNA).
- *putative_S.fontinalis_CCO5b.fa* – sequence identified in *Salvelinus fontinalis* mitochondrial genome to be similar to isoforms of *Salvelinus alpinus* cytochrome c oxidase subunit 5b. Used to design primers to measure CCO5B expression via RT-qPCR.
- *sfontinalis_salpinus_cco5b_alignment.mas* – alignment of three isoforms of *Salvelinus alpinus* cytochrome c oxidase subunit 5b to the mitochondrial genome of *Salvelinus fontinalis*. The conserved sequence (see below) was used to design primers to measure CCO5B expression via RT-qPCR. This is a MEGA file (software can be downloaded here https://www.megasoftware.net/).

- **statistical_results** – contains folders with files of all the statistical results produced in this study (including all non-significant results that were not presented in the results section of the paper). The files are split up by endpoint and contain tests for normality and lognormality, transformed data (as applicable), and the results of the appropriate statistical tests.
- *supplemental_results.xlsx* – Data collected in this experiment used for statistical analysis. Data are described at the top of each sheet. Includes plasma cortisol, glucose, and lactate values, as well as liver microRNA and mRNA Cq values, and enzyme activities.

Appendix E – Supplementary data for Chapter 5

The Supplementary Material for this article can be accessed online at:

https://www.frontiersin.org/articles/10.3389/fphys.2020.588313/full#supplementary-material

Appendix F – A selection of fish puns

(just because they are reel-y fin-tastic)

At some points of writing this thesis, I doubted if I **cod** do it, but I finally **fin**-ished it.

And though, sometimes I may **flounder** to find the perfect thing to say, I can always count on fish puns to be a good choice.

Walleye personally think fish puns are the best, there are those that disagree.

I am often **gill**-ty of spending too much time thinking of fish puns.

What do you call someone who inherited fillets from their relative? A bene-**fish**-iary

My friends will tell you that this is just **parr** for the course.

I **trout** you thought that you would be reading an appendix of fish puns.

It is amazing to think about the heights that **carp**-ets got to in the 70s. I mean there were carpets everywhere!

Let **minnow** if you're annoyed yet.

I'm not going to **sushi** said – and she hung up the phone on the lawyer.

Eel catch on eventually, either that or you can explain every single one of these puns.

When Canadians are asking if you if you are still hungry or not, they often ask if you've had your **fillet**.

In that Dr. Seuss book, **salmon** I initially have something in common, neither of us like green eggs and ham.

I hope to be **herring** from you soon about how much you enjoyed these puns.

Whale I know this is a mammal, but they still swim around in the water so don't **krill** me.

I hope these **mako** you smile.

Kind Re-**gar**-ds, Heather

Fin